frontiers | Research Topics

Emerging research organisms in regenerative biology

Edited by
Igor Schneider, Veronica Hinman, Mathilda Mommersteeg and Stefano Tiozzo

Published in
Frontiers in Ecology and Evolution
Frontiers in Cell and Developmental Biology

ISSN 1664-8714
ISBN 978-2-83251-650-8
DOI 10.3389/978-2-83251-650-8

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Emerging research organisms in regenerative biology

Topic editors

Igor Schneider — Federal University of Pará, Brazil
Veronica Hinman — Carnegie Mellon University, United States
Mathilda Mommersteeg — University of Oxford, United Kingdom
Stefano Tiozzo — Université Paris-Sorbonne, France

Citation

Schneider, I., Hinman, V., Mommersteeg, M., Tiozzo, S., eds. (2023). *Emerging research organisms in regenerative biology*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83251-650-8

Table of contents

frontiers | Frontiers in Ecology and Evolution

TYPE Editorial
PUBLISHED 30 January 2023
DOI 10.3389/fevo.2022.1127607

OPEN ACCESS

EDITED AND REVIEWED BY
Maria Ina Arnone,
Stazione Zoologica Anton Dohrn, Italy

*CORRESPONDENCE
Stefano Tiozzo
✉ stefano.tiozzo@imev-mer.fr

SPECIALTY SECTION
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 19 December 2022
ACCEPTED 21 December 2022
PUBLISHED 30 January 2023

CITATION
Tiozzo S and Schneider I (2023) Editorial: Emerging research organisms in regenerative biology. *Front. Ecol. Evol.* 10:1127607. doi: 10.3389/fevo.2022.1127607

Editorial: Emerging research organisms in regenerative biology

Stefano Tiozzo[1]* and Igor Schneider[2]

[1]Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), CNRS, Sorbonne University, Paris, France, [2]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, United States

KEYWORDS

regeneration, model organism, evolution, metazoan, wound healing, adaptation, development, cancer

Editorial on the Research Topic
Emerging research organisms in regenerative biology

To answer particular biological questions through mechanistic approaches, it seems sensible to adopt accessible and tractable organisms that simplify the experimental work. By electing a species as a model, the interest goes beyond the organism itself and the ultimate goal becomes a better understanding of a more general biological phenomenon. Then, around the chosen organism a scientific community takes shape, and the development of tools and resources comes along. Indeed, many of the established so-called "model organisms" are convenient for studying several aspects of biology but are not necessarily the best systems for others. In addition, when focusing on only one species, mainly if chosen for its lab amenability rather than for a specific trait, any evolutionary consideration should be taken with a grain of salt (Russell et al., 2017).

The field of regenerative biology seems to be a glaring example where the choice of one, or even a fistful of model organisms can limit or even mislead the comprehension of the whole phenomenon. For instance, the uneven distribution of regenerating capabilities across the whole metazoans seems to point toward multiple independent acquisitions, a scenario that is backed up by shreds of evidence against universal conserved cellular and molecular mechanisms behind regeneration (Carlson, 2007). At a finer phylogenetic resolution, the picture is even more complex. Variabilities in terms of regenerative capacity and regenerative mechanisms can be found at the taxonomic level of family, order, and even genus (Sinigaglia et al., 2022). Indeed, since Trembley's dissections of Hydra polyps, which helped to give birth to the field of regenerative biology, the descriptive and mechanistic study of animal regeneration has always been sourced from arrays of different organisms. Particularly T. H. Morgan, through his prominent work on regeneration, first advocated the importance of comparing the amplest diversity of organisms to recast the questions about regeneration and development in terms of experimentally testable hypotheses (Sinigaglia et al., 2022). Following Morgan's legacy, in the last decades, highly regenerating animal models such as a few species of flatworms, *Hydra*, zebrafish and axolotl, have significantly advanced the understanding of the cellular and molecular basis of their regeneration, highlighting both common and different mechanisms. Even more recently, thanks also to affordable sequencing techniques, cutting-edge imaging approaches, single-cell transcriptomics, and epigenetics, new research organisms for regenerative biology have emerged (Blanchoud and Gallio, 2022). Yet, some basic questions remain far from being answered. For instance, *how did regeneration evolve in metazoans, are there conserved cellular and molecular modules*? To better portray the complex evolutionary scenario that characterizes regeneration, it is fundamental to study as many possible organisms, and use their phylogenetic relationships as an interpretative fabric to formulate evolutionary hypotheses. When possible, multiple, closely related species should be compared as a strategy that can direct and facilitate the

search for potentially conserved modules (molecular and cellular toolkits) specific to each regenerative mode. In this issue, Chowdhury et al. compared the regeneration of two established model organisms from the same order of teleost fish but belonging to two different families: zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). The authors reviewed the different aspects of tissue regeneration and the established experimental tools in the two fish models, highlighting the importance of inter-species and inter-organ comparisons to reveal mechanistic insights for therapeutic strategies for human diseases. Shimizu and Kawasaki implemented such comparisons and used the same two Actinopterygii fishes to analyze the neural stem cell regenerative responses *via* activation of neural stem cells upon central nervous system injury. Their results revealed reduced neuronal differentiation and induction of pro-regenerative transcription factor expression in medaka when compared to zebrafish, uncovering significant differences in regenerative potential within these teleost species. Shifting to lungfishes, the sister group of tetrapods, Bothe et al. examined general morphological features of appendage regeneration. In their previous work, the same team observed that in salamanders, regeneration abnormalities are more frequently observed in limbs that were bitten in a natural habitat than in those amputated in a laboratory setting (Bothe et al., 2021), and in this issue, by examining regenerated fins resulting from natural bites, they reported also in lungfish various skeletal abnormalities similar to those observed in salamanders, further substantiating the hypothesis of a common origin of fin and limb regenerative abilities in Sarcopterygii. Another good example of the complex evolution of regenerative capabilities is the clade Tunicata, the sister group of vertebrates. This sub-phylum comprises species with limited regenerative capacities and species able to regenerate the entire body through different mechanisms. Their scattered distribution across the well-resolved tunicate phylogeny suggests many gains and losses of regenerative power (Alié et al., 2020). Ricci et al. described the first phases of whole-body regeneration in the tunicate species *Botryllus schlosseri* and pointed out potential differences in regenerative mechanisms with other species belonging to the same genus (Nourizadeh et al., 2021).

Indeed, despite the variety of mechanisms that, in different species, characterize the development of a particular regenerating unit, some similarities and conserved molecular pathways have been found also across relatively distant animals. One fairly conserved process that precedes injury-induced regeneration is wound healing (Fumagalli et al., 2018). Adamska et al. showed *via* comparative transcriptomics that conserved wound healing-related molecular players, such as FGF and Wnt signaling pathways, are expressed during the initial wound closure of injured colonies of *Acropora millepora* (phylum: Cnidaria Class: Anthozoa). The Acropora's ability to quickly regenerate upon mechanical and chemical damage is probably one of the strategies that made scleractinian corals as widespread and successful as niche-constructing organisms in coral reef ecosystems. Yet, such hypotheses are difficult to be tested and lead to another general and so far unanswered issue in regenerative biology, which is *why some species can regenerate while others cannot.* The question is tightly related to the single or multiple origins of regenerative capacities but it carries a more adaptationist flavor. The advantages of regenerating one part of the body, or the whole organism, may seem pretty evident. Yet, the real challenge is to explore what are the consequences of regeneration on the survival and/or reproductive fitness of individuals of a particular species. In other words, to test the adaptive role of regeneration, or the loss of it. In this issue, Elchaninov et al. reviewed different hypotheses that try to explain different trends in the evolution of regenerative capacity, putting the emphasis on the cost and benefits that regeneration has for the individual and notably for the species. While studying empirically the adaptive value of regeneration is not an easy task, it most likely requires an understanding of the ecological context in which the given species is in and how the species responds to it. Klein et al. analyzed the effect of common pollutants on the development and regeneration of the Anthozoa *Nematostella vectensis*, showing either inhibition or failure in the tentacle regeneration as well as observing a shifting in the microbiota composition. The observations on microbiota compositions and its role in different aspects of animal development and homeostasis have been a topic of particular interest in the last decades. Díaz-Díaz et al. summarized the recent studies on the relationship between microbiota and the regenerative processes of their hosts, focusing mainly on the potential influence on Echinoderm's regenerative capacity, but also reviewing possible roles of microorganisms during wound healing and regeneration in other models.

Besides the evolutionary and adaptation-driven questions, which demand the study and comparison of many species, there are also other compelling questions in regenerative biology where the use of one model *per se* can help to point out general aspects of regeneration in metazoans. For instance, *to what extent embryogenesis, asexual reproduction, cancer, and regeneration can be seen as different angles of the same phenomenon?* In other words, are the mechanisms of regeneration shared with, or co-opted from, other developmental phenomena? To explore these questions, different levels of comparisons can be done within one single species. For instance, mechanistic connections between uncontrolled cancerous growth, highly regulated embryonic development, and epimorphic regeneration have been theorized since the beginning of the last century by Waddington. More recent literature endorsed Waddington's theory and highlighted striking similarities between wound healing, regenerative phenomena and the progression of some tumors (Flier et al., 2010; Leigh et al., 2018). In this issue, Demirci et al. use the canonical model organism zebrafish to explore molecular mechanisms shared between early stages of brain regeneration, where cell proliferation activity spikes, and two brain cancers. By comparing transcriptomic profiles the authors highlighted early convergence and later divergence in the two phenomena, providing a trampoline dataset to further mechanistic studies and the development of target therapies for vertebrate brain cancers cancers (Demirci et al.).

In conclusion, this issue brings together original findings and reviews on very different aspects of regeneration, and that cover both established and less-established research organisms. The articles above-mentioned underscore the importance of broadening the scope beyond the study of the molecular and cellular processes of regeneration in a single species and also demonstrate the importance of studying imperfect, limited regeneration or even the absence of regenerative abilities in light of phylogenetic and ecological contexts. The current availability of thousands of animal genomes and the techniques allowing molecular studies at the single-cell level should only prompt the proliferation of comparative studies. The inclusion of understudied novel species in the roll of regeneration model

systems becomes a condition *sine qua non* to understanding the many mechanisms behind regeneration and their evolution.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Alié, A., Hiebert, L. S., Scelzo, M., and Tiozzo, S. (2020). The eventful history of nonembryonic development in tunicates. *J. Exp. Zool. Part B Mol. Dev. Evol.* 336, 250–266. doi: 10.1002/jez.b.22940

Blanchoud, S., and Galliot, B., eds. (2022). *Whole-Body Regeneration | SpringerLink. Methods in*. Springer US. Available online at: https://link.springer.com/book/9781071621714 (accessed December 20, 2021).

Bothe, V., Mahlow, K., and Fröbisch, N. B. (2021). A histological study of normal and pathological limb regeneration in the Mexican axolotl Ambystoma mexicanum. *J. Exp. Zool. Part B Mol. Dev. Evol.* 336, 116–128. doi: 10.1002/jez.b.22950

Carlson, B. M. (2007). *Principles of Regenerative Biology*. Burlington: Elsevier.

Flier, J. S., Underhill, L. H., and Dvorak, H. F. (2010). Tumors: wounds that do not heal. *N. Engl. J. Med.* 315, 1650–1659. doi: 10.1056/NEJM198612253152606

Fumagalli, M. R., Zapperi, S., and La Porta, C. A. M. (2018). Regeneration in distantly related species: common strategies and pathways. *npj Syst. Biol. Appl.* 4, 5. doi: 10.1038/s41540-017-0042-z

Leigh, N. D., Dunlap, G. S., Johnson, K., Mariano, R., Oshiro, R., Wong, A. Y., et al. (2018). Transcriptomic landscape of the blastema niche in regenerating adult axolotl limbs at single-cell resolution. *Nat. Commun.* 9, 1–14. doi: 10.1038/s41467-018-07604-0

Nourizadeh, S., Kassmer, S., Rodriguez, D., Hiebert, L. S., and De Tomaso, A. W. (2021). Whole body regeneration and developmental competition in two botryllid ascidians. *Evodevo* 12, 15. doi: 10.1186/s13227-021-00185-y

Russell, J. J., Theriot, J. A., Sood, P., Marshall, W. F., Landweber, L. F., Fritz-Laylin, L., et al. (2017). Non-model model organisms. *BMC Biol.* 15, 55. doi: 10.1186/s12915-017-0391-5

Sinigaglia, C., Alié, A., and Tiozzo, S. (2022). *The Hazards of Regeneration: From Morgan's Legacy to Evo-Devo*. New York, NY: Humana, 3–25. doi: 10.1007/978-1-0716-2172-1_1

frontiers in Ecology and Evolution

REVIEW
published: 05 March 2021
doi: 10.3389/fevo.2021.621686

Evolution of Regeneration in Animals: A Tangled Story

Andrey Elchaninov[1,2], Gennady Sukhikh[1] and Timur Fatkhudinov[3,4]*

[1] Laboratory of Regenerative Medicine, FSBI National Medical Research Center for Obstetrics, Gynecology and Perinatology Named after Academician V.I. Kulakov of Ministry of Healthcare of the Russian Federation, Moscow, Russia, [2] Histology Department, Pirogov Russian National Research Medical University, Ministry of Healthcare of the Russian Federation, Moscow, Russia, [3] Laboratory of Growth and Development, FSBSI Scientific Research Institute of Human Morphology, Moscow, Russia, [4] Histology Department, Medical Institute, Peoples' Friendship University of Russia (RUDN University), Moscow, Russia

The evolution of regenerative capacity in multicellular animals represents one of the most complex and intriguing problems in biology. How could such a seemingly advantageous trait as self-repair become consistently attenuated by the evolution? This review article examines the concept of the origin and nature of regeneration, its connection with the processes of embryonic development and asexual reproduction, as well as with the mechanisms of tissue homeostasis. The article presents a variety of classical and modern hypotheses explaining different trends in the evolution of regenerative capacity which is not always beneficial for the individual and notably for the species. Mechanistically, these trends are driven by the evolution of signaling pathways and progressive restriction of differentiation plasticity with concomitant advances in adaptive immunity. Examples of phylogenetically enhanced regenerative capacity are considered as well, with appropriate evolutionary reasoning for the enhancement and discussion of its molecular mechanisms.

Keywords: evolution, regeneration, morphallaxis, epimorphosis, blastema, dedifferentiation

OPEN ACCESS

Edited by:
Stefano Tiozzo,
Université Paris-Sorbonne, France

Reviewed by:
Eduardo E. Zattara,
Indiana University Bloomington, United States
Alexandra Bely,
University of Maryland, College Park, United States

****Correspondence:***
Andrey Elchaninov
elchandrey@yandex.ru

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

Received: *26 October 2020*
Accepted: *15 February 2021*
Published: *05 March 2021*

Citation:
Elchaninov A, Sukhikh G and Fatkhudinov T (2021) Evolution of Regeneration in Animals: A Tangled Story. Front. Ecol. Evol. 9:621686. doi: 10.3389/fevo.2021.621686

Nothing in biology makes sense except in the light of evolution
T.G. Dobzhansky
If there were no regeneration there could be no life
R.J. Goss

INTRODUCTION

Animal regeneration is a subject of continuous scientific interest. The first experimental studies on regeneration were carried out in the 18th century (Reaumur, 1712; Tremblay, 1744). Despite the remarkable progress in the field (Bely and Nyberg, 2010; Zattara et al., 2019), we have to face the fact that regenerative capacity varies colossally among the animal taxa. Despite the enormous amount of experimental data on regeneration, the mechanisms of its evolution remain largely uncertain.

The first attempts to understand the laws that drive the evolution of regenerative capacity in animals date to the 19th century. Since then, the so-called first rule of regeneration ("the regenerative capacity of animals decreases with an increase in anatomical complexity") was re-formulated by many authors independently (Vorontsova and Liosner, 1960). The first counter-examples of phylogenetically enhanced regenerative capacity in animals date back to the 19th century as well.

August Weismann (1834–1914) was the first to propose comprehensive evolutionary reasoning for the diverse regeneration potential in animals. He postulated that regenerative capacity is an adaptive trait that is subject to phylogenetic alterations and therefore may vary considerably among the taxa. According to Weismann, the regenerative capacity of a particular organ depends on three factors: anatomical and physiological complexity, the frequency of damage to the organ, and its significance for survival (Weismann, 1893, 1899). In the 20th century, similar views were expressed by Arthur Edwin Needham, who also emphasized the relevance of environmental conditions (for instance, he believed that aquatic environments are highly favorable for regeneration) (Needham, 1952). Needham's remarks on the adaptive value of high regenerative capacity, particularly on its ambiguous evolutionary feasibility and controversial impact on survival, represent an important addition to Weismann's concept. According to Needham, the routes of adaptation to the damaging factors are multiple. Even under conditions of frequent damage to an organ, its regeneration would not necessarily be the unique or least expensive adaptive mechanism; the compensations for the loss may include the enhanced breeding capacity, as well as the effective avoidance of the damage through enhanced mobility (Needham, 1952).

Despite the long history of the subject, the evolution of regenerative capacity in animals is far from being fully understood (Bely and Nyberg, 2010). In a broad sense, the problematics of contemporary experimental studies and theoretical investigations in the field have been set up by Weismann (1893, 1899) and Needham (1952). It includes the questions like whether regeneration is a primitive or adaptive trait, what is the role of damage frequency in the evolution of regenerative capacity, what is the role of the environment, what are the reasons for the dynamic evolutionary alterations in regenerative capacity, is it appropriate to consider regeneration as a direct correlate of asexual reproduction, etc. The answers to these and other old questions in their contemporary perspective are the subject of this review.

CONTRIBUTION OF RUSSIAN SCIENTISTS TO THE THEORY OF REGENERATION

The first comprehensive Russian studies in the field of regeneration date back to the early 20th century. We should mention the research by K. N. Davydov, performed on acorn worms *Ptychodera minuta* and *Ptychodera clavigera*. Davydov was one of the first to express the idea of the similarity between regeneration and embryonic development; his conclusions were based on the comparison of the process of anterior regeneration in *P. minuta* and *P. clavigera* with embryonic development (Davydov, 1903).

By the 1930s, several large scientific centers for the study of regeneration were formed in Russia. One of those was headed by academician A. A. Zavarzin. Scientific activities of his team had a pronounced evolutionary dimension; their principal findings include the archetypal similarity of skeletal muscle regeneration (with the involvement of myoblasts) in representatives of different taxa (Zavarzin, 1938).

Another famous team focused on studying regeneration in invertebrates (predominantly *Porifera*) was headed by B. P. Tokin (Tokin, 1969; Korotkova, 1988). B. P. Tokin reckoned that the term «regeneration» was historically coined as a generic notion encompassing multiple different phenomena. He believed that restoration of lost parts (extremities or organs) proceeds by a different scenario and obeys other laws than the so-called «somatic embryogenesis»—formation of a whole organism from a limited number of preserved cells or small tissue fragments. In this regard, B. P. Tokin and colleagues proposed a broader concept of «regulation» which was a unifying term for regeneration *per se* and «somatic embryogenesis» (Tokin, 1969). This idea was subsequently criticized by Liosner, who questioned the criteria for the distinction between the regeneration of body parts and «somatic embryogenesis». L. D. Liosner justly pointed that in many cases the distinction is vague, e.g., the restoration of body terminus in many invertebrates (cnidarians, planarians, annelids, etc.) satisfies the definitions of both regeneration and somatic embryogenesis (Liozner, 1975).

Another key term that B. P. Tokin was operating with was «integration»—a universal measure of adaptive fitness showing a tendency to a continuous increase in the course of phylogenesis. B. P. Tokin believed that the ability to regenerate body parts increases evolutionary along with "integration" (as indicated by the high regeneration rates characteristic of the integument and internal organs in vertebrates), while the capacity of asexual reproduction and somatic embryogenesis decreases (Tokin, 1969). Tokin's views on the origin of regenerative capacity should be mentioned as well: he believed that physiological regeneration arose very early based on the properties and metabolic needs of primitive living systems, while reparative regeneration evolved later, based on the principles of physiological regeneration and subsequent evolution of metabolic pathways and defense mechanisms of the body (Tokin, 1969).

Another influential Russian team working on fundamental problems of regeneration was the laboratory headed by M. A. Vorontsova and L. D. Liosner (the Laboratory of Growth and Development at the Institute of Human Morphology Russian Academy of Medical Sciences, Moscow). The scope of their scientific interest within the field of animal regeneration was extremely diverse. Initially, the model choice was confined to limb regeneration in amphibians, with the main focus on the balance of destruction and proliferation and the role of mitogenic radiation in these processes; a series of such studies was published in the *Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen* (Blacher et al., 1933; Liosner et al., 1936). Later on, the focus of scientific interest eventually shifted toward the regeneration of internal organs, notably parenchymal organs, in amphibians and ultimately in mammals. The vast experimental data on the regeneration of different organs (kidneys, liver, lungs, testes, ovaries, etc.) allowed a number of important fundamental generalizations (Vorontsova and Liosner, 1960; Liozner, 1974). In particular, Vorontsova found out that all parenchymal organs regenerate in a similar way; to describe this; the

term «regenerative hypertrophy» was introduced. Regenerative hypertrophy—compensation of the loss by, respectively, cell proliferation or the increase in the size of individual cells without restoration of the initial morphogenetic complexity (Vorontsova and Liosner, 1960; Liozner, 1974). V. F. Sidorova showed that cellular mechanisms of postnatal regeneration of parenchymal organs correspond to postnatal growth rather than embryonic development, as no additional structural units (lobules, acini, and nephrons) are formed after the resection (Sidorova, 1964, 1978). A. G. Babaeva demonstrated the key role of the immune system in regeneration, notably the ability of lymphocytes to stimulate or suppress the repair processes in mammals (Babaeva, 1989, 1990). In the works of G. B. Bolshakova and her co-workers began a new research area—the study of the regeneration of the internal organs of mammalian fetuses; it was shown that in the prenatal period, myocardial injury in 16-day-old rat fetuses causes an increase in the proliferation of cardiomyocytes away from the injury zone, while the formation of connective tissue in the damaged zone is slow, which turns out to be unfavorable on the survival of such animals in the postnatal period (Bolshakova, 2008). A. V. Elchaninov showed that after resection of the liver of rat fetuses, the proliferation of hepatocytes is also activated and the liver mass is restored, while, in contrast to the postnatal period, without an increase in the ploidy and size of hepatocytes (Elchaninov and Bolshakova, 2011a,b, 2012).

Findings of other Russian research teams that worked successfully on specific fundamental issues of animal regeneration should be mentioned as well. These include the influence of pigment epithelium of the retina in its regeneration in tailed amphibians studied by Mitashov (1996) and the role of polyploidy in liver regeneration/myocardium repair in mammals demonstrated by Brodsky and Uryvaeva (1977).

THE ORIGINS OF REGENERATION

From the very beginning of regeneration studies, two opposing opinions have been expressed about its origin. Some experts qualified regeneration as a primary property of living systems (A. E. Needham and T. H. Morgan adhered to this point) (Needham, 1952), while others believed that it had emerged as a trait in some primitive organisms along with multicellularity (Weismann, 1893, 1899; Bely and Nyberg, 2010). The second opinion implies the understanding of regeneration as an epiphenomenon—inducible re-play of a program, which underlies a particular morphogenetic process (asexual reproduction, growth, and embryogenesis) and is used repeatedly in the case of damage (Garza-Garcia et al., 2010; Tiozzo and Copley, 2015).

Except for the radically different interpretation of very early events, these two theories are mutually consistent, as both allow viewing regeneration in terms of fundamental homology and account for the employment of recognizable, highly conservative patterns (which can be loosely defined as intensive physiological maintenance of the remnant complemented by active reconstruction of the missing part). Repair processes in different organisms have much in common, for example, rapid re-epithelialization of the damaged site, activation of cell proliferation, activation of matrix metalloproteinases, scavenging and regulatory activities of macrophages and other cells of the immune system (Elchaninov et al., 2018, 2019), the impact of the nervous system, etc. Repair processes may involve dedifferentiation and transdifferentiation of cells and, notably, activation of a stereotype genetic program (Fumagalli et al., 2018; Darnet et al., 2019; Mehta and Singh, 2019).

Moreover, the diversity of views on the origin of regeneration is more of historical interest, as early studies considered this process only at the level of tissues and organs while understandably neglecting the corresponding phenomena at subcellular levels. With the current state of knowledge, it is difficult to ignore the events and processes of restoration and maintenance of intracellular integrity, including the continuous renewal of organelles, turnover of the membranes, duplication of centrioles, division of mitochondria, disassembly and reassembly of the nuclear envelope during mitosis, etc. A unicellular organism devoid of any ability to regenerate would be maladaptive if viable at all; therefore, the direct association of regenerative capacity with multicellularity is hardly reasonable.

Vorontsova and Liosner (1960) distinguished several types of regeneration which had evolved separately; this point has been reflected in recent studies (Bely and Nyberg, 2010). For example, the regeneration of various components of organs, the regeneration of whole organs, and the regeneration of the entire body from a fragment represent different types of regeneration. Some of these types are continuously preserved by evolution, while others become eliminated (for example, the regeneration of the entire body from a fragment).

Despite the distinct common features, repair processes in different animal taxa may take dramatically different ways (Alvarado and Tsonis, 2006). These ways are most commonly distinguished by the scale of damage-induced cell proliferation and its contribution to the morphogenesis, with the extremes called morphallaxis and epimorphosis (the terms were introduced by T. H. Morgan) (**Figures 1A–C**). Morphallaxis proceeds by a spatial reorganization of the remnant at the initial stages of repair; for example, *Hydra* regenerates by morphallaxis (**Figure 1A**) (Alvarado and Tsonis, 2006). The opposite way, epimorphosis, proceeds through the formation of regeneration blastema composed of low-differentiated cells with high proliferative capacity (**Figure 1C**). Epimorphosis is characteristic of limb regeneration in tailed amphibians (Caudata) and to a certain extent also of planarian regeneration (**Figure 1B**) (Gurley et al., 2008). Currently, most experts agree that the clear distinction between epimorphosis and morphallaxis hardly makes sense, as any real regeneration is usually a combination of both (Agata et al., 2007; Bely and Nyberg, 2010). For instance, the oral pole regeneration in *Hydra* is distinctly epimorphic (Chera et al., 2009; Galliot and Ghila, 2010).

The apparent phylogenetic primacy of morphallaxis is indirectly indicated by its broad representation in both bilaterians and non-bilaterians, whereas epimorphosis is specific for bilaterians (Bely and Nyberg, 2010). Considering their similarity, it can be assumed that epimorphosis evolved on the basis of morphallaxis (Agata et al., 2007; Ben Khadra et al., 2018; Ferrario et al., 2018).

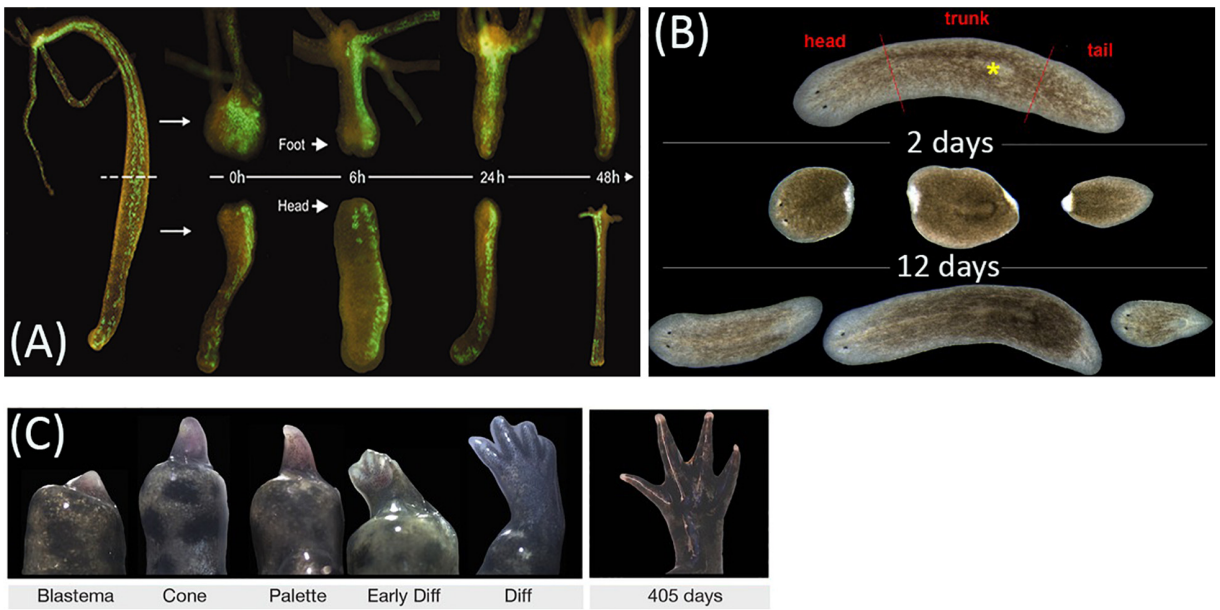


FIGURE 1 | Animal regeneration models. **(A)** Regeneration of head and foot in transgenic *Hydra vulgaris* by morphallaxis. **(B)** Regeneration timing in planarian *Schmidtea mediterranea*. **(C)** Epimorphic limb regeneration in axolotl *Ambystoma mexicanum*. Adapted from, respectively, Wittlieb et al. (2006); Adell et al. (2014), and Monaghan et al. (2014), under CC-BY. The asterisk labels the pharynx.

It should be noted that the overall homology of regeneration mechanisms in animals is not that obvious. The mechanisms of regeneration in distant taxa can differ beyond recognition, as can be illustrated by the diverse genesis of regeneration blastema in invertebrates (Das, 2015; Bertemes et al., 2020) and vertebrates (Seifert and Muneoka, 2018; Muneoka and Dawson, 2020).

In planarians, the formation of blastema results from the proliferation of neoblasts in response to amputation (Bertemes et al., 2020); in crustaceans and insects, wound blastema is formed from the migrating epidermal cells that undergo dedifferentiation (Mito et al., 2002; Das, 2015; Bando et al., 2018).

Phylogenetic plasticity of regeneration mechanisms in Caudata, with optional stem cell involvement and varying contributions of dedifferentiation and transdifferentiation, should be noted (Seifert and Muneoka, 2018; Muneoka and Dawson, 2020). For instance, in newts, myoblasts are formed by fragmentation of muscle fibers, whereas in axolotls, they form by differentiation of myosatellite cells found within the blastema (Seifert and Muneoka, 2018; Muneoka and Dawson, 2020).

Based on these findings, K. Muneoka et al. reckon that regenerative capacity in vertebrates evolved independently in different taxa originating from a hypothetical common tetrapod ancestor incapable of limb regeneration. The authors use this concept to describe the evolution of epimorphic limb regeneration in amphibians (Seifert and Muneoka, 2018; Muneoka and Dawson, 2020) and suggest a similar scenario for the evolution of regenerative capacity in mammals, with their ability to partially restore the terminal phalanx of a finger by forming a blastema-like structure through remodeling and growth of bone tissue, which is different from the mechanisms of blastema formation in amphibians (Seifert and Muneoka, 2018; Muneoka and Dawson, 2020).

It should also be noted that, in mammals, cellular sources of the wound blastema of the terminal phalanx differ in an age-dependent manner. In mouse embryos at advanced developmental stages, wound blastema is a derivative of chondrogenic cells of the terminal phalanx, which express *Msx1, Msx2, Dlx5,* and *Bmp4* markers. A similar amputation performed in the neonatal period promotes the formation of the wound blastema as a derivative of mesenchymal cells located predominantly beneath the nail organ and expressing *Msx1*, while the blastema cells express *Bmp2* and *Bmp7* (Seifert and Muneoka, 2018; Muneoka and Dawson, 2020).

The diversity of cellular mechanisms of blastema formation has been emphasized by Brockes et al. whose theory of regeneration origin and evolution is based on two assumptions: (1) regeneration employs the highly conservative principal mechanisms of growth, development, and maintenance of tissue homeostasis universally found in animals, and ensuring the capability of self-repair in certain species/taxa and (2) these highly conservative cellular mechanisms are governed and regulated by a relatively small number of taxon-specific genes responsible for the pronounced regenerative capacity (Garza-Garcia et al., 2010).

The first of these points is consistent with the evidence on the molecular invariance of morphogenetic processes (i.e., various types of morphogenesis involve similar regulatory cascades) (Cary et al., 2019; Mehta and Singh, 2019). The second point (existence of "principal regulator" genes) is less evident; notable examples include *fgf20* proposed as a primary regulator of fin regeneration in *Danio rerio* (Whitehead et al., 2005;

Poss, 2010). A taxon-specific protein Prod1 (Geng et al., 2015), found in newts and salamanders but missing in *D. rerio*, *Xenopus*, and mammals, participates in the neural control over regeneration and patterning (Garza-Garcia et al., 2010; Geng et al., 2015; Muneoka and Dawson, 2020). The presence of *Prod1* orthologs in *Ambystoma mexicanum* and *Ambystoma maculatum* places its origin before the divergence of Salamandridae and Ambystomidae (Garza-Garcia et al., 2010). In a planarian *Schmidtea mediterranea*, 15% of 1065 genes associated with homeostasis and regeneration have no homologs in other organisms and are considered taxon-specific (Reddien et al., 2005). According to Brockes et al., this group of genes is likely to comprise principal regulators that determine the ability to regenerate (Garza-Garcia et al., 2010).

The concept of principal regulators has also been indirectly supported by a comparative genomic study encompassing 132 species of multicellular animals with different regeneration capacities. A group of 118 highly conservative genes, 96% of which encoded Jumonji C (JmjC) domain-containing proteins, have been found specific for the «highly regenerative» species. The evolutionary loss of such genes has been associated with a dramatic decrease in regenerative capacity (Cao et al., 2019).

The evolutionary relationship between morphallaxis and epimorphosis is disputable. The assumption on their intrinsic homology was expressed by Bely and Nyberg (2010). This point of view is supported by the non-random incidence of both regeneration modes among animal taxa, as well as the fundamental similarity of the cellular processes underlying them. However, the depth of this similarity varies, and the mechanisms can be fundamentally different. Moreover, the terms «morphallaxis» and «epimorphosis», in the sense that Morgan (who coined them) put into them, do not take into account the overall mechanistic diversity of regenerative processes in the animal kingdom; as a result, phenomena of different nature are combined under one term. In this regard, some authors propose to abandon the use of terms «morphallaxis» and «epimorphosis». For instance, K. Agata suggested new terms «distalization» and «intercalation» (Agata et al., 2007; Tiozzo and Copley, 2015). Recent findings indicate striking diversity of regulation and implementation of regenerative processes at molecular and cellular levels; even within a taxonomic group, the mechanisms of regenerative response may vary significantly. In this regard, the concept of homology as related to regeneration becomes a distinct complex problem (Tiozzo and Copley, 2015).

The question of the origin of reparative regeneration is closely related to the problem of how physiological regeneration (i.e., the *non-injury-induced restorative processes*) and reparative regeneration relate to each other. In general, physiological regeneration is defined as the restoration of organs, tissues, cells, and subcellular structures lost during their normal life cycle or when performing their functions (Vorontsova and Liosner, 1960). In modern understanding, physiological regeneration is inherent in all tissues and cells; however, it proceeds in different forms. The phenomena of physiological regeneration include desquamation of epidermal cells, renewal of the intestinal epithelium, restoration of the uterine mucosa during the menstrual cycle, etc. (Carlson, 2007). B. P. Tokin viewed physiological regeneration as a mechanistic basis and direct evolutionary precursor to reparative processes. In an extreme interpretation (currently only of historical interest), reparative regeneration is an enhanced version of physiological regeneration. This simplification is due to the fact that cell proliferation, observed in some tissues under normal conditions and activated after injury, was the only measurable sign of regeneration. Currently, it is obvious that reparative regeneration differs in mechanisms from physiological regeneration and according to some views evolves as epiphenomenon which partially employs both the principles of physiological regeneration and the highly conserved molecular and cellular mechanisms of embryonic development and growth (Goss, 1992; Tiozzo and Copley, 2015).

Anyway, there is no doubt that regeneration as a process arose very early in the evolution and therefore involves highly conserved cellular mechanisms of morphogenesis. The intrinsic similarity of regeneration processes with asexual reproduction (Vorontsova and Liosner, 1960; Martinez et al., 2005; Kawamura et al., 2008; Burton and Finnerty, 2009; Zattara and Bely, 2016), growth (Bely and Wray, 2001; Gurley et al., 2008), and embryonic development (Martin and Parkhurst, 2004; Ghosh et al., 2008; Vogg et al., 2019) has been repeatedly noted.

REGENERATION AND ASEXUAL REPRODUCTION

Indeed, it is quite difficult not to link regeneration with asexual reproduction (Vorontsova and Liosner, 1960; Martinez et al., 2005; Brockes and Kumar, 2008; Kawamura et al., 2008; Burton and Finnerty, 2009; Zattara and Bely, 2016). In many organisms, regeneration can be morphologically indistinguishable from asexual reproduction by budding or fission. The mechanisms of asexual reproduction could be "easily" adapted for regeneration; the key difference is the stimuli that trigger these processes. Such concept has been supported by molecular studies of regeneration and asexual reproduction in hydras, planarians, annelids, and other invertebrates (Martinez et al., 2005; Mehta and Singh, 2019; Reddy et al., 2019a,b) revealing specific involvement of stem cells and generically similar roles of Wnt-signaling in these two processes (Mehta and Singh, 2019).

Ultimately, the phenomenon of restoration of the entire body from a fragment can be considered as asexual reproduction (Tokin, 1969). B. P. Tokin viewed the decreasing capacity for asexual reproduction as a direct correlate (and reflection) of the loss in regenerative capacity.

The resemblance of asexual reproduction with regeneration in invertebrates is remarkable. However, despite the rich recent history of comparative studies on the histological level, only a limited number of specific molecular findings support the intrinsic similarity of the two processes. The positive examples include similar expression of *Pl-en* in the nervous system, as well as *Pl-Otx1* and *Pl-Otx2* in the anterior body wall, foregut, and nervous system, of the annelid worm *Pristina leidyi* during regeneration and asexual reproduction (Bely and Wray, 2001). Also, *Hydra* shows a similar expression of *HyBMP5-8b*, a

BMP5-8 ortholog involved in axial patterning and formation of tentacles, in budding and regeneration (Reinhardt et al., 2004). However, despite the outward similarity of asexual reproduction with regeneration, these two processes evolved separately. For instance, the closest common ancestor of *Annelida* was probably capable of regenerating the anterior and posterior ends of the body but was devoid of the ability to reproduce itself asexually (Zattara and Bely, 2011, 2016). In *Nematostella vectensis*, molecular markers expressed during asexual reproduction and regeneration significantly overlap; however, no expression of regeneration markers *Nv-otxC* and *anthox1* is observed during asexual reproduction (Burton and Finnerty, 2009).

REGENERATION AND EMBRYOGENESIS

K. N. Davydov was one of the first to express the idea of the similarity between regeneration and embryonic development; his conclusions were based on the comparison of the process of anterior regeneration in *P. minuta* and *P. clavigera* with embryonic development (Davydov, 1903). The relationship between regeneration and embryogenesis is of particular importance for evolutionary biology, as it allows experimental investigation of the emergence of new structures. Sánchez Alvarado and coauthors developed an original view of this problem (Sánchez Alvarado, 2000; Elliott and Sánchez Alvarado, 2013). According to his opinion, the limb development in arthropods and vertebrates is governed by similar molecular cascades. However, the closest common ancestors of arthropods and vertebrates had no limbs at all. What factors, then, predetermined the homology? (Sánchez Alvarado, 2000; Elliott and Sánchez Alvarado, 2013).

The answer to this question can be obtained by studying regeneration. The similarity of embryonic limb buds with regeneration blastema is evident both histologically and at the level of molecular signaling cascades (Galis et al., 2003). In planarians, the blastema contains key components of molecular pathways regulating the establishment of anterior–posterior (Wnt-signaling), dorsal–ventral (BMP-pathway), and medial–lateral polarities (Sánchez Alvarado, 2000; Elliott and Sánchez Alvarado, 2013; Karami et al., 2015). According to Sánchez Alvarado and coauthors opinion, «the molecular processes underlying blastema formation and regeneration have been co-opted by sexually reproducing animals for the production of new structures such as limbs during the evolution of their developmental processes» (Sánchez Alvarado, 2000; Elliott and Sánchez Alvarado, 2013).

In molecular terms, embryonic development and regeneration are very different. *N. vectensis* shows no asymmetric expression of Hox-like genes (characteristic of embryogenesis) during asexual reproduction or regeneration (Burton and Finnerty, 2009). In zebrafish, the epimorphic regeneration of fins requires *fgf20a* expression, which is not required for fin development (Whitehead et al., 2005). In *Xenopus*, three Abdominal B-type Hox genes *XHoxc10*, *XHoxa13*, and *XHoxd13* show different expression patterns in regenerating and developing limbs (Christen et al., 2003). The similarities and differences of embryonic development, asexual reproduction, and regeneration are consistent with the idea that the capacities of asexual reproduction and regeneration evolved on the basis of signaling pathways of growth and development; however, the "borrowing" was selective and proceeded in a variety of ways.

Apparently, signaling pathways governing regeneration and asexual reproduction in primitive animals were eventually redirected for the performance of other tasks, e.g., limb development (Sánchez Alvarado, 2000; Elliott and Sánchez Alvarado, 2013).

EVOLUTIONARY MAINTENANCE OF REGENERATIVE CAPACITY

Regardless of the character of regeneration origins at the most ancient stages of evolution (whether it was a primary or secondary property of animals), this property was propagated in diverse forms throughout the animal kingdom.

The problem of maintaining regenerative capacity during evolution is one of the key ones. However, there are very few specific experimental studies. Initially, the very idea of maintaining the ability to regenerate, the role of the frequency of damage in this process was developed by Weismann (1893, 1899), and further tested in the works of Morgan T.H. (1901). Further insight into the role of injury and the value of regeneration in the fitness of a species was developed by Needham (1952) and Goss (1969).

According to the classical reasoning, frequent damage to an organ is favorable for the maintenance of its regenerative capacity (Weismann, 1893, 1899), given that its loss will significantly reduce the individual's fitness and the overall costs are not detrimental for the species (Needham, 1952; Goss, 1969).

At the initial stages of evolution, aggressive environmental conditions apparently played a principal role in maintaining the regenerative capacity (Wulff, 2006). Indeed, a high frequency of damage is typical for some groups of highly regenerative organisms in natural environments, to the extent that the majority of individuals in wild populations show distinct signs of damage and repair (Clark et al., 2007; Bely and Nyberg, 2010). However, the high regenerative capacity may be preserved even at low frequencies of damage. T. H. Morgan, in his classical studies on hermit crabs, showed that the rudimentary hind limbs, hidden in the shell and rarely damaged unless the shell is broken (in which case the animal would likely perish), regenerate in the same way as front limbs (exposed to the environment and frequently damaged or autotomized) (Morgan T., 1901; Morgan T.H., 1901; Sunderland, 2010). Noteworthy, hydras, and planarians, with their remarkable regenerative capacities, show no signs of active repair in the wild (Bely and Nyberg, 2010). As emphasized by Needham, regeneration would never be the only adaptive response to frequent damage. Instead, the species may enhance its reproductive potential; the animals may also develop mobility, protective coloration, exoskeleton, etc. (Needham, 1952).

Theoretically, as already noted, the severity of damage must be balanced by the cost of the regenerative process. Excessive severity of damage will kill the animal, whereas its

insignificance for the normal functioning (due to dispensability or redundancy of the damaged structure) will eliminate the need for regeneration. However, in practice, it is rather difficult to determine the cost of damage, as well as the cost of regeneration for a particular organism (Tiozzo and Copley, 2015). Several studies indicate that regeneration is indeed associated with significant energy expenditures (Naya et al., 2007) and functional opportunity costs that affect the survival and reproductive capacity of the organism (Bernardo and Agosta, 2005; Maginnis, 2006; Suzuki et al., 2019). Complex adaptive reactions (e.g., autotomy, which helps to minimize the loss of biological fluids and tissues when attacked by predators) can reduce the cost of damage thus increasing the feasibility of regeneration (Maginnis, 2006; Mcgaw, 2006; Bateman et al., 2008). In the general case, the regeneration is feasible when its benefits and rates override the possible negative effects from the existence of functionally immature and burdensome intermediate structures (Ramos et al., 2004; Dupont and Thorndyke, 2006; Barr et al., 2019) or incomplete/deviant recovery in cases of atypical regeneration (Lailvaux et al., 2009; Bely and Nyberg, 2010).

Due to the difficulties and contradictions of adaptationism (when applied on its own), alternative hypotheses were proposed to explain the evolutionary maintenance of regenerative capacity. In this regard, pleiotropic effects and phylogenetic inertia represent particularly important factors that should be discussed separately.

In an evolutionary context, the term «pleiotropy» refers to the maintenance of regenerative capacity of an organ in close association with some other important morphogenetic process, for example, asexual reproduction, growth, embryogenesis, or regeneration of another organ (possibly regulated by the same genetic frameworks). Pleiotropy implies default activation of related morphogenetic processes; for instance, in cnidarians and flatworms, the mechanisms of regeneration and normal growth are intrinsically similar (Alvarado and Tsonis, 2006; Bosch, 2007).

The concept of phylogenetic inertia refers to cases when regenerative capacity confers no distinct selective advantages to the species, nor shows distinct associations with any other morphogenetic process. In such cases, regeneration is preserved for the reason of insufficient selection pressure (or time) for its elimination. This concept provides a valuable description for the evolution of regenerative capacity in annelids, some of which retained the capacity while others lost it (Bely and Wray, 2001; Bely, 2006).

EVOLUTIONARY ENHANCEMENT OF REGENERATIVE CAPACITY

It should be noted that evolutionary enhancement of regenerative capacity is rare. Nevertheless, the distinct minor trends can be illustrated by the enhanced regenerative capacity of muscle liver tissues and in mammals and birds compared with amphibians (Liozner, 1974; Carlson, 2005) and the enhanced regeneration of extremities in arthropods compared with other ecdysozoans (Maruzzo and Bortolin, 2013). Another famous example is the regeneration of the tail in lizards (Garza-Garcia et al., 2010) and high skin regeneration in the spiny mouse, Acomys (Brant et al., 2016). Despite these impressive examples of the enhanced regenerative capacity, their mutual relationship is too distant to allow comprehensive investigation of common evolutionary patterns.

One of the most productive strategies in tracing the evolutionary dynamics of regenerative capacity is to compare closely related species with different regenerative capacities (Bely and Sikes, 2010; Zattara et al., 2019). Phylum Nemertea is one of the most promising in this aspect, as all of its studied species are capable of regenerating the posterior portion of the body, while only some of them can regenerate the anterior terminus (Bely et al., 2014; Zattara and Bely, 2016). The findings indicate that the common ancestor of Nemertea was capable of regenerating the posterior portion, but not the anterior terminus. In the evolution of Nemertea, this capacity was reinforced in at least four instances, as revealed by facile regeneration of the anterior terminus in corresponding species (one among Palaeonemertea and three among Pilidiophora; Zattara et al., 2019). The repeated events of enhancement were apparently promoted by repeated emergence of certain traits which allowed the transition (probably, the long-term survival of decapitated individuals) (Zattara et al., 2019). Mechanistically, the enhancement may result from the activation of some embryonic developmental programs in adults. Such assumption is consistent with the experiments on the embryos of *Nemertopsis bivittata*, which, after being cut into two parts, develop into two individuals (whereas the adults of this species are non-regenerative) (Martindale and Henry, 1995). Such mechanisms can be highly conserved; *cf.* the organizing roles of Wnt/β-catenin signaling during apical regeneration in *Hydra* and early development in vertebrates (Guder et al., 2006; Reddy et al., 2019a; Vogg et al., 2019).

AN EVOLUTIONARY DECLINE IN REGENERATIVE CAPACITY

The decline in regenerative capacity is a very strong phylogenetic trend, the examples of which can be found in any phylum (Bely and Nyberg, 2010; Lai and Aboobaker, 2018). However, its accurate comparative assessment in different groups of animals is complicated (Bely, 2010; Bely and Sikes, 2010).

Meanwhile, mechanistic reasons for the decline, though much discussed, remain understudied. In the view of adaptationists, regenerative capacity may be alleviated as a direct consequence of low damage frequency (Baumiller and Gahn, 2004). However, this view has not been supported by experimental findings, efficient regeneration of rudimentary limbs in hermit crabs reported by T. Morgan. The same applies to the regeneration of internal organs, which, according to A. Weismann, should regenerate poorly (Weismann, 1893, 1899). In the 20th century, this concept was criticized by M. A. Vorontsova, L. D. Liosner, and their followers (Vorontsova and Liosner, 1960; Liozner, 1974).

In addition, a decline in regenerative capacity may occur as a result of a significant change in the adaptive value of the organ. In case of dramatic gain in adaptive value, damage to the organ

may kill the individual without giving regeneration a chance. However, a decrease in the adaptive value of an organ may also promote a decline in its regenerative capacity, as it happens with a multiplication of identical or similar structures, e.g., the alleviated capacity of limb regeneration in certain arachnids (Brautigam and Persons, 2003).

Regenerative capacity may also decrease in a pleiotropic manner. Galis et al. (2003) suggest that the regenerative capacity of vertebrate limbs evolves in connection with their embryonic development. In the case of the early onset of limb development, its formation coincides with basic morphogenetic events involving complex interactions of multiple embryonic structures. As a consequence, the limb develops under powerful inducing effects of somites, lateral plate mesoderm, etc., but not as a self-organizing structure. Accordingly, the regenerative capacity of the definitive limb is reduced (Galis et al., 2003).

When the onset of limb development is delayed until the completion of fundamental inductive interactions between the primary germ layer derivatives (somites, neural tube, etc.), the autonomously developing limbs will be regenerative. This concept can be illustrated by the delayed limb development in Caudata (whose capacity for limb regeneration is renowned). Opposite examples include the fins of sharks and lungfish, as well as the limbs of birds and mammals, which develop from early anlagen and regenerate poorly. At the same time, the concept does not account for the poor limb regeneration in Anura, whose limbs develop fairly late, but regenerate well in larvae only (Galis et al., 2003). However, adult Anura are not completely devoid of the ability to regenerate limbs: in *Rana temporaria* and *Rana clamitans*, limb regeneration can be obtained after additional damaging effects on the wound surface (Polezhaev, 1946), while in *Xenopus laevis*, the same effect can be achieved by blocking proton channels and limiting the duration of local immune responses (Adams et al., 2007; Fukazawa et al., 2009).

Close to the concept under consideration is the concept of modules, a network of genes that control the behavior of cells taken from evo-devo. Defining the concept of modularity is not a trivial task. In developmental biology, the hypothesis of modules assumes the division of a developing organism into functional or organizational subunits that have pronounced morphological isolation, for example, somites, or correspond to a certain part of the body of an adult, such as a limb kidney (Bolker, 2000). Raff (1996) listed the following module characteristics: it should have a discrete genetic specification, hierarchical organization, interactions with other modules, a particular physical location within a developing organism, and the ability to undergo transformations on both developmental and evolutionary time scales (Raff, 1996).

In connection with the problem of the evolution of regeneration, this concept implies the idea of developmental constraint, i.e., restraints on phenotype production due to limited interaction among modules. For example, an increase in the complexity of the structure at the histological level can prevent the propagation of gradients of morphogens or bioelectric signals, which can lead to a decrease in the regenerative capacity (Tiozzo and Copley, 2015).

The interplay of regeneration and immunity represents a special issue (Mescher et al., 2017). The advent of adaptive immunity apparently collided with the pronounced regenerative capacity. In the highly regenerative Caudata, many components of adaptive immunity are underdeveloped; for example, compared with tailless amphibians, they lack antiviral immunity (Cotter et al., 2008; Murawala et al., 2012). Significant upgrade of the adaptive immune system during metamorphosis in Anura is consistent with the observed decline in the regenerative capacity of the adult individuals compared with the larvae (Robert and Ohta, 2009; Godwin and Rosenthal, 2014). In Anura, the immune system undergoes significant developmental changes. Prior to metamorphosis, it is functionally immature, as indicated by larval repertoires of T cell and B cell receptors, low expression of MHC I, low levels of B cell-mediated responses and antibody production, the negligible activity of natural killer cells, and low activity of helper and killer T cells. Metamorphosis is associated with a significant upgrade of these indicators; in addition, it brings the capacity of MHC II-dependent activation of helper T cells (Robert and Ohta, 2009). The increase in activity of natural killer cells and T cells in tailless amphibians leads to enhanced antitumor and antiviral immunity, which apparently costs them their regenerative potential.

Similar patterns are observed in mammals, with the pronounced regenerative capacity (manifested in scarless wound healing and myocardial regeneration) confined to certain stages of fetal development (Porrello et al., 2011; Vivien et al., 2016). The pronounced regenerative capacity of fetal skin and myocardium can be associated with certain functional properties of the developing immune system. It has been demonstrated that during this period the body more readily develops a Th2-mediated anti-inflammatory response than pro-inflammatory reactions (Sattler and Rosenthal, 2016). The shifted balance apparently favors a full-value compensation of the defect in line with its immediate tissue environment rather than its replacement with fibrous tissue. Apart from the plausible role of T cell-mediated responses, the influence of innate immunity should be considered as well. The development of organs is accompanied by their colonization with macrophages of bone marrow origin as opposed to primary populations of embryonic macrophages, which may also affect the regenerative capacity (Epelman et al., 2014; Elchaninov et al., 2019, 2020). Apparently similar reasons explain the high skin regeneration in the spiny mouse, Acomys. So they have an almost complete absence of macrophages and a low level of pro-inflammatory cytokines in their skin wounds (Brant et al., 2016).

Thus, it can be noted that evolutionary maturation of the immune system leads to a decrease in the regenerative potential, as illustrated by the inability of frogs to regenerate limbs after metamorphosis, as well as the extinction of scarless healing of skin wounds in mammals.

The reverse correlation between adaptive immunity and regenerative capacity (Godwin et al., 2017) may reflect the important role of under-, trans-, or dedifferentiated cells in regeneration (considered in the next section). It has been suggested that the advanced adaptive immunity (characteristic of Anura, birds, and mammals) is poorly compatible with the presence of non-differentiated cells, which are considered

compromised and become eliminated along with foreign cells. The constant immune pressure on the populations of cells with high differentiation potential negatively affects the regenerative capacity (Godwin et al., 2017).

Another reason for the decline in regenerative capacity may be the high energy cost of this process. In animals with a short lifespan, individuals invest more resources in reproduction, which leads to a decrease in regenerative potential; this apparently has happened to certain species of lizards (Fox and McCoy, 2000; Bernardo and Agosta, 2005). A similar relationship between reproduction and regeneration can be observed in species with asexual reproduction, e.g., annelids who have lost the capacity of anterior regeneration (Bely and Wray, 2001; Bely, 2010; Zattara and Bely, 2013). Regeneration may affect the development; for instance, it significantly delays the metamorphosis in fruit flies, cockroaches, butterflies, and crabs, which can also adversely affect survival (Suzuki et al., 2019). Another possible cause for the decline in regenerative capacity is warm-bloodedness (Goss, 1969), which is closely related to the evolution of adaptive immunity, hard skeleton (Wulff, 2006), and finite growth (Bely and Wray, 2001; Bely, 2010).

Elucidation of mechanisms that determine the decline of regenerative capacity is challenging, especially given the varying degree of such effects in the evolution. It was noted that in certain groups of animals, e.g., annelids, regeneration is reduced to wound healing, amphibians and fish tend to exhibit hypomorphic regeneration, whereas reptiles may show either decreased rates of recovery or confinement of repair to certain stages of ontogeny (Vorontsova and Liosner, 1960; Han et al., 2003, 2008; Seifert and Muneoka, 2018). In planarian *Dendrocoelum lacteum*, cross-cut at a certain level, tail fragments are incapable of regenerating the head. It has been found that the restriction is due to the uninhibited Wnt/b-catenin signaling in such fragments and that ectopic suppression of Wnt/b-catenin signaling makes them capable of anterior regeneration (Liu et al., 2013; Maden, 2018). Similarly, the lack of anterior regeneration observed in certain annelids has been associated with low expression of *nanos* (Bely and Sikes, 2010).

DIFFERENTIATION STATUS AS A CORRELATE OF REGENERATIVE CAPACITY

According to Weismann's theory, the regenerative capacity decreases as the structural and functional organization becomes more complex. In other words, Weismann believed that complex structural patterns are poorly compatible with regeneration, which requires pronounced tissue plasticity and a sufficient degree of freedom for the reconstruction.

Despite the vagueness and controversy of the term "organization complexity" as applied to animals, differentiation plasticity of cells is certainly connected with regeneration capacity.

The terms «transdifferentiation», «dedifferentiation», and «redifferentiation» have a rich history of scientific usage. The issue of their exact meanings and, in general, whether their use makes sense, is still open. Despite the long controversy, the definitions vary. Literally, dedifferentiation is the loss of structural and functional specialization; accordingly, redifferentiation may be understood as reacquisition of its previous differentiated phenotype by a particular cell (Odelberg, 2004, 2005; Grigoryan, 2016). «Transdifferentiation» is a particularly controversial term. Some experts use it loosely, even to describe a transition between derivatives of the same germ layer, for example, the transition between cholangiocyte and hepatocyte (Michalopoulos, 2011). Others use it in a narrower sense, to describe a transition between germ layers; the examples include the transition of the coelomic epithelium into gut epithelium during gut regeneration in holothurians (Dolmatov et al., 2019) and the transition of pigment cells of the iris into epithelial cells of the lens (Grigoryan, 2016). «Dedifferentiation» implies explicit transition to a low-differentiated state with high proliferative activity. A classic example of dedifferentiation is observed during regeneration of the retina from the pigment epithelium in newts, during which the epithelial cells lose melanin granules, enter proliferation, and differentiate into neurons (Mitashov, 1996); the whole sequence, however, can be justly classified as redifferentiation or even transdifferentiation. Formation of the wound blastema during regeneration of newt limbs also involves dedifferentiation, with muscle fibers losing their striation and undergoing fragmentation to become myoblasts (Odelberg, 2005).

Differentiation plasticity of cells at the site of injury (or directed to it) is closely related to the extent of remodeling in response to damage, with the extremes termed morphallaxis («blastema-less» regeneration) and epimorphosis (which involves the formation of blastema). For instance, the diploblastic *Hydra* can be considered as an organism that is constantly in a state of regeneration (Sánchez Alvarado, 2000; Martínez and Bridge, 2012). In *Hydra*, non-differentiated pluripotent cells of the gastric column are constantly proliferating and changing their location within the body (Sánchez Alvarado, 2000; Bosch, 2007; Vogg et al., 2019). According to some expert opinions, these cells may be considered as a hidden permanent analog of the blastema. The constant «circulation» of such cells in *Hydra*'s body provides a reasonable alternative to their emergency accumulation at the site of damage (which would be an epimorphic feature). Moreover, the constant presence of non-differentiated progenitors enables the triggering of determination and differentiation processes immediately after damage, which is typical for morphallaxis (Sánchez Alvarado, 2000).

In triploblastic animals, the evolution of an expanded system of cell differentiation checkpoints posed critical restrictions on the pluripotency. In planarians (considered as the most primitive triploblastic animals), the only pluripotent cells are neoblasts. In the case of damage to the planarian body, neoblasts actively proliferate and form blastema. It is believed that the cells involved in the restoration of the entire body from a fragment have similar properties in different groups of animals (endowed with such capacity). These cells are marked with RNA/protein-rich structures referred to as nuage, germ plasm, or chromatoid bodies (nuGPCB) which typically contain the expression products of germline-associated genes of *Vasa, Nanos, Piwi, Tudor, Pumilio,*

and *Bruno* families. In invertebrates, non-differentiated cells are also typically marked by high expression of PIWI/piRNA genes, which ensures genome stability (Tiozzo and Copley, 2015; Lai and Aboobaker, 2018).

In more complex triploblastic animals, e.g., tailed amphibians, the pluripotency is restricted even further. These animals lack a reserve of pluripotent cells, which emerge during regeneration as a result of dedifferentiation and transdifferentiation of the pre-existing differentiated cells (Alvarado and Tsonis, 2006; Brockes and Kumar, 2008; Li et al., 2015). In tailless amphibians and salamanders, the potency of accumulating non-differentiated cells in response to injury is dramatically reduced or restricted to the larval stages (Agata and Inoue, 2012). Relative contributions of dedifferentiation and transdifferentiation to regeneration remain disputable, partly due to the pluralism of definitions for these processes in different settings (Galliot and Ghila, 2010). The majority of experts agree that dedifferentiation and transdifferentiation characteristically occur during regeneration in *Hydra*, as well as during Wolffian regeneration of the lens in Caudata (Galliot and Ghila, 2010; Henry and Hamilton, 2018). Transdifferentiation of coelomic epithelial cells into enterocytes can be observed during regeneration in sea cucumbers (Dolmatov et al., 2019; Boyko et al., 2020). At the same time, the cells of regenerating limbs in tailed amphibians have been shown to retain their key differentiation determinants (Kragl et al., 2009; Slack, 2017).

According to a number of authors, the ability of cells to return to the cell cycle is closely related to the concept of cell plasticity (Galliot and Ghila, 2010). In the course of evolution in some animals, the regulation of the cell cycle became more complicated, the appearance of additional checkpoints, which in turn could cause a decrease in the regenerative capacity.

In the course of a comparative study of the mechanisms of regulation of the cell cycle, it was found that 23 cyclins are encoded in the *Saccharomyces cerevisiae* genome, which regulates six proline-directed serine/threonine protein kinases. Cdc28 is required for driving the cell cycle. The multifunctional kinase Pho85 regulates G1 progression and other intracellular processes. In humans, 13 members of the CDK-family (cyclin-dependent kinase) have been found to interact with 29 cyclins and cyclin-related proteins (Malumbres and Barbacid, 2005). A family of five proteins (known as Ringo or Speedy) has been found in vertebrates but not in *S. cerevisiae*, *Caenorhabditis elegans*, or *Drosophila melanogaster* (Nebreda, 2006).

It has been found that CDK7, CDK8, and CDK9 are not very different from their yeast orthologs. CDK4 and CDK6 first appeared in multicellular organisms. The increased number of cyclins in the mammalian genome has resulted in a large variety of CDK–cyclin complexes. However, only 10 cyclins (three D-type, two E-type, two A-type, and three B-type cyclins) are known to be directly involved in driving the mammalian cell cycle (Malumbres and Barbacid, 2009).

The control of the mitotic cycle in the nuclei of muscle fibers in Anamnia and mammals is carried out with the involvement of different amounts of regulatory proteins. It was found that in non-amniotic vertebrates, one *INK4* gene functions, which is responsible for the synthesis of cyclin-dependent kinase inhibitor 2 (p16Ink4). At the same time, mammals have two *Ink4* genes (*Ink4a* which produces p16INK4a, and ARF, and *Ink4b* which produces p15INK4b). p16INK4a and p15INK4b block cyclin-dependent kinases 4 and 6 (CDK4,6) activity under normal conditions. In mammals, there is an additional mechanism of inhibition of the cell cycle re-entry by alternate open reading frame (ARF) through tumor protein p53. Under normal conditions, maintenance of chromosomes 2 (MCM2) ubiquitinates p53 and targets it for destruction (Seifert et al., 2012).

Despite the limitations in proliferative potential and phenotypic plasticity, mammalian tissues present with certain examples of dedifferentiation. However, these examples are most often associated with pathological processes, to leave alone tumorigenesis. For example, under conditions of severe viral or toxic liver damage, cholangiocytes are prone to dedifferentiation, with subsequent redifferentiation to cholangiocytes or transdifferentiation to hepatocytes (Michalopoulos, 2011). Another effect of viral or toxic liver damage on cell differentiation status is the loss of lipid droplets by Ito cells and their transition to myofibroblasts (Unanue, 2007).

CONCLUSION

In the course of the evolution of certain animal taxa, more and more checkpoints were added to the regulation of the cell cycle and exit from it. These checkpoints are maintained by the expanded system of cyclins and cyclin-dependent kinases with associated gene-and-protein networks and circuits (Malumbres and Barbacid, 2009; Seifert et al., 2012). The establishment of complex multilevel control of the mitotic cycle was inevitably coupled to enhanced control of the differentiation status; this association represents a major cause for the decline in regenerative capacity in vertebrates. An eventual increase in the activity of metabolic processes in warm-blooded animals allowed neither the preservation of non-differentiated cells in sufficiently high numbers nor the massive waves of dedifferentiation fraught with tumorigenesis (Sánchez Alvarado, 2000; Li et al., 2015).

Regeneration is a complex and diversified process inherent to the life at different levels of its organization. For obvious reasons, morphologically advanced cases of regeneration (such as restoration of the entire body from a fragment or regeneration of amputated limbs) draw more attention than others. As a consequence, a limited number of regeneration model organisms are used for research: zebrafish, newts, hydra, and planaria. In this case, the same type of damage is very often used—amputation, which narrows our understanding of regeneration and its evolution. Almost nothing is known about the mechanisms of regeneration in such animals after toxic damage, viral or bacterial. This is often considered in the relevant sections of microbiology, toxicology, and is not taken into account by regeneration researchers.

The evolution of regeneration can be studied by various approaches (Vorontsova and Liosner, 1960; Bely and Nyberg, 2010). The methodology involves a reduction of the phenomenon

to particular events assigned to different levels of the organization and classified accordingly, with appropriate accounting for their relative contributions in a single model. Moreover, it is obvious that the evolution of regeneration is not a unidirectional process. Despite a major trend of the decline in regenerative capacity with the increasing organizational complexity, the phenomenon is modified in a variety of ways and never completely eliminated. For instance, mammals, who have suffered a pronounced phylogenetic decline in regenerative capacity, are capable of restoring neither amputated limbs nor other external appendages (the repair is limited to wound healing). At the same time, regeneration of certain organs and structures in mammals is morphologically consistent and results in complete functional recovery; characteristic examples include the restoration of the auricle tissue after a perforating wound (Williams-Boyce and Daniel, 1986) and restoration of the liver mass after massive resections (Bangru and Kalsotra, 2020).

Evolutionary studies on regeneration involve overcoming certain biases. Regrettably, the studies on regenerative capacity are still linked to a limited number of animal models and species. Importantly, in natural habitats, the organs may be damaged by disease rather than mechanically, which dramatically affects the course of regeneration. Regeneration of pathologically altered organs has been experimentally studied in mammals; for other animal taxa, the corresponding data are fragmentary or missing.

AUTHOR CONTRIBUTIONS

AE, GS, and TF contributed the text. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by the Russian Science Foundation (Grant No. 17-15-01419).

REFERENCES

Adams, D. S., Masi, A., and Levin, M. (2007). H+ pump-dependent changes in membrane voltage are an early mechanism necessary and sufficient to induce *Xenopus* tail regeneration. *Development* 134, 1323–1335. doi: 10.1242/dev.02812

Adell, T., Saló, E., Van Loon, J. J. W. A., and Auletta, G. (2014). Planarians sense simulated microgravity and hypergravity. *Biomed Res. Int.* 2014:679672. doi: 10.1155/2014/679672

Agata, K., and Inoue, T. (2012). Survey of the differences between regenerative and non-regenerative animals. *Dev. Growth Differ.* 54, 143–152. doi: 10.1111/j.1440-169X.2011.01323.x

Agata, K., Saito, Y., and Nakajima, E. (2007). Unifying principles of regeneration I: epimorphosis versus morphallaxis. *Dev. Growth Differ.* 49, 73–78. doi: 10.1111/j.1440-169X.2007.00919.x

Alvarado, A. S., and Tsonis, P. A. (2006). Bridging the regeneration gap: genetic insights from diverse animal models. *Nat. Rev. Genet.* 7, 873–884. doi: 10.1038/nrg1923

Babaeva, A. G. (1989). Cellular and humoral immunity factors as regulators of regenerative morphogenesis. *Ontogenez* 20, 453–460.

Babaeva, A. G. (1990). Limfotsity kak reguliatory proliferatsii i differentsirovki kletok nelimfoidnykh organov. *Vestn. Akad. Med. Nauk. SSSR* 2, 43–45.

Bando, T., Mito, T., Hamada, Y., Ishimaru, Y., Noji, S., and Ohuchi, H. (2018). Molecular mechanisms of limb regeneration: insights from regenerating legs of the cricket gryllus bimaculatus. *Int. J. Dev. Biol.* 62, 559–569. doi: 10.1387/ijdb.180048ho

Bangru, S., and Kalsotra, A. (2020). Cellular and molecular basis of liver regeneration. *Semin. Cell Dev. Biol.* 100, 74–87. doi: 10.1016/j.semcdb.2019.12.004

Barr, J. I., Boisvert, C. A., Somaweera, R., Trinajstic, K., and Bateman, P. W. (2019). Re-regeneration to reduce negative effects associated with tail loss in lizards. *Sci. Rep.* 9:18717. doi: 10.1038/s41598-019-55231-6

Bateman, P. W., Fleming, P. A., Bateman, P. W., and Bennett, N. (2008). To cut a long tail short: a review of lizard caudal autotomy studies carried out over the last 20 years. *J. Zool.* 277, 1–14. doi: 10.1111/j.1469-7998.2008.00484.x

Baumiller, T. K., and Gahn, F. J. (2004). Testing predator-driven evolution with *Paleozoic crinoid* arm regeneration. *Science* 305, 1453–1455. doi: 10.1126/science.1101009

Bely, A. E. (2006). Distribution of segment regeneration ability in the *Annelida*. *Integr. Comp. Biol.* 46, 508–518. doi: 10.1093/icb/icj051

Bely, A. E. (2010). Evolutionary loss of animal regeneration: pattern and process. *Integr. Comp. Biol.* 50, 515–527. doi: 10.1093/icb/icq118

Bely, A. E., and Nyberg, K. G. (2010). Evolution of animal regeneration: re-emergence of a field. *Trends Ecol. Evol.* 25, 161–170. doi: 10.1016/j.tree.2009.08.005

Bely, A. E., and Sikes, J. M. (2010). Latent regeneration abilities persist following recent evolutionary loss in asexual annelids. *Proc. Natl. Acad. Sci. U.S.A.* 107, 1464–1469. doi: 10.1073/pnas.0907931107

Bely, A. E., and Wray, G. A. (2001). Evolution of regeneration and fission in annelids: insights from engrailed- and orthodenticle-class gene expression. *Development* 128, 2781–2791.

Bely, A. E., Zattara, E. E., and Sikes, J. M. (2014). Regeneration in spiralians: evolutionary patterns and developmental processes. *Int. J. Dev. Biol.* 58, 623–634. doi: 10.1387/ijdb.140142ab

Ben Khadra, Y., Sugni, M., Ferrario, C., Bonasoro, F., Oliveri, P., Martinez, P., et al. (2018). "Regeneration in stellate echinoderms: Crinoidea, Asteroidea and Ophiuroidea," in *Results and Problems in Cell Differentiation*, eds M. Kloc and J. Kubiak (Cham: Springer), 285–320. doi: 10.1007/978-3-319-92486-1_14

Bernardo, J., and Agosta, S. J. (2005). Evolutionary implications of hierarchical impacts of nonlethal injury on reproduction, including maternal effects. *Biol. J. Linn. Soc.* 86, 309–331. doi: 10.1111/j.1095-8312.2005.00532.x

Bertemes, P., Grosbusch, A. L., and Egger, B. (2020). No head regeneration here: regeneration capacity and stem cell dynamics of *Theama mediterranea* (Polycladida, Platyhelminthes). *Cell Tissue Res.* 379, 301–321. doi: 10.1007/s00441-019-03094-8

Blacher, L. J., Irichimowitsch, A. I., Liosner, L. D., and Woronzowa, M. A. (1933). Resorptionsprozesse als quelle der formbildung - IX. Einfluss der mitogenetischen Strahlen auf die Geschwindigkeit der regeneration. *Wilhelm Roux. Arch. Entwickl. Mech. Org.* 127, 339–352. doi: 10.1007/BF01390722

Bolker, J. A. (2000). Modularity in development and why it matters to evo-devo. *Am. Zool.* 40, 770–776. doi: 10.1093/icb/40.5.770

Bolshakova, G. B. (2008). Cardiomyocyte proliferation in rat fetuses under normal conditions and after heart injury. *Bull. Exp. Biol. Med.* 145, 487–489. doi: 10.1007/s10517-008-0125-3

Bosch, T. C. G. (2007). Why polyps regenerate and we don't: towards a cellular and molecular framework for Hydra regeneration. *Dev. Biol.* 303, 421–433. doi: 10.1016/j.ydbio.2006.12.012

Boyko, A. V., Girich, A. S., Tkacheva, E. S., and Dolmatov, I. Y. (2020). The *Eupentacta fraudatrix* transcriptome provides insights into regulation of cell transdifferentiation. *Sci. Rep.* 10:1522. doi: 10.1038/s41598-020-58470-0

Brant, J. O., Yoon, J. H., Polvadore, T., Barbazuk, W. B., and Maden, M. (2016). Cellular events during scar-free skin regeneration in the spiny mouse, *Acomys*. *Wound Repair Regen.* 24, 75–88. doi: 10.1111/wrr.12385

Brautigam, S. E., and Persons, M. H. (2003). The effect of limb loss on the courtship and mating behavior of the wolf spider *Pardosa milvina*

(Araneae: Lycosidae). *J. Insect Behav.* 16, 571–587. doi: 10.1023/A:1027311625059

Brockes, J. P., and Kumar, A. (2008). Comparative aspects of animal regeneration. *Annu. Rev. Cell Dev. Biol.* 24, 525–549. doi: 10.1146/annurev.cellbio.24.110707.175336

Brodsky, W. Y., and Uryvaeva, I. V. (1977). Cell polyploidy: its relation to tissue growth and function. *Int. Rev. Cytol.* 50, 275–332. doi: 10.1016/S0074-7696(08)60100-X

Burton, P. M., and Finnerty, J. R. (2009). Conserved and novel gene expression between regeneration and asexual fission in *Nematostella vectensis*. *Dev. Genes Evol.* 219, 79–87. doi: 10.1007/s00427-009-0271-2

Cao, P.-L., Kumagai, N., Inoue, T., Agata, K., and Makino, T. (2019). JmjC domain-encoding genes are conserved in highly regenerative metazoans and are associated with planarian whole-body regeneration. *Genome Biol. Evol.* 11, 552–564. doi: 10.1093/gbe/evz021

Carlson, B. M. (2005). Some principles of regeneration in mammalian systems. *Anat. Rec. B. New Anat.* 287, 4–13. doi: 10.1002/ar.b.20079

Carlson, B. M. (2007). *Principles of Regenerative Biology*, 1st Edn, Michigan: Elsevier Inc.

Cary, G. A., Wolff, A., Zueva, O., Pattinato, J., and Hinman, V. F. (2019). Analysis of sea star larval regeneration reveals conserved processes of whole-body regeneration across the metazoa. *BMC Biol.* 17:16. doi: 10.1186/s12915-019-0633-9

Chera, S., Ghila, L., Dobretz, K., Wenger, Y., Bauer, C., Buzgariu, W., et al. (2009). Apoptotic cells provide an unexpected source of Wnt3 signaling to drive hydra head regeneration. *Dev. Cell* 17, 279–289. doi: 10.1016/j.devcel.2009.07.014

Christen, B., Beck, C. W., Lombardo, A., and Slack, J. M. W. (2003). Regeneration-specific expression pattern of three posterior *Hox* genes. *Dev. Dyn.* 226, 349–355. doi: 10.1002/dvdy.10231

Clark, M. S., Dupont, S., Rossetti, H., Burns, G., Thorndyke, M. C., and Peck, L. S. (2007). Delayed arm regeneration in the antarctic brittle star *Ophionotus victoriae*. *Aquat. Biol.* 1, 45–53. doi: 10.3354/ab00004

Cotter, J. D., Storfer, A., Page, R. B., Beachy, C. K., and Voss, S. R. (2008). Transcriptional response of Mexican axolotls to *Ambystoma tigrinum* virus (ATV) infection. *BMC Genom.* 9:493. doi: 10.1186/1471-2164-9-493

Darnet, S., Dragalzew, A. C., Amaral, D. B., Sousa, J. F., Thompson, A. W., Cass, A. N., et al. (2019). Deep evolutionary origin of limb and fin regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 116, 15106–15115. doi: 10.1073/pnas.1900475116

Das, S. (2015). Morphological, molecular, and hormonal basis of limb regeneration across pancrustacea. *Integr. Compar. Biol.* 55, 869–877. doi: 10.1093/icb/icv101

Davydov, K. N. (1903). Observations of the regeneration processes at *Enteropneumata*. *Notes Acad. Sci.* 22, 1–10.

Dolmatov, I. Y., Shulga, A. P., Ginanova, T. T., Eliseikina, M. G., and Lamash, N. E. (2019). Metalloproteinase inhibitor GM6001 delays regeneration in holothurians. *Tissue Cell* 59, 1–9. doi: 10.1016/j.tice.2019.05.006

Dupont, S., and Thorndyke, M. C. (2006). Growth or differentiation? Adaptive regeneration in the brittlestar *Amphiura filiformis*. *J. Exp. Biol.* 209, 3873–3881. doi: 10.1242/jeb.02445

Elchaninov, A., Lokhonina, A., Nikitina, M., Vishnyakova, P., Makarov, A., Arutyunyan, I., et al. (2020). Comparative analysis of the transcriptome, proteome, and miRNA profile of kupffer cells and monocytes. *Biomedicines* 8:627. doi: 10.3390/biomedicines8120627

Elchaninov, A. V., and Bolshakova, G. B. (2011a). Dynamics of hepatocyte proliferation in regenerating fetal rat liver. *Bull. Exp. Biol. Med.* 151, 374–377. doi: 10.1007/s10517-011-1334-8

Elchaninov, A. V., and Bolshakova, G. B. (2011b). Reparative regeneration of rat fetal liver after partial hepatectomy. *Bull. Exp. Biol. Med.* 150, 383–386. doi: 10.1007/s10517-011-1148-8

Elchaninov, A. V., and Bolshakova, G. B. (2012). Proliferation and cell death of hepatocytes in regenerating fetal rat liver. *Cell Tissue Biol.* 6, 485–489. doi: 10.1134/S1990519X12050070

Elchaninov, A. V., Fatkhudinov, T. K., Usman, N. Y., Kananykhina, E. Y., Arutyunyan, I. V., Makarov, A. V., et al. (2018). Dynamics of macrophage populations of the liver after subtotal hepatectomy in rats. *BMC Immunol.* 19:23. doi: 10.1186/s12865-018-0260-1

Elchaninov, A. V., Fatkhudinov, T. K., Vishnyakova, P. A., Lokhonina, A. V., and Sukhikh, G. T. (2019). Phenotypical and functional polymorphism of liver resident macrophages. *Cells* 8:32. doi: 10.3390/cells8091032

Elliott, S. A., and Sánchez Alvarado, A. (2013). The history and enduring contributions of planarians to the study of animal regeneration. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 301–326. doi: 10.1002/wdev.82

Epelman, S., Lavine, K. J., and Randolph, G. J. (2014). Origin and functions of tissue macrophages. *Immunity* 41, 21–35. doi: 10.1016/j.immuni.2014.06.013

Ferrario, C., Ben Khadra, Y., Czarkwiani, A., Zakrzewski, A., Martinez, P., Colombo, G., et al. (2018). Fundamental aspects of arm repair phase in two echinoderm models. *Dev. Biol.* 433, 297–309. doi: 10.1016/j.ydbio.2017.09.035

Fox, S. F., and McCoy, J. K. (2000). The effects of tail loss on survival, growth, reproduction, and sex ratio of offspring in the lizard *Uta stansburiana* in the field. *Oecologia* 122, 327–334. doi: 10.1007/s004420050038

Fukazawa, T., Naora, Y., Kunieda, T., and Kubo, T. (2009). Suppression of the immune response potentiates tadpole tail regeneration during the refractory period. *Development* 136, 2323–2327. doi: 10.1242/dev.033985

Fumagalli, M. R., Zapperi, S., and La Porta, C. A. M. (2018). Regeneration in distantly related species: common strategies and pathways. *NPJ Syst. Biol. Appl.* 4:5. doi: 10.1038/s41540-017-0042-z

Galis, F., Wagner, G. P., and Jockusch, E. L. (2003). Why is limb regeneration possible in amphibians but not in reptiles, birds, and mammals? *Evol. Dev.* 5, 208–220. doi: 10.1046/j.1525-142X.2003.03028.x

Galliot, B., and Ghila, L. (2010). Cell plasticity in homeostasis and regeneration. *Mol. Reprod. Dev.* 77, 837–855. doi: 10.1002/mrd.21206

Garza-Garcia, A. A., Driscoll, P. C., and Brockes, J. P. (2010). Evidence for the local evolution of mechanisms underlying limb regeneration in salamanders. *Integr. Comp. Biol.* 50, 528–535. doi: 10.1093/icb/icq022

Geng, J., Gates, P. B., Kumar, A., Guenther, S., Garza-Garcia, A., Kuenne, C., et al. (2015). Identification of the orphan gene Prod 1 in basal and other salamander families. *Evodevo* 6:9. doi: 10.1186/s13227-015-0006-6

Ghosh, S., Roy, S., Séguin, C., Bryant, S. V., and Gardiner, D. M. (2008). Analysis of the expression and function of Wnt-5a and Wnt-5b in developing and regenerating axolotl (*Ambystoma mexicanum*) limbs. *Dev. Growth Differ.* 50, 289–297. doi: 10.1111/j.1440-169X.2008.01000.x

Godwin, J. W., Pinto, A. R., and Rosenthal, N. A. (2017). Chasing the recipe for a pro-regenerative immune system. *Semin. Cell Dev. Biol.* 61, 71–79. doi: 10.1016/j.semcdb.2016.08.008

Godwin, J. W., and Rosenthal, N. (2014). Scar-free wound healing and regeneration in amphibians: immunological influences on regenerative success. *Differentiation* 87, 66–75. doi: 10.1016/j.diff.2014.02.002

Goss, R. J. (1969). *Principles of Regeneration*, 1st Edn, New York, NY: Academic Press.

Goss, R. J. (1992). The evolution of regeneration: adaptive or inherent? *J. Theor. Biol.* 159, 241–260. doi: 10.1016/S0022-5193(05)80704-0

Grigoryan, E. N. (2016). High regenerative ability of tailed amphibians (*Urodela*) as a result of the expression of juvenile traits by mature animals. *Russ. J. Dev. Biol.* 47, 83–92. doi: 10.1134/S1062360416020041

Guder, C., Pinho, S., Nacak, T. G., Schmidt, H. A., Hobmayer, B., Niehrs, C., et al. (2006). An ancient Wnt-dickkopf antagonism in Hydra. *Development* 133, 901–911. doi: 10.1242/dev.02265

Gurley, K. A., Rink, J. C., and Sánchez Alvarado, A. (2008). Beta-catenin defines head versus tail identity during planarian regeneration and homeostasis. *Science* 319, 323–327. doi: 10.1126/science.1150029

Han, M., Yang, X., Farrington, J. E., and Muneoka, K. (2003). Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. *Development* 130, 5123–5132. doi: 10.1242/dev.00710

Han, M., Yang, X., Lee, J., Allan, C. H., and Muneoka, K. (2008). Development and regeneration of the neonatal digit tip in mice. *Dev. Biol.* 315, 125–135. doi: 10.1016/j.ydbio.2007.12.025

Henry, J. J., and Hamilton, P. W. (2018). Diverse evolutionary origins and mechanisms of lens regeneration. *Mol. Biol. Evol.* 35, 1563–1575. doi: 10.1093/molbev/msy045

Karami, A., Tebyanian, H., Goodarzi, V., and Shiri, S. (2015). Planarians: an in vivo model for regenerative medicine. *Int. J. Stem Cells* 8, 128–133. doi: 10.15283/ijsc.2015.8.2.128

Kawamura, K., Sugino, Y., Sunanaga, T., and Fujiwara, S. (2008). Multipotent epithelial cells in the process of regeneration and asexual reproduction in colonial tunicates. *Dev. Growth Differ.* 50, 1–11. doi: 10.1111/j.1440-169X.2007.00972.x

Korotkova, G. P. (1988). Integratsionnye mekhanizmy i morfogenez (k probleme évoliutsii ontogeneza). *Zh. Obshch. Biol.* 49, 464–475.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., et al. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* 460, 60–65. doi: 10.1038/nature08152

Lai, A. G., and Aboobaker, A. A. (2018). EvoRegen in animals: time to uncover deep conservation or convergence of adult stem cell evolution and regenerative processes. *Dev. Biol.* 433, 118–131. doi: 10.1016/j.ydbio.2017.10.010

Lailvaux, S. P., Reaney, L. T., and Backwell, P. R. Y. (2009). Dishonest signalling of fighting ability and multiple performance traits in the fiddler crab *Uca mjoebergi*. *Funct. Ecol.* 23, 359–366. doi: 10.1111/j.1365-2435.2008.01501.x

Li, Q., Yang, H., and Zhong, T. P. (2015). Regeneration across metazoan phylogeny: lessons from model organisms. *J. Genet. Genom.* 42, 57–70. doi: 10.1016/j.jgg.2014.12.002

Liosner, L. D., Woronzowa, M. A., and Kusmina, N. A. (1936). Regenerationspotenz der Knochenlosen Extremität. *Wilhelm Roux. Arch. Entwickl. Mech. Org.* 134, 738–750. doi: 10.1007/BF00576070

Liozner, L. D. (1975). Ob évoliutsii regeneratsionnoĭ sposobnosti zhivotnykh. *Usp. Sovrem. Biol.* 79, 459–467.

Liozner, L. D. (ed.) (1974). *Organ Regeneration: A Study of Developmental Biology in Mammals (Studies in Soviet Science)*, 1st Edn, Berlin: Springer, doi: 10.1007/978-1-4684-8456-4

Liu, S.-Y., Selck, C., Friedrich, B., Lutz, R., Vila-Farré, M., Dahl, A., et al. (2013). Reactivating head regrowth in a regeneration-deficient planarian species. *Nature* 500, 81–84. doi: 10.1038/nature12414

Maden, M. (2018). The evolution of regeneration - Where does that leave mammals? *Int. J. Dev. Biol.* 62, 369–372. doi: 10.1387/ijdb.180031mm

Maginnis, T. L. (2006). The costs of autotomy and regeneration in animals: a review and framework for future research. *Behav. Ecol.* 17, 857–872. doi: 10.1093/beheco/arl010

Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. *Trends Biochem. Sci.* 30, 630–641. doi: 10.1016/j.tibs.2005.09.005

Malumbres, M., and Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nat. Rev. Cancer* 9, 153–166. doi: 10.1038/nrc2602

Martin, P., and Parkhurst, S. M. (2004). Parallels between tissue repair and embryo morphogenesis. *Development* 131, 3021–3034. doi: 10.1242/dev.01253

Martindale, M. Q., and Henry, J. Q. (1995). Modifications of cell fate specification in equal-cleaving nemertean embryos: alternate patterns of Spiralian development. *Development* 121, 3175–3185.

Martínez, D. E., and Bridge, D. (2012). Hydra, the everlasting embryo, confronts aging. *Int. J. Dev. Biol.* 56, 479–487. doi: 10.1387/ijdb.113461dm

Martinez, V. G., Menger, G. J., and Zoran, M. J. (2005). Regeneration and asexual reproduction share common molecular changes: upregulation of a neural glycoepitope during morphallaxis in *Lumbriculus*. *Mech. Dev.* 122, 721–732. doi: 10.1016/j.mod.2004.12.003

Maruzzo, D., and Bortolin, F. (2013). "Arthropod regeneration," in *Arthropod Biology and Evolution: Molecules, Development, Morphology*, ed. A. Minelli (Berlin: Springer-Verlag), 149–169. doi: 10.1007/978-3-642-36160-9_7

Mcgaw, I. J. (2006). Cardiovascular and respiratory responses associated with limb autotomy in the blue crab, *Callinectes sapidus*. *Mar. Freshw. Behav. Physiol.* 39, 131–141. doi: 10.1080/10236240600563321

Mehta, A. S., and Singh, A. (2019). Insights into regeneration tool box: an animal model approach. *Dev. Biol.* 453, 111–129. doi: 10.1016/j.ydbio.2019.04.006

Mescher, A. L., Neff, A. W., and King, M. W. (2017). Inflammation and immunity in organ regeneration. *Dev. Comp. Immunol.* 66, 98–110. doi: 10.1016/j.dci.2016.02.015

Michalopoulos, G. K. (2011). Liver regeneration: alternative epithelial pathways. *Int. J. Biochem. Cell Biol.* 43, 173–179. doi: 10.1016/j.biocel.2009.09.014

Mitashov, V. I. (1996). Mechanisms of retina regeneration in *Urodeles*. *Int. J. Dev. Biol.* 40, 833–844. doi: 10.1387/ijdb.8877458

Mito, T., Inoue, Y., Kimura, S., Miyawaki, K., Niwa, N., Shinmyo, Y., et al. (2002). Involvement of hedgehog, wingless, and dpp in the initiation of proximodistal axis formation during the regeneration of insect legs, a verification of the modified boundary model. *Mech. Dev.* 114, 27–35. doi: 10.1016/S0925-4773(02)00052-7

Monaghan, J. R., Stier, A. C., Michonneau, F., Smith, M. D., Pasch, B., Maden, M., et al. (2014). Experimentally induced metamorphosis in axolotls reduces regenerative rate and fidelity. *Regeneration* 1, 2–14. doi: 10.1002/reg2.8

Morgan, T. (1901). *Regeneration*. New York: Macmillan.

Morgan, T. H. (1901). Regeneration and liability to injury. *Science* 14, 235–248. doi: 10.1126/science.14.346.235

Muneoka, K., and Dawson, L. A. (2020). Evolution of epimorphosis in mammals. *J. Exp. Zool. Part B Mol. Dev. Evol.* 2020:jez.b.22925. doi: 10.1002/jez.b.22925

Murawala, P., Tanaka, E. M., and Currie, J. D. (2012). Regeneration: the ultimate example of wound healing. *Semin. Cell Dev. Biol.* 23, 954–962. doi: 10.1016/j.semcdb.2012.09.013

Naya, D. E., Veloso, C., Muñoz, J. L. P., and Bozinovic, F. (2007). Some vaguely explored (but not trivial) costs of tail autotomy in lizards. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 146, 189–193. doi: 10.1016/j.cbpa.2006.10.014

Nebreda, A. R. (2006). CDK activation by non-cyclin proteins. *Curr. Opin. Cell Biol.* 18, 192–198. doi: 10.1016/j.ceb.2006.01.001

Needham, A. E. (1952). *Regeneration and Wound Healing*, 1st Edn, London: Methuen.

Odelberg, S. J. (2004). Unraveling the molecular basis for regenerative cellular plasticity. *PLoS Biol.* 2:20232. doi: 10.1371/journal.pbio.0020232

Odelberg, S. J. (2005). Cellular plasticity in vertebrate regeneration. *Anat. Rec. Part B New Anat.* 287, 25–35. doi: 10.1002/ar.b.20080

Polezhaev, L. W. (1946). The loss and restoration of regenerative capacity in the limbs of tailless amphibia. *Biol. Rev.* 21, 141–147. doi: 10.1111/j.1469-185X.1946.tb00319.x

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., et al. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* 331, 1078–1080. doi: 10.1126/science.1200708

Poss, K. D. (2010). Advances in understanding tissue regenerative capacity and mechanisms in animals. *Nat. Rev. Genet.* 11, 710–722. doi: 10.1038/nrg2879

Raff, R. (1996). *The Shape of Life: Genes, Development, and the Evolution of Animal Form*, 1st Edn, Chicago: University of Chicago Press.

Ramos, M., Irschick, D. J., and Christenson, T. E. (2004). Overcoming an evolutionary conflict: removal of a reproductive organ greatly increases locomotor performance. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4883–4887. doi: 10.1073/pnas.0400324101

Reaumur, R.-A. F. D. (1712). Sur les diverses reproductions qui se fontdans les Ecrivisses, les Omars, les Crabes, etc. et entre Autressur celles de leurs jambes et de *Leurs ecailles*. *Mem. R. Sci.* 12, 226–245.

Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R., and Sánchez Alvarado, A. (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in *Planaria*. *Dev. Cell* 8, 635–649. doi: 10.1016/j.devcel.2005.02.014

Reddy, P. C., Gungi, A., Ubhe, S., Pradhan, S. J., Kolte, A., and Galande, S. (2019a). Molecular signature of an ancient organizer regulated by Wnt/β-catenin signalling during primary body axis patterning in Hydra. *Commun. Biol.* 2:434. doi: 10.1038/s42003-019-0680-3

Reddy, P. C., Gungi, A., and Unni, M. (2019b). Cellular and molecular mechanisms of hydra regeneration. *Results Prob. Cell Differ.* 68, 259–290. doi: 10.1007/978-3-030-23459-1_12

Reinhardt, B., Broun, M., Blitz, I. L., and Bode, H. R. (2004). HyBMP5-8b, a BMP5-8 orthologue, acts during axial patterning and tentacle formation in hydra. *Dev. Biol.* 267, 43–59. doi: 10.1016/j.ydbio.2003.10.031

Robert, J., and Ohta, Y. (2009). Comparative and developmental study of the immune system in *Xenopus*. *Dev. Dyn.* 238, 1249–1270. doi: 10.1002/dvdy.21891

Sánchez Alvarado, A. (2000). Regeneration in the metazoans: why does it happen? *Bioessays* 22, 578–590.

Sattler, S., and Rosenthal, N. (2016). The neonate versus adult mammalian immune system in cardiac repair and regeneration. *Biochim. Biophys. Acta Mol. Cell Res.* 183, 1813–1821. doi: 10.1016/j.bbamcr.2016.01.011

Seifert, A. W., Monaghan, J. R., Smith, M. D., Pasch, B., Stier, A. C., Michonneau, F., et al. (2012). The influence of fundamental traits on mechanisms controlling appendage regeneration. *Biol. Rev.* 87, 330–345. doi: 10.1111/j.1469-185X.2011.00199.x

Seifert, A. W., and Muneoka, K. (2018). The blastema and epimorphic regeneration in mammals. *Dev. Biol.* 433, 190–199. doi: 10.1016/j.ydbio.2017.08.007

Sidorova, V. F. (1964). Ob Osobennostiakh Vosstanovleniia i postnatal'nogo rosta nekotroykh. *Usp. Sovrem. Biol.* 57, 283–299.

Sidorova, V. F. (1978). *The Postnatal Growth and Restoration of Internal Organs in Vertebrates*. Littleton, MA: PSG Pub. Co.

Slack, J. M. (2017). Animal regeneration: ancestral character or evolutionary novelty? *EMBO Rep.* 18, 1497–1508. doi: 10.15252/embr.201643795

Sunderland, M. E. (2010). Regeneration: Thomas Hunt Morgan's window into development. *J. Hist. Biol.* 43, 325–361. doi: 10.1007/s10739-009-9203-2

Suzuki, Y., Chou, J., Garvey, S. L., Wang, V. R., and Yanes, K. O. (2019). Evolution and regulation of limb regeneration in arthropods. *Results Probl. Cell Differ.* 68, 419–454. doi: 10.1007/978-3-030-23459-1_17

Tiozzo, S., and Copley, R. R. (2015). Reconsidering regeneration in metazoans: an evo-devo approach. *Front. Ecol. Evol.* 3:67. doi: 10.3389/fevo.2015.00067

Tokin, B. P. (1969). Asexual reproduction, somatic embryogenesis and regeneration. *Zh. Obs. Biol.* 30, 18–32.

Tremblay, A. (1744). *Mémoires Pour Servir à l'histoire d'un Genre de Polypes d'eau Douce à bras en Forme de Cornes*, 1st Edn, eds C. Jean and H. Verbeek Leyde (London: Nabu Press).

Unanue, E. R. (2007). Ito cells, stellate cells, and myofibroblasts: new actors in antigen presentation. *Immunity* 26, 9–10. doi: 10.1016/j.immuni.2007.01.001

Vivien, C. J., Hudson, J. E., and Porrello, E. R. (2016). Evolution, comparative biology and ontogeny of vertebrate heart regeneration. *NPJ Regen. Med.* 1:12. doi: 10.1038/npjregenmed.2016.12

Vogg, M. C., Beccari, L., Iglesias Ollé, L., Rampon, C., Vriz, S., Perruchoud, C., et al. (2019). An evolutionarily-conserved Wnt3/β-catenin/Sp5 feedback loop restricts head organizer activity in Hydra. *Nat. Commun.* 10:312. doi: 10.1038/s41467-018-08242-2

Vorontsova, M., and Liosner, L. (1960). *Asexual Propagation and Regeneration*. New York, NY: Pergamon Press.

Weismann, À (1893). *The Germ-Plasm. A Theory of Heredity*. New York, NY: Charles Scribner's Sons.

Weismann, À (1899). Tatsachen und auslegungen in bezug auf regeneration. *Anat. Anz.* 23:456.

Whitehead, G. G., Makino, S., Lien, C.-L., and Keating, M. T. (2005). Fgf20 Is essential for initiating Zebrafish fin regeneration supporting online material. *Science* 310, 1957–1960. doi: 10.1126/science.1117637

Williams-Boyce, P. K., and Daniel, J. C. (1986). Comparison of ear tissue regeneration in mammals. *J. Anat.* 149, 55–63.

Wittlieb, J., Khalturin, K., Lohmann, J. U., Anton-Erxleben, F., and Bosch, T. C. G. (2006). Transgenic Hydra allow in vivo tracking of individual stem cells during morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6208–6211. doi: 10.1073/pnas.0510163103

Wulff, J. L. (2006). Resistance vs recovery: morphological strategies of coral reef sponges. *Funct. Ecol.* 20, 699–708. doi: 10.1111/j.1365-2435.2006.01143.x

Zattara, E. E., and Bely, A. E. (2011). Evolution of a novel developmental trajectory: fission is distinct from regeneration in the annelid *Pristina leidyi*. *Evol. Dev.* 13, 80–95. doi: 10.1111/j.1525-142X.2010.00458.x

Zattara, E. E., and Bely, A. E. (2013). Investment choices in post-embryonic development: quantifying interactions among growth, regeneration, and asexual reproduction in the annelid *Pristina leidyi*. *J. Exp. Zool. Part B Mol. Dev. Evol.* 320, 471–488. doi: 10.1002/jez.b.22523

Zattara, E. E., and Bely, A. E. (2016). Phylogenetic distribution of regeneration and asexual reproduction in *Annelida*: regeneration is ancestral and fission evolves in regenerative clades. *Invertebr. Biol.* 135, 400–414. doi: 10.1111/ivb.12151

Zattara, E. E., Fernandez-Alvarez, F. A., Hiebert, T. C., Bely, A. E., and Norenburg, J. L. (2019). A phylum-wide survey reveals multiple independent gains of head regeneration in nemertea. *Proc. R. Soc. B Biol. Sci.* 286:20182524. doi: 10.1098/rspb.2018.2524

Zavarzin, A. A. (1938). Studies in comparative histology of muscle tissue. Regeneration of somatic muscles in fish (cottus cottus). *Arch. Anat. Histol. Embryol.* 3, 353–381.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 29 June 2021
doi: 10.3389/fcell.2021.686755

Differential Regenerative Capacity of the Optic Tectum of Adult Medaka and Zebrafish

Yuki Shimizu[1,2]* *and Takashi Kawasaki*[1]

[1] *Functional Biomolecular Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Osaka, Japan,* [2] *DBT-AIST International Laboratory for Advanced Biomedicine, National Institute of Advanced Industrial Science and Technology, Osaka, Japan*

Zebrafish have superior regenerative capacity in the central nervous system (CNS) compared to mammals. In contrast, medaka were shown to have low regenerative capacity in the adult heart and larval retina, despite the well-documented high tissue regenerative ability of teleosts. Nevertheless, medaka and zebrafish share similar brain structures and biological features to those of mammals. Hence, this study aimed to compare the neural stem cell (NSC) responses and regenerative capacity in the optic tectum of adult medaka and zebrafish after stab wound injury. Limited neuronal differentiation was observed in the injured medaka, though the proliferation of radial glia (RG) was induced in response to tectum injury. Moreover, the expression of the pro-regenerative transcriptional factors ascl1a and oct4 was not enhanced in the injured medaka, unlike in zebrafish, whereas expression of sox2 and stat3 was upregulated in both fish models. Of note, glial scar-like structures composed of GFAP^{+} radial fibers were observed in the injured area of medaka at 14 days post injury (dpi). Altogether, these findings suggest that the adult medaka brain has low regenerative capacity with limited neuronal generation and scar formation. Hence, medaka represent an attractive model for investigating and evaluating critical factors for brain regeneration.

Keywords: radial glia, stab wound injury, optic tectum, neuronal differentiation, reactive gliosis, zebrafish, medaka

OPEN ACCESS

Edited by:
Igor Schneider,
Federal University of Pará, Brazil

Reviewed by:
Katharina Lust,
Research Institute of Molecular Pathology (IMP), Austria
Sepand Rastegar,
Karlsruhe Institute of Technology (KIT), Germany
Karen Mruk,
University of Wyoming, United States

***Correspondence:**
Yuki Shimizu
yuki.shimizu@aist.go.jp

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *27 March 2021*
Accepted: *01 June 2021*
Published: *29 June 2021*

Citation:
Shimizu Y and Kawasaki T (2021) Differential Regenerative Capacity of the Optic Tectum of Adult Medaka and Zebrafish. Front. Cell Dev. Biol. 9:686755. doi: 10.3389/fcell.2021.686755

INTRODUCTION

Zebrafish have a superior ability to regenerate various tissues, including the central nervous system (CNS) and heart, compared with mammals (Becker et al., 1997; Poss et al., 2002; Raymond et al., 2006; März et al., 2011). Recently, to better understand the molecular mechanisms underlying the high regenerative capacity of zebrafish, comparative analyses of tissue regeneration in the retina and heart between zebrafish and mice have been performed, given their similarities in cell type and tissue structure (Kang et al., 2016; Hoang et al., 2020; Simões et al., 2020). Comparative studies using next-generation sequencing technology have revealed differences in the immune response or expression of transcriptional factors associated with tissue regeneration (Hoang et al., 2020; Simões et al., 2020). In contrast, the brain structure and cell types between zebrafish and mice are quite different (Kizil et al., 2012; Alunni and Bally-Cuif, 2016; Diotel et al., 2020;

Labusch et al., 2020). Despite the efforts made to explore and compare the brain regeneration mechanisms in zebrafish and mice, comparative studies with omics approaches have not been well examined (Llorens-Bobadilla et al., 2015; Zhong et al., 2016; Arneson et al., 2018; Yu and He, 2019; Demirci et al., 2020). To investigate the mechanisms that contribute to the high regenerative capacity of the zebrafish brain, non-regenerative animal models with similar brain structures and biological features are warranted.

Medaka (*Oryzias latipes*) is a popular experimental model among freshwater teleosts that has been extensively used for tissue regeneration analysis. Despite its high regenerative capacity in the fin and pancreas (Akimenko et al., 1995; Katogi et al., 2004; Moss et al., 2009; Otsuka and Takeda, 2017), similar to zebrafish, medaka have a low capacity for heart and retina regeneration (Ito et al., 2014; Lai et al., 2017; Lust and Wittbrodt, 2018). Comparative analysis of heart regeneration between adult medaka and zebrafish, cardiac cryoinjury results in less cardiomyocyte proliferation and scar formation in medaka (Ito et al., 2014; Lai et al., 2017), whereas zebrafish show induced cardiomyocyte proliferation and injured tissues are filled with newborn cardiomyocytes, with little or no scar tissue formation (Poss et al., 2002; Kikuchi and Poss, 2012). Regenerative capacity in the retina has also been compared between larval medaka and zebrafish, indicating that retinal injury induces Müller glia proliferation in both models; however, Müller glia in medaka have less multipotency, with photoreceptors being generated, but not retinal ganglion cells (Lust and Wittbrodt, 2018). Moreover, overexpression of sox2 in Müller glia was found to promote the regenerative potential of these cells in the medaka retina. However, the CNS regenerative capacity in the adult medaka remains unclear.

Medaka and zebrafish have similar brain structures and niches of adult neural stem cells (NSCs) (Adolf et al., 2006; Grandel et al., 2006; Alunni et al., 2010; Kuroyanagi et al., 2010). Stab wound injury models affecting various regions of the adult zebrafish brain, including the optic tectum, have been developed to investigate brain regeneration (Kroehne et al., 2011; März et al., 2011; Kishimoto et al., 2012; Kaslin et al., 2017; Shimizu et al., 2018; Lindsey et al., 2019; Yu and He, 2019). The optic tectum of both zebrafish and medaka harbors two types of NSCs—neuroepithelial-like stem (NE) and radial glia (RG) cells—that express stem cell markers, such as *sox2* and *msi1*. NE cells are proliferative cells that produce neurons, RG, and oligodendrocytes, whereas most of RG are quiescent (Alunni et al., 2010; Ito et al., 2010; Takeuchi and Okubo, 2013; Galant et al., 2016; Dambroise et al., 2017). Previous studies showed that RG proliferation and differentiation into newborn neurons are induced in response to injury in young adult zebrafish (2–4 months old) (Shimizu et al., 2018; Ueda et al., 2018; Yu and He, 2019; Kiyooka et al., 2020). In contrast, the regenerative responses in the medaka tectum remain to be elucidated.

Herein, the proliferation and differentiation of RG and NE in injured medaka and zebrafish were examined to evaluate the regenerative capacity of the medaka brain. The present study highlights the potential of medaka as a useful experimental non-regenerative model to investigate and identify pro-regenerative factors that mediate CNS regeneration.

MATERIALS AND METHODS

Animals

Medaka (*O. latipes*) and zebrafish (*Danio rerio*), specifically the Kyoto-Cab and RIKEN Wako wild-type strains, respectively, were maintained at 27.0 ± 1°C under a 14/10 h light/dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology (2021-0276). Animals with 3–7 months old were used for all experiments, except for the analysis of newborn neurons after tectum injury, which 3–5-months-old medaka and zebrafish were used.

Stab Wound Injury Protocol

To induce a stab wound injury in the adult optic tectum, medaka and zebrafish were anesthetized with 0.02% tricaine (pH 7.0; Nacalai Tesque, Kyoto, Japan) and a 30 G needle was vertically inserted into the medial region of the right hemisphere, as previously described (Shimizu et al., 2018). The contralateral uninjured hemisphere was used as internal control for each animal. For quantitative real-time polymerase chain reaction (PCR) analysis, both hemispheres were injured.

5-Bromo-2-Deoxyuridine (BrdU) Administration

To label proliferating cells, injured medaka and zebrafish were kept in 5 mM BrdU (Wako, Osaka, Japan). Injured medaka and zebrafish were treated with BrdU for 48 h, from 1 to 3 days post injury (dpi).

Histological and Immunohistochemical Analysis

Medaka and zebrafish were anesthetized using 0.02% tricaine and intracardially perfused with phosphate-buffered saline. Brains were dissected and stored in 4% paraformaldehyde (Wako) solution overnight at 4°C. The fixed brains were stored in 30% sucrose solution overnight at 4°C, and whole brains were then embedded in a 2:1 mixture of 30% sucrose and Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan). For fluorescence immunohistochemistry, 14 μm cryosections were prepared using a Leica CM1960 cryostat (Leica Biosystems, Wetzlar, Germany). Fluorescence immunohistochemistry was performed as described previously, using the following primary antibodies: mouse anti-HuC (1:100 dilution, A21271; Invitrogen, Waltham, MA, United States) as a pan-neuronal marker, mouse anti-proliferating cell nuclear antigen (PCNA) (1:200, sc-56; Santa Cruz Biotechnology, Dallas, TX, United States) as a proliferating cell marker, mouse anti-glial fibrillary acid protein (GFAP) (1:500, G3893; Sigma-Aldrich, St. Louis, MO, United States), and rabbit anti-brain lipid binding protein (BLBP) (1:500, ABN14; Millipore, Burlington, MA, United States) as RG cell markers, and sheep anti-BrdU (1:500, ab1893; Abcam,

Cambridge, United Kingdom). Alexa Fluor 488- and 546-conjugated subclass-specific antibodies (1:500, Invitrogen) were used as secondary antibodies. For PCNA antigen retrieval, sections were incubated with 10 mM sodium citrate for 30 min at 85°C prior to primary antibody incubation. For BrdU antigen retrieval, sections were incubated with 2N HCl (Wako) for 30 min at 37°C. For nuclear staining, the sections were incubated with Hoechst 33258 (1:500; Dojindo, Kumamoto, Japan) for 30 min following immunohistochemistry.

Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR, both hemispheres of the optic tectum were injured. After anesthesia with 0.02% tricaine, both hemispheres of the optic tectum were dissected from one fish and homogenized in TRIzol reagent (Invitrogen). Total RNA was purified using the Directozol RNA Miniprep (Zymo Research, Irvine, CA, United States), and cDNA was synthesized using RevaTra Ace (Toyobo, Osaka, Japan). The gene-specific primers used for *ascl1a, oct4, sox2, stat3*, and *tbp* are listed in **Supplementary Table 1**. The expression of tbp was used as endogenous control.

Cell Quantification

To quantify proliferating RG after the stab injury, the number of $BLBP^+PCNA^+$ cells was counted in 5–10 sections, including the center of the injury. To quantify NE proliferation, the number of $PCNA^+$ cells located in the tectal marginal zone was counted in 5–10 sections, including the center of the injury. To quantify the number of newborn neurons after the stab injury, the number of $BrdU^+HuC^+$ cells in five sections, including the center of the injury, was counted. The number of $BrdU^+HuC^+$ cells in the tectal marginal zone was also counted in five sections after the tectum injury. The corresponding contralateral regions were examined as internal controls.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM), and sample numbers are indicated in each figure legend. Statistical analysis in two experimental groups was performed using paired and unpaired Student's *t*-tests. In three or more groups, one-way analysis of variance was performed, followed by Tukey's *post hoc* test. *P*-values were calculated using Prism software (GraphPad Software, San Diego, CA, United States) and statistical significance was defined as *** and † † † if $P < 0.001$; ** and †† if $P < 0.01$; * and † if $P < 0.05$.

RESULTS

Increase in the Proliferation of Radial Glia in Response to Stab Injury

In the adult zebrafish optic tectum, most RG are quiescent under physiological conditions, but stab wound injury can induce their proliferation (Shimizu et al., 2018; Lindsey et al., 2019; Yu and He, 2019). To examine that this regenerative mechanism was also present in medaka, stab wound injury was induced in the right hemisphere of the optic tectum of medaka and RG proliferation was quantified by counting BLBP (RG marker), and PCNA (proliferating cell marker) double-positive cells. At 2 dpi, the number of proliferative RG cells ($BLBP^+PCNA^+$ cells) was significantly increased in the injured hemisphere than in the contralateral (internal control) uninjured side (**Figures 1A,B**). Additional analysis between 6 h post injury (hpi) to 7 dpi (**Figures 1C–F**) further revealed that the number of proliferative RG significantly increased from 1 dpi and peaking at around 2 dpi, with no significant difference being observed at 7 dpi (**Figure 1G**), which follows the same response trend observed in the injured zebrafish (Shimizu et al., 2018; Yu and He, 2019). Moreover, we quantified $BLBP^-PCNA^+$ cells except for proliferative NE known as $PCNA^+$ cells located in the tectal marginal zone to analyze the cell proliferation of another type of cell. These $BLBP^-PCNA^+$ cells which may include oligodendrocytes, microglia, neutrophils, and endothelial cells also significantly increased in response to the injury (**Figure 1H**). Although the contribution of NE to tectum regeneration is controversial (Shimizu et al., 2018; Lindsey et al., 2019), NE proliferation after stab wound injury was also evaluated by counting the $PCNA^+$ cells in the tectal marginal zone (**Supplementary Figures 1A–L**). This analysis confirmed that the stab wound injury had no significant effect on the proliferation of NE (**Supplementary Figure 1M**), which is consistent with previous injured zebrafish (Shimizu et al., 2018). Taken together, these results suggest that RG in the medaka and zebrafish tectum have similar proliferative potential after injury.

Limited Generation of Newborn Neurons After Stab Injury of Optic Tectum

Previous studies showed that newborn neurons around the injured site after the tectum injury in young adult zebrafish are mainly derived from RG (Shimizu et al., 2018; Yu and He, 2019). To analyze whether newborn neurons were similarly generated in tectum injured medaka, BrdU-labeled proliferative cells (including RG and NE) in the injured zebrafish and medaka were evaluated at 7 dpi (**Figure 2A**). We confirmed that RG incorporated BrdU at 3 dpi (**Supplementary Figures 2A–E**). Then, the number of newborn neurons ($BrdU^+HuC^+$ cells) at 7 dpi was quantified (**Figures 2B,C**), revealing that were not significantly increased in the injured hemisphere in the medaka unlike in the zebrafish (**Figure 2D**). $BrdU^+$ cells around the injured periventricular gray zone (PGZ) in the medaka optic tectum are $BLBP^+$ (**Supplementary Figures 2F–I**). Moreover, the number of $BrdU^+$ cells observed in PGZ was not significantly different in both fish models (**Figure 2E**). These results suggest that post-proliferating RG in injured medaka have limited capacity for neuronal differentiation. As NE can also generate neuronal cells in the optic tectum, the differentiation potential of $BrdU^+$ cells in the tectal marginal zone after tectum injury was also evaluated (**Supplementary Figure 3A**). However, no significant differences were observed in the $BrdU^+HuC^+$ cells around the tectal marginal zone between injured and uninjured hemispheres in both medaka and zebrafish (**Supplementary Figures 3B–G**). Overall, these results suggest that post-proliferating RG in the injured medaka tectum have

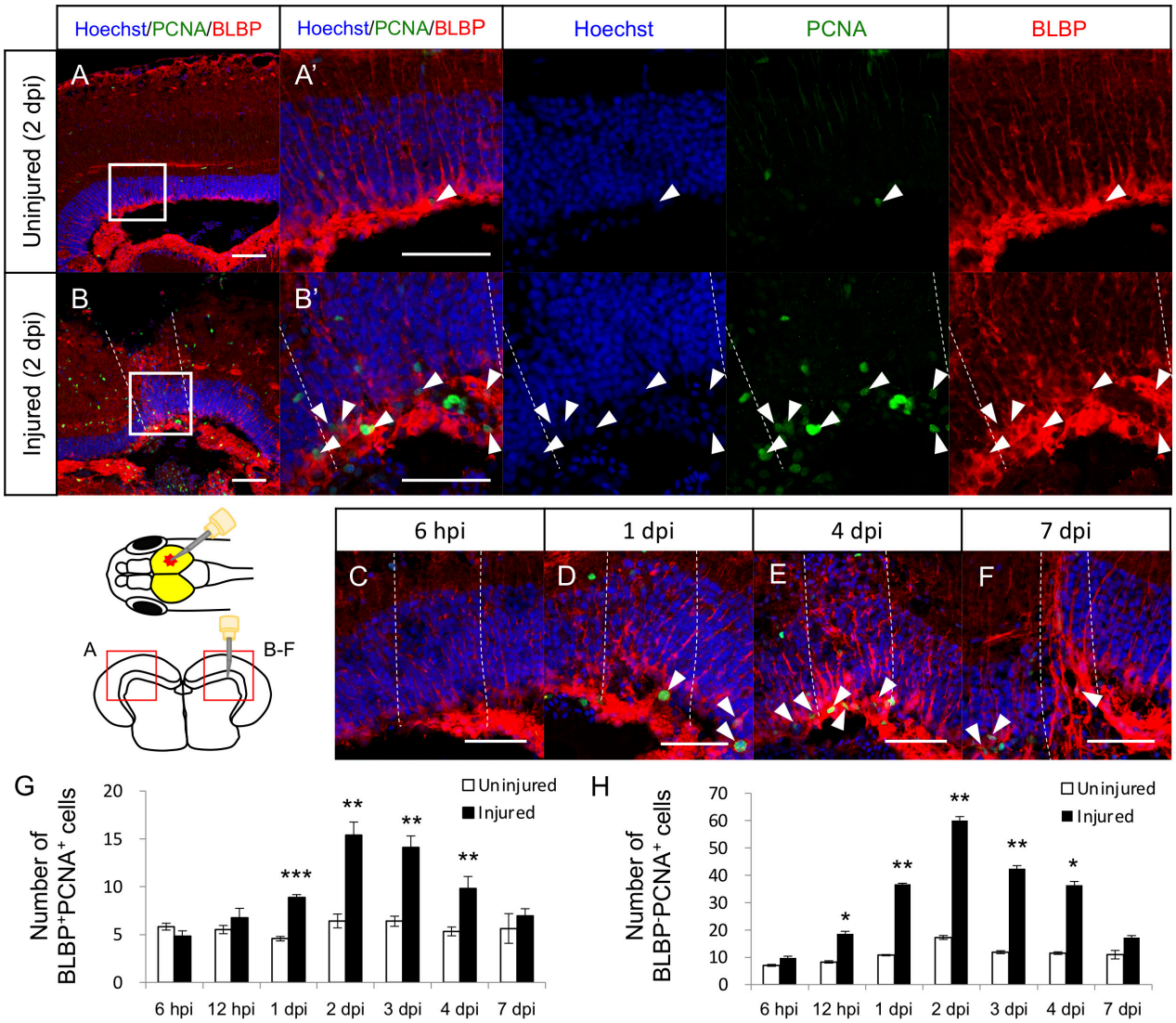


FIGURE 1 | Proliferation of radial glia (RG) is increased in response to stab wound injury. Representative images of proliferative RG ($BLBP^+PCNA^+$ cells) in the uninjured **(A)** and injured **(B)** hemispheres at 2 days post injury (dpi). **(A',B')** Magnified images of the boxed area in **(A,B)**. **(C–F)** Representative images of proliferative RG in the injured hemisphere at 6 h post injury (hpi) and at 1, 4, and 7 dpi. White arrowheads indicate $BLBP^+PCNA^+$ cells, and dashed lines indicate injured areas. Scale bar: 50 μm in **(A–F)** and **(A',B')**. Schematic drawing of the stab injury in the right hemisphere of the optic tectum and cross-section. **(G)** Quantification of proliferative RG in both uninjured and injured hemispheres at 6 (n = 5) and 12 (n = 3) hpi, and 1 (n = 5), 2 (n = 5), 3 (n = 4), 4 (n = 5), and 7 (n = 4) dpi. **(H)** Quantification of proliferative cells ($BLBP^-PCNA^+$ cells) except NE in both uninjured and injured hemispheres at 6 (n = 5) and 12 (n = 3) hpi, and 1 (n = 5), 2 (n = 5), 3 (n = 4), 4 (n = 5), and 7 (n = 4) dpi. Statistical analyses between uninjured and injured hemispheres at each time point were evaluated using paired Student's t-tests. Statistical significance was defined as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

limited neuronal differentiation, whereas stab wound injury in the optic tectum does not affect NE differentiation into neurons.

Differential Expression of Transcriptional Factors Between Medaka and Zebrafish After Tectum Injury

Molecular mechanisms related to *ascl1a* during zebrafish retina regeneration have been well studied (Fausett et al., 2008; Ramachandran et al., 2010). In particular, the expression of this pro-regenerative transcriptional factor was shown to be induced the optic tectum of zebrafish. Moreover, induction of *sox2, stat3*, and *oct4* expression was also shown to be required for NSC proliferation and differentiation into neurons (Fausett et al., 2008; Ramachandran et al., 2010; Nelson et al., 2012; Zhao et al., 2014; Gorsuch et al., 2017; Sharma et al., 2019). Herein, the expression of these transcriptional factors was also evaluated to assess potential changes induced in response to the tectum injury. Thus, *ascl1a, oct4* (*pou5f1* in medaka and *pou5f3* in zebrafish), *sox2*, and *stat3* were evaluated at 6, 24, 96, and

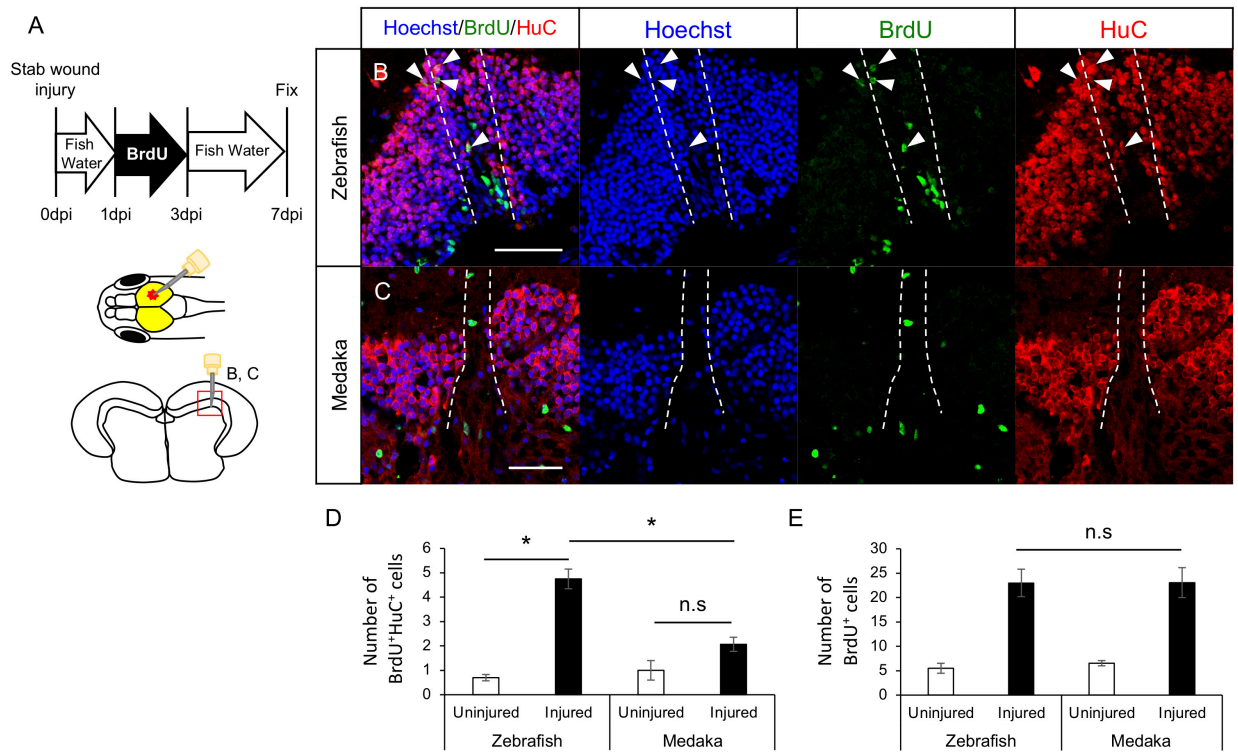


FIGURE 2 | Generation of newborn neurons in the injured medaka is limited compared with zebrafish. **(A)** Schematic drawing of bromodeoxyuridine (BrdU)-labeling and stab injury in the right hemisphere of the optic tectum. Representative images of newborn neurons ($BrdU^+HuC^+$ cells) in both injured zebrafish **(B)** and medaka **(C)**. White arrowheads indicate $BrdU^+HuC^+$ cells, and dashed lines indicate the injured areas. Scale bar: 50 μm. **(D)** Quantification of $BrdU^+HuC^+$ cells in the uninjured and injured hemispheres in zebrafish ($n = 4$) and medaka ($n = 4$). **(E)** Quantification of total $BrdU^+$ cells in both uninjured and injured zebrafish ($n = 4$) and medaka ($n = 4$). Statistical analyses were evaluated using one-way analysis of variance followed by Tukey's *post hoc* test. Statistical significance was defined as $^*P < 0.05$.

168 hpi. Expression changes of *sox2* and *stat3* showed similar patterns, significantly increasing from 6 hpi (**Figures 3A,B**). At 168 hpi, *stat3* expression in zebrafish remained significantly elevated though *stat3* expression in medaka returned to baseline. Interestingly, upregulation of *ascl1a* and *oct4* was observed in the injured zebrafish (**Figures 3C,D**), whereas it was not induced in the injured medaka. Expression of *oct4* was decreased at 6 hpi in both injured medaka and zebrafish, subsequently increasing in the injured zebrafish at 24 and 96 hpi, but not in the injured medaka (**Figure 3D**). These results suggest that differential expression of pro-regenerative factors may contribute for the limited neuronal differentiation potential of RG in medaka during tectum regeneration.

Glial Scar-Like Structures Persist in the Injured Medaka Tectum

In the adult mammalian brain, stab wound injury increases GFAP immunoreactivity in astrocytes, called reactive gliosis, and these reactive astrocytes are shown to contribute to the $GFAP^+$ scar formation, called glial scar (Feeney et al., 1981; Hozumi et al., 1990; Smith et al., 1995; Xiong et al., 2013; Burda et al., 2016). Although stab wound injury in the zebrafish telencephalon also increases GFAP immunoreactivity in the injured hemisphere, scar formation has not been observed (Kroehne et al., 2011; März et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012). Hence, the reactive gliosis after the tectum injury was herein assessed by comparing GFAP immunoreactivity in injured medaka and zebrafish at 7, 14, and 30 dpi (**Figures 4A–H**). At 7 dpi, GFAP expression increased in both injured fishes (**Figures 4B,F**). In particular, the GFAP immunoreactivity remained activated in the injured zebrafish at 14 dpi (**Figures 4I–P**), compared with the uninjured tectum (**Figure 4C**); however, its levels were relatively weak and no obvious scar-like structure was observed at 30 dpi (**Figure 4D**). Surprisingly, $GFAP^+$ scar-like structures were formed in the injured medaka at 14 dpi (**Figure 4G**), which persisted at 30 dpi (**Figure 4H**). Moreover, at 14 dpi (**Figures 4I–P**), the injured medaka lacked cell layer in the injured PGZ indicated by dashed lines (**Figure 4N**), and $GFAP^+$ fibers covered the area of this missing cell layer (**Figure 4O**). This $GFAP^+$ scar-like structure elongated from the basal layer of the PGZ to the apical side (**Figure 4O**) and this injury-induced $GFAP^+$ structures were co-expressed with BLBP (**Figures 4O,P**), suggesting that RG in the medaka optic tectum could form this scar-like structure in response to injury. Of note, in the injured zebrafish optic tectum, a disturbed cell layer due to the injury was also observed (**Figure 4J**), but no obvious lack of layer and no accumulation of $GFAP^+$

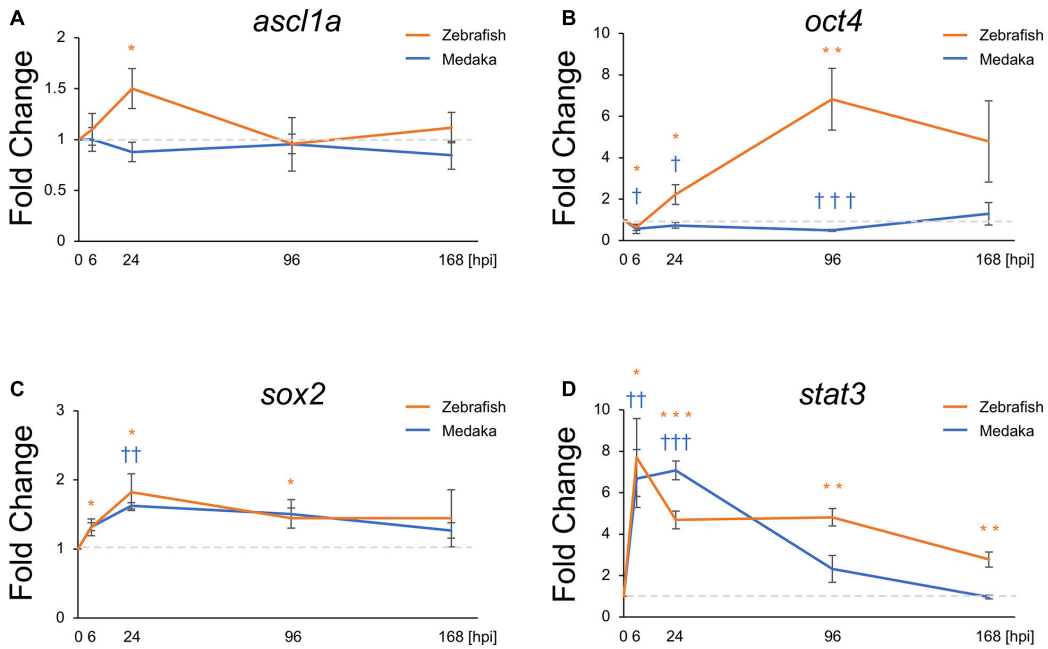


FIGURE 3 | Pro-regenerative transcriptional factors are differentially expressed between the injured medaka and zebrafish. Quantitative polymerase chain reaction analysis of the pro-regenerative transcriptional factors ascl1a **(A)**, oct4 **(B)**, sox2 **(C)**, and stat3 **(D)**. Graphs indicate the relative gene expression in the injured tectum from 6 to 168 h post injury (hpi) compared to the uninjured tectum ($n = 4$). Statistical analyses between the uninjured and injured hemispheres at each time point were evaluated by unpaired Student's *t*-tests. We used * for zebrafish and † for medaka to indicate significant difference. Statistical significance was defined as * and †$P < 0.05$; ** and ††$P < 0.01$; *** and ††† $P < 0.001$.

or BLBP$^+$ radial fibers around the injured area were noted (**Figures 4J–L**). These results suggest that RG with GFAP$^+$ scar-like structures in the injured medaka tectum have reactive astrocytic characteristics.

DISCUSSION

Zebrafish have higher CNS regeneration capacity, including of the brain, retina, and spinal cord, compared with mammals (Kizil et al., 2012; Alunni and Bally-Cuif, 2016). Medaka and zebrafish share similar biological features, such as brain structure, body size, and lifespan; nevertheless, medaka have different regenerative capacities in heart and retina (Ito et al., 2014; Lai et al., 2017; Lust and Wittbrodt, 2018). The present study showed that stab wound injury could induce the proliferation of RG in the medaka, but with limited generation of newborn neurons in the injured site compared with the response observed in similarly injured zebrafish. Therefore, this is the first report indicating the limited capacity of neuronal regeneration in the teleost young adult brain. We also confirmed that there was no induction of transcriptional factors, *ascl1a* and *oct4* in the injured medaka. Moreover, we observed injury-induced GFAP$^+$ radial fibers from RG at 14 dpi and found that this glial scar-like structure covered the injured area with lack of cell layer in medaka. Taken together, our findings suggest that medaka have low regenerative ability in the tectum compared to zebrafish because RG in the injured medaka tectum may have reactive astrocytic characteristics rather than neurogenic NSCs.

In the adult zebrafish CNS, the optic tectum and retina share similar features regarding NSCs. For example, RG in the optic tectum and Müller glia in the retina are quiescent under physiological conditions, whereas proliferation and differentiation of these NSCs are activated upon injury (Raymond et al., 2006; Ito et al., 2010; Shimizu et al., 2018). The optic tectum and retina also have NE cells that continuously proliferate and generate newborn neurons throughout life (Raymond et al., 2006; Ito et al., 2010). Comparative analyses of retinal regeneration showed that Müller glia in the larval medaka have limited neuronal differentiation compared with larval zebrafish despite the proliferative response induced by retinal injury (Lust and Wittbrodt, 2018), which is consistent with here observed limited capacity of RG in the medaka tectum. Furthermore, although Müller glia in the medaka only contribute for the generation of photoreceptors, induction of *sox2* expression in Müller glia after retinal injury can restore their multi-potency. Although *sox2* and *stat3* expression increased in both medaka and zebrafish after the tectum injury, that of *ascl1a* and *oct4* did not increase in the injured medaka. In the zebrafish, the transcriptional factors ascl1a (also known as Ascl1/Mash1 in mammals) and *oct4* are known to play important roles in retinal regeneration (Fausett et al., 2008; Ramachandran et al., 2010; Sharma et al., 2019). Furthermore, during zebrafish retinal regeneration from light damage, *stat3* expression may precede *ascl1a* expression (Nelson et al., 2012; Goldman, 2014), whereas N-methyl-*d*-asparate-injured mouse retina showed the lack of *Ascl1* expression (Karl et al., 2008) despite the upregulation of phosphorylated Stat3 (Jorstad et al., 2020).

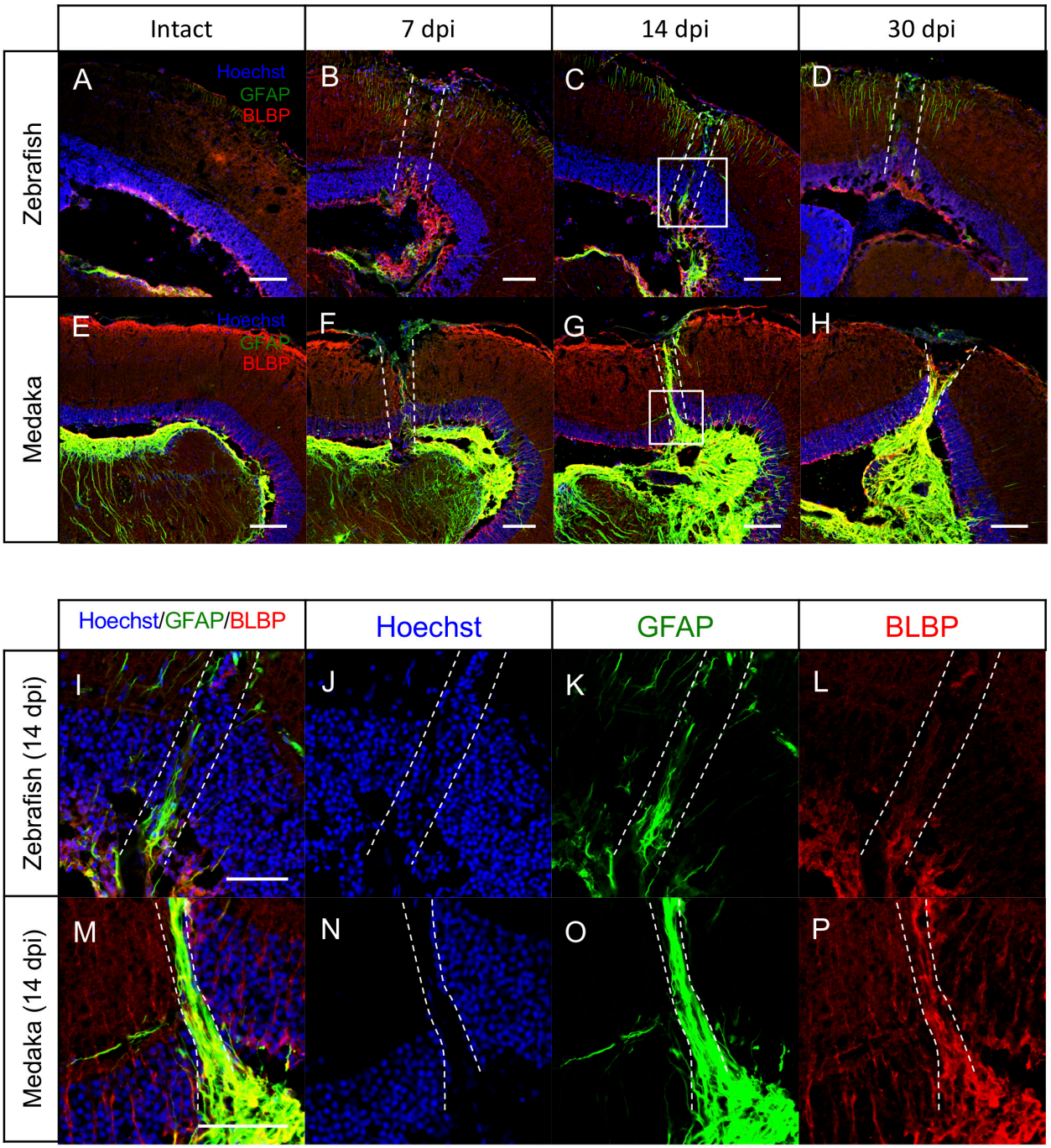


FIGURE 4 | Persistent glial scar-like structure is observed in the injured medaka tectum. **(A–H)** Representative images of immunostaining with anti-GFAP and anti-BLBP antibodies on the uninjured (**A:** zebrafish and **B:** medaka) and injured hemisphere at 7, 14, and 30 days post injury (dpi) [**(B–D)**: zebrafish and **(E–H)**: medaka]. **(I–P)** Magnified images of the boxed area at 14 dpi [**(I–L)**: zebrafish and **(M–P)**: medaka]. The dashed lines indicate the injured areas. Scale bar: 100 **(A–H)** and 50 **(I,M)** μm.

These findings suggest that upregulation of STAT3-mediated signaling is a shared feature in both injured medaka and zebrafish, but that lack of *ascl1a* expression in the injured medaka may result in low neurogenic capacity of RG in the medaka tectum.

In addition to limited neuronal generation after medaka tectum injury, persistent $GFAP^{+}BLBP^{+}$ scar-like structures were clearly observed from 14 to 30 dpi. In contrast, in the zebrafish adult brain, stab wound injury in the telencephalon induced reactive gliosis with upregulation of GFAP immunoreactivity, but

no scar formation was observed (März et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012). In the injured zebrafish optic tectum, although upregulation of GFAP immunoreactivity was also observed, obvious scar formation like medaka has not been observed. These findings suggest that scar-like structures with radial fibers in the injured medaka tectum are similar to glial scar formed by reactive astrocytes in the damaged mammalian CNS (Burda et al., 2016). Glial scar in the injured rodent CNS includes GFAP and other extracellular matrices, such as chondroitin sulfate proteoglycan and collagen IV (McKeon et al., 1991). The role of glial scar in the tissue regeneration is well investigated, but the findings remain inconclusive (Anderson et al., 2016; Adams and Gallo, 2018; Yang et al., 2020). Glial scar is shown to prevent acute inflammation spreading; however, large scar is an obstacle for neuronal and axonal regeneration. Whether glial scar-like structure in the injured medaka shares these features remain to be explored. Furthermore, Stat3 activation in astrocytes is involved in glial scar formation after spinal cord injury in mice (Herrmann et al., 2008; Anderson et al., 2016), suggesting that the activated stat3 signaling in the medaka RG may contribute to scar formation rather than neuronal generation unlike zebrafish.

Teleost species are shown to have a high regenerative capacity of various tissues, including the CNS. In addition to zebrafish, goldfish (*Carassius auratus*) and brown ghost knifefish (*Apteronotus leptorhynchus*) are known to have high CNS regeneration potential (Bernstein, 1964; Stevenson and Yoon, 1978; Zupanc, 1999; Sîrbulescu et al., 2009). In addition to these teleosts, recently, various other species including salmonoids (masu and chum salmon) (Pushchina et al., 2017, 2020) and killifish (mummichog, *Aphaniops hormuzensis*, and *Nothobranchius furzeri*) (Bisese et al., 2019; Soltani et al., 2020; Van houcke et al., 2021) have been explored as models to assess the mechanisms regulating the CNS regenerative potential. Previous studies showed that only medaka have low CNS regenerative potential, regardless of age and health condition (Lust and Wittbrodt, 2018). For comparative analyses of tissue regeneration, compatible injury models and similar biological properties, except regenerative capacity, are important. Hence, medaka represent an attractive non-regenerative model to investigate and identify pro-regenerative factors that mediate CNS regeneration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the National Institute of Advanced Industrial Science and Technology.

AUTHOR CONTRIBUTIONS

YS and TK designed the experiments and wrote and revised the manuscript. YS performed histological and molecular experiments. Both authors approved the submitted version of the manuscript.

FUNDING

This work was supported by JSPS KAKENHI Grant Numbers 18K14824 and 21K15195, and internal grant of AIST, Japan.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.686755/full#supplementary-material

REFERENCES

Adams, K. L., and Gallo, V. (2018). The diversity and disparity of the glial scar. *Nat. Neurosci.* 21, 9–15. doi: 10.1038/s41593-017-0033-9

Adolf, B., Chapouton, P., Lam, C. S., Topp, S., Tannhauser, B., Strahle, U., et al. (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev. Biol.* 295, 278–293. doi: 10.1016/j.ydbio.2006.03.023

Akimenko, M. A., Johnson, S. L., Westerfield, M., and Ekker, M. (1995). Differential induction of four msx homeobox genes during fin development and regeneration in zebrafish. *Development* 121, 347–357.

Alunni, A., and Bally-Cuif, L. (2016). A comparative view of regenerative neurogenesis in vertebrates. *Development* 143, 741–753. doi: 10.1242/dev.122796

Alunni, A., Hermel, J. M., Heuzé, A., Bourrat, F., Jamen, F., and Joly, J. S. (2010). Evidence for neural stem cells in the medaka optic tectum proliferation zones. *Dev. Neurobiol.* 70, 693–713. doi: 10.1002/dneu.20799

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., et al. (2016). Astrocyte scar formation aids central nervous system axon regeneration. *Nature* 532, 195–200. doi: 10.1038/nature17623

Arneson, D., Zhang, G., Ying, Z., Zhuang, Y., Byun, H. R., Ahn, I. S., et al. (2018). Single cell molecular alterations reveal target cells and pathways of concussive brain injury. *Nat. Commun.* 9:3894. doi: 10.1038/s41467-018-06222-0

Baumgart, E. V., Barbosa, J. S., Bally-Cuif, L., Götz, M., and Ninkovic, J. (2012). Stab wound injury of the zebrafish telencephalon: a model for comparative analysis of reactive gliosis. *Glia* 60, 343–357. doi: 10.1002/glia.22269

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., and Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.* 377, 577–595.

Bernstein, J. J. (1964). Relation of spinal cord regeneration to age in adult goldfish. *Exp. Neurol.* 1964, 161–174. doi: 10.1016/0014-4886(64)90014-7

Bisese, E. C., Ciuba, C. M., Davidson, A. L., Kaushik, A., Mullen, S. M., Barth, J. L., et al. (2019). The acute transcriptome response of the midbrain/diencephalon to injury in the adult mummichog (*Fundulus heteroclitus*). *Mol. Brain* 12:119. doi: 10.1186/s13041-019-0542-4

Burda, J. E., Bernstein, A. M., and Sofroniew, M. V. (2016). Astrocyte roles in traumatic brain injury. *Exp. Neurol.* 275, 305–315. doi: 10.1016/j.expneurol.2015.03.020

Dambroise, E., Simion, M., Bourquard, T., Bouffard, S., Rizzi, B., Jaszczyszyn, Y., et al. (2017). Postembryonic fish brain proliferation zones exhibit

neuroepithelial-type gene expression profile. *Stem Cells* 35, 1505–1518. doi: 10.1002/stem.2588

Demirci, Y., Cucun, G., Poyraz, Y. K., Mohammed, S., Heger, G., Papatheodorou, I., et al. (2020). Comparative transcriptome analysis of the regenerating zebrafish telencephalon unravels a resource with key pathways during two early stages and activation of wnt/β-catenin signaling at the early wound healing stage. *Front. Cell Dev. Biol.* 8:584604. doi: 10.3389/fcell.2020.584604

Diotel, N., Lübke, L., Strähle, U., and Rastegar, S. (2020). Common and distinct features of adult neurogenesis and regeneration in the telencephalon of zebrafish and mammals. *Front. Neurosci.* 14:568930. doi: 10.3389/fnins.2020.568930

Fausett, B. V., Gumerson, J. D., and Goldman, D. (2008). The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. *J. Neurosci.* 28, 1109–1117. doi: 10.1523/JNEUROSCI.4853-07.2008

Feeney, D. M., Boyeson, M. G., Linn, R. T., Murray, H. M., and Dail, W. G. (1981). Responses to cortical injury: I. Methodology and local effects of contusions in the rat. *Brain Res.* 211, 67–77. doi: 10.1016/0006-8993(81)90067-6

Galant, S., Furlan, G., Coolen, M., Dirian, L., Foucher, I., and Bally-Cuif, L. (2016). Embryonic origin and lineage hierarchies of the neural progenitor subtypes building the zebrafish adult midbrain. *Dev. Biol.* 420, 120–135. doi: 10.1016/j.ydbio.2016.09.022

Goldman, D. (2014). Müller glial cell reprogramming and retina regeneration. *Nat. Rev. Neurosci.* 15, 431–442. doi: 10.1038/nrn3723

Gorsuch, R. A., Lahne, M., Yarka, C. E., Petravick, M. E., Li, J., and Hyde, D. R. (2017). Sox2 regulates Muller glia reprogramming and proliferation in the regenerating zebrafish retina via Lin28 and Ascl1a. *Exp. Eye Res.* 161, 174–192. doi: 10.1016/j.exer.2017.05.012

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Dev. Biol.* 295, 263–277. doi: 10.1016/j.ydbio.2006.03.040

Herrmann, J. E., Imura, T., Song, B., Qi, J., Ao, Y., Nguyen, T. K., et al. (2008). STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. *J. Neurosci.* 28, 7231–7243. doi: 10.1523/JNEUROSCI.1709-08.2008

Hoang, T., Wang, J., Boyd, P., Wang, F., Santiago, C., Jiang, L., et al. (2020). Gene regulatory networks controlling vertebrate retinal regeneration. *Science* 370:eabb8598. doi: 10.1126/science.abb8598

Hozumi, I., Chiu, F. C., and Norton, W. T. (1990). Biochemical and immunocytochemical changes in glial fibrillary acidic protein after stab wounds. *Brain Res.* 524, 64–71. doi: 10.1016/0006-8993(90)90492-t

Ito, K., Morioka, M., Kimura, S., Tasaki, M., Inohaya, K., and Kudo, A. (2014). Differential reparative phenotypes between zebrafish and medaka after cardiac injury. *Dev. Dyn.* 243, 1106–1115. doi: 10.1002/dvdy.24154

Ito, Y., Tanaka, H., Okamoto, H., and Ohshima, T. (2010). Characterization of neural stem cells and their progeny in the adult zebrafish optic tectum. *Dev. Biol.* 342, 26–38. doi: 10.1016/j.ydbio.2010.03.008

Jorstad, N. L., Wilken, M. S., Todd, L., Finkbeiner, C., Nakamura, P., Radulovich, N., et al. (2020). STAT signaling modifies Ascl1 chromatin binding and limits neural regeneration from muller glia in adult mouse retina. *Cell Rep.* 30, 2195–2208. doi: 10.1016/j.celrep.2020.01.075

Kang, J., Hu, J., Karra, R., Dickson, A. L., Tornini, V. A., Nachtrab, G., et al. (2016). Modulation of tissue repair by regeneration enhancer elements. *Nature* 532, 201–206. doi: 10.1038/nature17644

Karl, M. O., Hayes, S., Nelson, B. R., Tan, K., Buckingham, B., and Reh, T. A. (2008). Stimulation of neural regeneration in the mouse retina. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19508–19513. doi: 10.1073/pnas.0807453105

Kaslin, J., Kroehne, V., Ganz, J., Hans, S., and Brand, M. (2017). Distinct roles of neuroepithelial-like and radial glia-like progenitor cells in cerebellar regeneration. *Development* 144, 1462–1471. doi: 10.1242/dev.144907

Katogi, R., Nakatani, Y., Shin-i, T., Kohara, Y., Inohaya, K., and Kudo, A. (2004). Large-scale analysis of the genes involved in fin regeneration and blastema formation in the medaka *Oryzias latipes*. *Mech. Dev.* 121, 861–872. doi: 10.1016/j.mod.2004.03.015

Kikuchi, K., and Poss, K. D. (2012). Cardiac regenerative capacity and mechanisms. *Annu. Rev. Cell Dev. Biol.* 28, 719–741. doi: 10.1146/annurev-cellbio-101011-155739

Kishimoto, N., Shimizu, K., and Sawamoto, K. (2012). Neuronal regeneration in a zebrafish model of adult brain injury. *Dis. Model Mech.* 5, 200–209. doi: 10.1242/dmm.007336

Kiyooka, M., Shimizu, Y., and Ohshima, T. (2020). Histone deacetylase inhibition promotes regenerative neurogenesis after stab wound injury in the adult zebrafish optic tectum. *Biochem. Biophys. Res. Commun.* 529, 366–371. doi: 10.1016/j.bbrc.2020.06.025

Kizil, C., Kaslin, J., Kroehne, V., and Brand, M. (2012). Adult neurogenesis and brain regeneration in zebrafish. *Dev. Neurobiol.* 72, 429–461. doi: 10.1002/dneu.20918

Kroehne, V., Freudenreich, D., Hans, S., Kaslin, J., and Brand, M. (2011). Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. *Development* 138, 4831–4841. doi: 10.1242/dev.072587

Kuroyanagi, Y., Okuyama, T., Suehiro, Y., Imada, H., Shimada, A., Naruse, K., et al. (2010). Proliferation zones in adult medaka (*Oryzias latipes*) brain. *Brain Res.* 1323, 33–40. doi: 10.1016/j.brainres.2010.01.045

Labusch, M., Mancini, L., Morizet, D., and Bally-Cuif, L. (2020). Conserved and divergent features of adult neurogenesis in zebrafish. *Front. Cell Dev. Biol.* 8:525. doi: 10.3389/fcell.2020.00525

Lai, S. L., Marín-Juez, R., Moura, P. L., Kuenne, C., Lai, J. K. H., Tsedeke, A. T., et al. (2017). Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. *eLife* 6:e25605. doi: 10.7554/eLife.25605

Lindsey, B. W., Aitken, G. E., Tang, J. K., Khabooshan, M., Douek, A. M., Vandestadt, C., et al. (2019). Midbrain tectal stem cells display diverse regenerative capacities in zebrafish. *Sci. Rep.* 9:4420. doi: 10.1038/s41598-019-40734-z

Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwadlo, K., and Martin-Villalba, A. (2015). Single-cell transcriptomics reveals a population of dormant neural stem cells that become activated upon brain injury. *Cell Stem Cell* 17, 329–340. doi: 10.1016/j.stem.2015.07.002

Lust, K., and Wittbrodt, J. (2018). Activating the regenerative potential of Müller glia cells in a regeneration-deficient retina. *eLife* 7:e32319. doi: 10.7554/eLife.32319

März, M., Schmidt, R., Rastegar, S., and Strähle, U. (2011). Regenerative response following stab injury in the adult zebrafish telencephalon. *Dev. Dyn.* 240, 2221–2231. doi: 10.1002/dvdy.22710

McKeon, R. J., Schreiber, R. C., Rudge, J. S., and Silver, J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J. Neurosci.* 11, 3398–3411. doi: 10.1523/JNEUROSCI.11-11-03398.1991

Moss, J. B., Koustubhan, P., Greenman, M., Parsons, M. J., Walter, I., and Moss, L. G. (2009). Regeneration of the pancreas in adult zebrafish. *Diabetes* 58, 1844–1851. doi: 10.2337/db08-0628

Nelson, C. M., Gorsuch, R. A., Bailey, T. J., Ackerman, K. M., Kassen, S. C., and Hyde, D. R. (2012). Stat3 defines three populations of Muller glia and is required for initiating maximal muller glia proliferation in the regenerating zebrafish retina. *J. Comp. Neurol.* 520, 4294–4311. doi: 10.1002/cne.23213

Otsuka, T., and Takeda, H. (2017). Targeted ablation of pancreatic beta cells in medaka. *Zoolog. Sci.* 34, 179–184. doi: 10.2108/zs170004

Poss, K. D., Wilson, L. G., and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* 298, 2188–2190. doi: 10.1126/science.1077857

Pushchina, E. V., Kapustyanov, I. A., and Varaksin, A. A. (2020). Neural Stem cells/neuronal precursor cells and postmitotic neuroblasts in constitutive neurogenesis and after, traumatic injury to the mesencephalic tegmentum of juvenile chum salmon, oncorhynchus keta. *Brain Sci.* 10:65. doi: 10.3390/brainsci10020065

Pushchina, E. V., Zharikova, E. I., and Varaksin, A. A. (2017). Persistent and reparative neurogenesis in the juvenile masu salmon *Oncorhynchus masou* telencephalon after mechanical injury. *Russ. J. Dev. Biol.* 48, 307–320.

Ramachandran, R., Fausett, B. V., and Goldman, D. (2010). Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nat. Cell Biol.* 2010, 1101–1107. doi: 10.1038/ncb2115

Raymond, P. A., Barthel, L. K., Bernardos, R. L., and Perkowski, J. J. (2006). Molecular characterization of retinal stem cells and their niches in adult zebrafish. *BMC Dev. Biol.* 6:36. doi: 10.1186/1471-213X-6-36

Sharma, P., Gupta, S., Chaudhary, M., Mitra, S., Chawla, B., Khursheed, M. A., et al. (2019). Oct4 mediates Muller glia reprogramming and cell cycle exit during retina regeneration in zebrafish. *Life Sci. Alliance* 2:e201900548. doi: 10.26508/lsa.201900548

Shimizu, Y., Ueda, Y., and Ohshima, T. (2018). Wnt signaling regulates proliferation and differentiation of radial glia in regenerative processes after stab injury in the optic tectum of adult zebrafish. *Glia* 66, 1382–1394. doi: 10.1002/glia.23311

Simões, F. C., Cahill, T. J., Kenyon, A., Gavriouchkina, D., Vieira, J. M., Sun, X., et al. (2020). Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. *Nat. Commun.* 11:600. doi: 10.1038/s41467-019-14263-2

Sîrbulescu, R. F., Ilieş, I., and Zupanc, G. K. (2009). Structural and functional regeneration after spinal cord injury in the weakly electric teleost fish, *Apteronotus leptorhynchus*. *J. Comp. Physiol. Neuroethol. Sens. Neural Behav. Physiol.* 195, 699–714. doi: 10.1007/s00359-009-0445-4

Smith, D. H., Soares, H. D., Pierce, J. S., Perlman, K. G., Saatman, K. E., Meaney, D. F., et al. (1995). A model of parasagittal controlled cortical impact in the mouse: cognitive and histopathologic effects. *J. Neurotrauma* 12, 169–178. doi: 10.1089/neu.1995.12.169

Soltani, A. R., Motamedi, M., and Teimori, A. (2020). Adult neuronal regeneration in the telencephalon of the killifish *Aphaniops hormuzensis*. *J. Exp. Zool. B Mol. Dev. Evol.* 334, 350–361. doi: 10.1002/jez.b.23008

Stevenson, J. A., and Yoon, M. G. (1978). Regeneration of optic nerve fibers enhances cell proliferation in the goldfish optic tectum. *Brain Res.* 153, 345–351. doi: 10.1016/0006-8993(78)90413-4

Takeuchi, A., and Okubo, K. (2013). Post-proliferative immature radial glial cells female-specifically express aromatase in the medaka optic tectum. *PLoS One* 8:e73663. doi: 10.1371/journal.pone.0073663

Ueda, Y., Shimizu, Y., Shimizu, N., Ishitani, T., and Ohshima, T. (2018). Involvement of sonic hedgehog and notch signaling in regenerative neurogenesis in adult zebrafish optic tectum after stab injury. *J. Comp. Neurol.* 526, 2360–2372. doi: 10.1002/cne.24489

Van houcke, J., Mariën, V., Zandecki, C., Vanhunsel, S., Moons, L., Ayana, R., et al. (2021). Aging impairs the essential contributions of non-glial progenitors to neurorepair in the dorsal telencephalon of the Killifish *N. furzeri*. *Biorxiv* [Preprint] doi: 10.1101/2021.02.26.433041

Xiong, Y., Mahmood, A., and Chopp, M. (2013). Animal models of traumatic brain injury. *Nat. Rev. Neurosci.* 14, 128–142. doi: 10.1038/nrn3407

Yang, T., Dai, Y., Chen, G., and Cui, S. (2020). Dissecting the dual role of the glial scar and scar-forming astrocytes in spinal cord injury. *Front. Cell Neurosci.* 14:78. doi: 10.3389/fncel.2020.00078

Yu, S., and He, J. (2019). Stochastic cell-cycle entry and cell-state-dependent fate outputs of injury-reactivated tectal radial glia in zebrafish. *eLife* 8:e48660. doi: 10.7554/eLife.48660

Zhao, X. F., Wan, J., Powell, C., Ramachandran, R., Myers, M. G. Jr., and Goldman, D. (2014). Leptin and IL-6 family cytokines synergize to stimulate Muller glia reprogramming and retina regeneration. *Cell Rep.* 9, 272–284. doi: 10.1016/j.celrep.2014.08.047

Zhong, J., Jiang, L., Cheng, C., Huang, Z., Zhang, H., Liu, H., et al. (2016). Altered expression of long non-coding RNA and mRNA in mouse cortex after traumatic brain injury. *Brain Res.* 1646, 589–600. doi: 10.1016/j.brainres.2016.07.002

Zupanc, G. K. (1999). Neurogenesis, cell death and regeneration in the adult gymnotiform brain. *J. Exp. Biol.* 202, 1435–1446.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 02 December 2021
doi: 10.3389/fevo.2021.784828

A Morphological and Histological Investigation of Imperfect Lungfish Fin Regeneration

Vivien Bothe[1], Igor Schneider[2] and Nadia B. Fröbisch[1,3]*

[1] Museum für Naturkunde Berlin, Leibnitz Institute for Research on Evolution and Biodiversity, Berlin, Germany, [2] Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, United States, [3] Department of Biology, Humboldt University, Berlin, Germany

Regeneration, the replacement of body parts in a living animal, has excited scientists for centuries and our knowledge of vertebrate appendage regeneration has increased significantly over the past decades. While the ability of amniotes to regenerate body parts is very limited, members of other vertebrate clades have been shown to have rather high regenerative capacities. Among tetrapods (four-limbed vertebrates), only salamanders show unparalleled capacities of epimorphic tissue regeneration including replacement of organ and body parts in an apparently perfect fashion. The closest living relatives of Tetrapoda, the lungfish, show regenerative abilities that are comparable to those of salamanders and recent studies suggest that these high regenerative capacities may indeed be ancestral for bony fish (osteichthyans) including tetrapods. While great progress has been made in recent years in understanding the cellular and molecular mechanisms deployed during appendage regeneration, comparatively few studies have investigated gross morphological and histological features of regenerated fins and limbs. Likewise, rather little is known about how fin regeneration compares morphologically to salamander limb regeneration. In this study, we investigated the morphology and histology of regenerated fins in all three modern lungfish families. Data from histological serial sections, 3D reconstructions, and x-ray microtomography scans were analyzed to assess morphological features, quality and pathologies in lungfish fin regenerates. We found several anomalies resulting from imperfect regeneration in regenerated fins in all investigated lungfish species, including fusion of skeletal elements, additional or fewer elements, and distal branching. The similarity of patterns in regeneration abnormalities compared to salamander limb regeneration lends further support to the hypothesis that high regenerative capacities are plesiomorphic for sarcopterygians.

Keywords: regeneration, lungfish, pathologies, salamander, axolotl

OPEN ACCESS

Edited by:
Alessandro Minelli, University of Padua, Italy

Reviewed by:
Ryan Robert Kerney, Gettysburg College, United States
Renata Freitas, Universidade do Porto, Portugal
Karen Echeverri, Marine Biological Laboratory (MBL), United States

****Correspondence:***
Vivien Bothe
Vivien.Bothe@mfn.berlin

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

Received: *28 September 2021*
Accepted: *08 November 2021*
Published: *02 December 2021*

Citation:
Bothe V, Schneider I and Fröbisch NB (2021) A Morphological and Histological Investigation of Imperfect Lungfish Fin Regeneration. Front. Ecol. Evol. 9:784828. doi: 10.3389/fevo.2021.784828

INTRODUCTION

The capability to replace lost organ and body parts, better known as regeneration, has fascinated scientists for several centuries (Reaumur, 1712). While this ability varies widely among Metazoa (e.g., Morgan, 1898; Lenhoff and Lenhoff, 1991; Bely and Nyberg, 2010), epimorphic regeneration was considered exceptional among extant vertebrates (e.g., Alvarado, 2000; Tsonis, 2000). This

form of regeneration, considered to be true regeneration, is characterized by the formation of two crucial structures, the blastema and the apical epithelial cap (or AEC) (Londono et al., 2018). Full regeneration of limbs after loss by injury has been reported only in salamanders and frogs, albeit appendage regeneration in frogs is limited to tadpole stages before metamorphic climax and is lost in adults (Dent, 1962; Girvan et al., 2002). Hence, urodeles are the only living tetrapods capable of fully regenerating limbs throughout their whole lifespan, even though larval salamanders show regenerates with less abnormalities than adults (Bothe et al., 2021). Through intensive research, especially on the model organism axolotl (*Ambystoma mexicanum*) the underlying processes of epimorphic regeneration in salamanders are quite well understood (amongst others: Kragl et al., 2009; McCusker et al., 2015; Bryant et al., 2017a). More recently, salamander lineage-specific genes (LSGs) were identified and shown to play a role in limb development as well as regeneration in salamanders, which led to the proposal that certain features in salamander limb development and their capacities to fully regenerate limbs may indeed be lineage specific for urodeles (Garza-Garcia et al., 2010; Brockes and Gates, 2014). However, data from fossil shows that limb regeneration already occurred in ancient amphibians long before the emergence of salamanders (Fröbisch et al., 2014, 2015), suggesting that the high regenerative capacities of body appendages are an ancient feature of tetrapods that was lost in the amniote lineage. This was later supported by molecular data that demonstrated that the genetic toolkit playing a role in lungfish tail regeneration is very similar to that seen in axolotl (Verissimo et al., 2020). Moreover, high regenerative capacities of the endochondral appendage skeleton were also demonstrated for several clades of osteichthyians, including paddle fish, gar, *Polypterus,* and several members of teleost fish (Cuervo et al., 2012; Amaral and Schneider, 2018; Darnet et al., 2019). Darnet et al. (2019) therein also showed that osteichthyians deploy a similar genetic toolkit for appendage regeneration, lending support to an ancient origin of epimorphic regeneration in vertebrates. However, many aspects of the evolution of vertebrate regeneration remain poorly understood and will require more detailed molecular, morphological, evolutionary, and ecological investigations of non-model as well as model organisms to gain a better understanding of the drivers of epimorphic regeneration in vertebrates.

Lungfish (Dipnoi) play a central role in this context, as they display a high degree of tissue regeneration in body appendages comparable to modern salamanders. Dipnoi are an ancient lineage of osteichthyan fish (bony fish) and, next to coelacanths, the only extant sarcopterygian (lobe-finned) fish. They first appeared in the Early Devonian period, about 419.2–393.3 million years ago (Chang and Yu, 1984) and were widespread and common in both marine and freshwater habitats. Several phylogenomic analyses and genome sequencing over the past few years have revealed that lungfish, rather than coelacanths, are the closest extant relatives of tetrapods (Amemiya et al., 2013; Biscotti et al., 2016; Irisarri and Meyer, 2016).

Although Dipnoi were notably abundant during the Devonian, most lungfish went extinct after the end Permian mass extinction (Nelson et al., 2016). Only three freshwater genera survived until now, represented by six species: South American lungfish (*Lepidosiren*, one species), African lungfish (*Protopterus*, four species), and the Australian lungfish (*Neoceratodus*, one species).

Lepidosiren was the first lungfish to be discovered in the 1830s (Bischoff, 1840) and have been the focus of many studies on sarcopterygian and vertebrate evolution, the transition from fishes to land vertebrates, genome size studies and the evolution of tetrapod feeding systems (Reilly and Lauder, 1990; Ericsson et al., 2010; Boisvert et al., 2013; Ziermann et al., 2018). Moreover, their high regenerative abilities, which rival those of salamanders, make them a highly relevant subject for regeneration research (Conant, 1970; Darnet et al., 2019; Verissimo et al., 2020).

Anatomically, paired lungfish fins rest on a single cartilaginous girdle element and are constructed according to the archipterygial fin type, in which the metapterygial stem consists of a projecting series of endoskeletal basal fin elements running along the middle of the fin. From this central axis, preaxial (anterior) and postaxial (posterior) radials proceed outward to sides of the fin for further support (Kardong, 1997). Among the modern taxa, the Australian lungfish resembles most closely the ancestral fin anatomy of ancient lobe-finned fishes (Kardong, 1997). Viewed from the outside, the fin appears leaf-shaped and narrow at its base. Cartilaginous, serially arranged elements comprising the fin main axis, called mesomeres, follow the pectoral or pelvic girdle, respectively, and become increasingly smaller toward the fin tip. The first mesomere is considered homologous to the femur/humerus of tetrapods, the second to radius and ulna or tibia and fibula, respectively (Romer et al., 1959; Kardong, 1997). Numerous fin radials are articulated dorsally (preaxially) and ventrally (postaxially) from the second mesomere onward, whereas the amount and arrangement of the fin radials is variable (Braus, 1900). The second mesomere usually has one pre-axial side radial on the pectoral and two or more on the pelvic fin, as well as about 4–5 postaxial lateral radials on the pectoral and about three on the pelvic fin. Subsequent mesomeres each carry one or two side radials postaxially and preaxially.

By contrast, the fins of South American and African lungfish species are significantly reduced. Preaxial and postaxial radials are missing altogether in *Lepidosiren*, whereas vestigial post-axial radials are present in *Protopterus*, which is why the fins look thin and thread-like, a conformation considered to be derived (Johanson et al., 2007). A striking difference between the two genera is that the pelvic fins of the South American male lungfish are covered with a unique array of filaments. These structures are not found in fins of other lungfish species. The function of these fin attachments has not yet been fully clarified. Suggestions that they serve as "limb gills" for the release or uptake of oxygen could not be directly confirmed (Lima et al., 2017).

Despite its great potential as a model organism for regenerative research very few studies of tail or fin regeneration in lungfish were published in the following decades after initial observations by Traquair (1871) and Conant (1970).

One cause for this lies in the difficulties of lungfish housing and breeding for research and the unavailability of embryos. Despite this, lungfish have attracted great scientific interest in

the field of regeneration in only recently, yielding important insights into the regeneration process and its evolution, via comparisons of lungfish and salamanders regeneration. While it has been known that lungfish develop a blastema at the wound site during the regeneration process that is comparable to those in salamander appendage regeneration (Conant, 1970), recent studies detected further similarities in the formation of a proliferative blastema, development of an Apical Epithelial Cap (AEC), and self-replacement of original structures including muscles, skeleton, and spinal cord (Verissimo et al., 2020). Transcriptome and differential gene expression analyses identified strong parallels in gene regulation and transcriptional profiles applied in lungfish and salamander appendage blastema formation (Nogueira et al., 2016). These commonalities highlight the importance of lungfish as model for regenerative research of body appendages and lend further support for the hypothesis of a deep evolutionary origin of regenerative capacities.

Apart from studies on regeneration in controlled laboratory experiments, investigations of naturally occurring regeneration provide crucial insights into the ecological and evolutionary parameters that may have influenced and directed regeneration in sarcopterygians. Since lungfish are very territorial animals (Curry-Lindahl, 1956), conspecific biting of fins and tails occur frequently both in the wild but also especially in captivity. In the Australian lungfish, primarily the juvenile individuals behave aggressive toward conspecifics, especially in view of food consumption and shelter areas. However, dominance hierarchies are also know among adults (Kind, 2002; Department of the Environment, Water, Heritage and the Arts, 2009; Jorgensen and Joss, 2016). In these settings, whole appendages or only parts may be bitten off and repeated biting may occur. As a result, fin pathologies and anomalies can often be observed in natural regenerates.

The main aim of our study was to compare the natural regenerative abilities of pectoral and pelvic fins of the three modern lungfish genera, the South American lungfish *Lepidosiren paradoxa*, the African lungfish *Protopterus* spp., as well as the Australian lungfish, *Neoceratodus forsteri*.

We present morphological and histological data of natural fin regenerates in all three modern taxa based to understand overall regeneration abilities as well as occurring anomalies and pathologies after failed regeneration. For this purpose, we have analyzed data from gross morphology, via histological serial sections, cleared, and double stained specimens, as well as x-ray computed tomography (μCT-scanning) and 3D reconstructions. The results are discussed in comparison to natural limb regenerates in the salamander model *Ambystoma mexicanum*, the Mexican axolotl.

MATERIALS AND METHODS

Material

Two fins of *Lepidosiren paradoxa* (ZMB_Pisces_37121, ZMB_Pisces_37122) were provided by Igor Schneider. These specimens were adults collected as wild caught in the city of Breves, state of Para, Brazil. The six specimens of *Protopterus* were obtained from the Royal Belgian Institute of Natural Science (RBINS 148, RBINS 8112) and the Royal Museum for Central Africa (RMCA_Vert_1973.015.P.0001, RMCA_Vert_1991.024.P.0001-0002, RMCA_Vert_P.124855-124859, RMCA_Vert_P.165214-165235). Three living specimens of *Neoceratodus forsteri* used for external gross observation were imported by Jindalee International Pty Ltd from an Australian lungfish farm, where they were reared in groups and obtained bite wounds on their appendages by conspecifics. In the animal husbandry of the Museum für Naturkunde Berlin individuals were kept separate in order to investigate the regeneration process of the fins without continued biting. *Neoceratodus* Specimen (ZMB_Pisces_33693) used for x-ray microtomography and histology is housed at the Museum für Naturkunde Berlin.

Gross morphology of specimen was examined with a Leica (MZ12) binocular microscope using ordinary transmitted light in magnifications ranging from 8x to 50x. Images were taken with a Leica DFC 495 Digital Color Microscope Camera (Leica Application Suite V4.2. software) and a Nikon D3100 digital camera.

Methods

Before using material for any analytical method, samples were fixed in fresh 4% paraformaldehyde in PBS (Phosphate Buffered Saline) for about 48 h. No information on the original fixation method of the specimens was available, but all collection material has been stored in 70% ethanol for long periods of time (several years to decades).

Because availability of material for destructive investigation was limited, we applied as many methods as possible to any given sample to maximize informative outcome. As μCT (X-ray microtomography) scanning without prior tissue staining produced low inherent contrast of non-mineralized soft tissues, a new suitable staining protocol using PTA (phosphotungstic acid) and/or iodine was developed to produce images with better tissue-specific gray contrasts. Unfortunately, these staining agents exert negative effects on subsequently performed histological serial sections and cleared and double staining methods.

Contrast-Enhanced Micro-CT Imaging and Analysis

Due to restrictions for the use of invasive methods on some of the collection material, different staining protocols were applied to the individual specimens. All specimens were stained at room temperature on a plate stirrer. Therein, the two most important staining parameters are the concentration of the staining solution and the duration of time that the specimen remains in solution. The concentration of the staining solutions was gradually increased within the first days to protect the tissue while achieving the best possible staining results. To check the progress of the staining and to avoid overstaining, test scans were carried out in the concomitantly.

Both fins of *Lepidosiren paradoxa* (ZMB_Pisces_37121, ZMB_Pisces_37122) were stained in a 10% solution of Lugol's iodine (I_2KI) in distilled water for 7 days. Fins

of the genus *Protopterus* (RMCA_Vert_P.165214-165235, RMCA_Vert_1991.024.P.0001-0002, RMCA_Vert_1973.015.P.0001, RMCA_Vert_P.124855-124859) were stained with 1.25% phosphotungstic acid (PTA) in distilled water for 3 weeks with the PTA solution changed twice during this period. A double staining protocol with iodine and PTA was tested on the fins of *Neoceratodus forsteri* (ZMB_Pisces_33693) with the first 7 days of staining with a 10% solution of Lugol's iodine (I_2KI) in distilled water followed by 2 weeks with 1.5% PTA in distilled water. The fins were examined through micro-tomographic analysis by using a YXLON FF35 CT. Scan settings varied depending on the object and among other things, were dependent on the size of the objects. *Lepidosiren paradoxa* (ZMB_Pisces_37121, ZMB_Pisces_37122) were scanned at 100 kV and 120 μA, generating 1,440 projections with 1,250 ms per picture. Effective voxel size was 6 μm. *Protopterus* specimen (RBINS 8112, RMCA_Vert_P.165214-165235, RMCA_Vert_1991.024.P.0001-0002, RMCA_Vert_1973.015.P.0001, RMCA_Vert_P.124855-124859) were scanned at 90 kV and 150 μA, generating 1,440 projections with 750 ms per picture. Effective voxel size ranged between 14 and 15 μm. Fins of *Neoceratodus forsteri* (ZMB_Pisces_33693) were scanned at 90/120 kV and 100/150 μA, generating 1,440 projections with 750 ms per picture. Effective voxel size ranged between 16 and 20μm. The cone beam reconstruction was performed using the datos| x-reconstruction software (GE Sensing and Inspection Technologies GMBH phoenix| x-ray datos| x 2) and the three-dimensional reconstructions were visualized in VGStudio Max 3.1. (Volume Graphics Inc., Germany). Fin skeletons were segmented manually from the stained scans.

Clearing and Double Staining

After micro CT scanning the left pelvic fin of *Neoceratodus forsteri* (ZMB_Pisces_33693) was skinned and cleared and double stained (Alcian blue and Alizarin red) for visualizing cartilage and bone. The protocol was modified according to Ovchinnikov (2009). The cartilaginous skeletal elements of the fins were stained in a 0.015%-Alcian-blue-solution for approximately 12 h and washed afterward in an ethanol series. Maceration was performed in trypsin (0.1%, Sigma) for 2 weeks at 37°C. Bony skeletal elements were stained in 0.01%-Alizarin-red-solution for approximately 6 h and washed afterward in a 30%-glycerin solution. For final storage, the sample was transferred to a 100% solution of glycerin.

Histological Staining

Lungfish fins were decalcified in 20% EDTA solution for about 2 weeks. Afterward, tissue was dehydrated by means of an ascending alcohol series (80–96–100%) and cleared with Xylene. Finally, the preparations were soaked and embedded in paraffin. The samples were serial sectioned at a thickness of 7–10 μm with a microtome. First, slides were deparaffinized with xylenes and thereafter rehydrated in a graded series of decreasing ethanol concentrations and distilled water. The serial sections were each stained alternately with Alcian Blue/Nuclear Fast Red or Heidenhain's Azan. Subsequently, the sections were dehydrated through an ascending series of ethanol and xylene and covered with Entellan (Merck KGaA) and a cover glass (detailed protocols for staining are attached in **Supplementary Materials**). Sections were viewed with a transmitted light microscope and photographed by using the Leica DFC495 Digital Color Microscope Camera mounted on the Axioskop and the Leica Application Suite V 4.2. Software.

RESULTS

Notably, in the majority of cases it is difficult to identify a regenerated fin from the outer morphology once the regeneration process is finished. The most evident indication for regeneration are fin abnormalities such as stark morphological deformations, bifurcations, but also constrictions, foreshortened fins and paired fins of uneven lengths.

Lepidosiren paradoxa (South American Lungfish)

In their original state, South American lungfish fins consist of only one fin radial composed of numerous serially repeated cartilaginous fin radial elements. For this study we investigated two regenerated pectoral fins of this taxon in detail (ZMB_Pisces_37121, ZMB_Pisces_37122).

The pectoral fin of specimen number ZMB_Pisces_37121 does not show any obvious signs of regeneration in its outer morphology (**Figure 1A**), except for a noticeable narrowing in the very proximal region of the fin (**Figure 1A**, dashed box). CT scanning and the 3D reconstruction revealed constrictions of two proximal radial elements (**Figures 1B–D**, arrow) and an amalgamation with the adjacent distal elements (**Figures 1B–D**, arrow). Additionally, several fused elements were identified in the more distal region of the fin (**Figures 1B**, dashed box; 1E, arrow). Histological investigation revealed detailed information on the cellular level and showed that the constrictions of the skeletal fin radial elements affect all types of tissue at this position of the fin, including the perichondrium, the cartilaginous matrix, and musculature (**Figures 1F,G**). In addition, the epidermis is not fully regenerated (**Figures 1D,F**, asterisks). Contrary to the assumption that the epidermis regenerates pretty fast in order to protect the wound healing area from infection while internal parts regenerate slower, in this specimen it is unusually thin in some places. At the narrowest point of the constriction, it seems to be completely absent. This type of pathology is not known from salamander limb regeneration, but may be a sign of repeated biting. However, causes other than regeneration for this anomaly are also possible, as for example and infection or skin disease. Nevertheless, it appears likely that the restriction coincides with the location of the bite sites and sections also show jointed individual fin radial elements in several regions of the fin (**Figure 1G**). Despite the greater resolution on the cellular level, it remains unclear whether these are fused elements, for which complete separation failed during initial fin development, or whether they were also caused by constrictions of the periosteum and the cartilaginous matrix of a single fin radial element during the regeneration process.

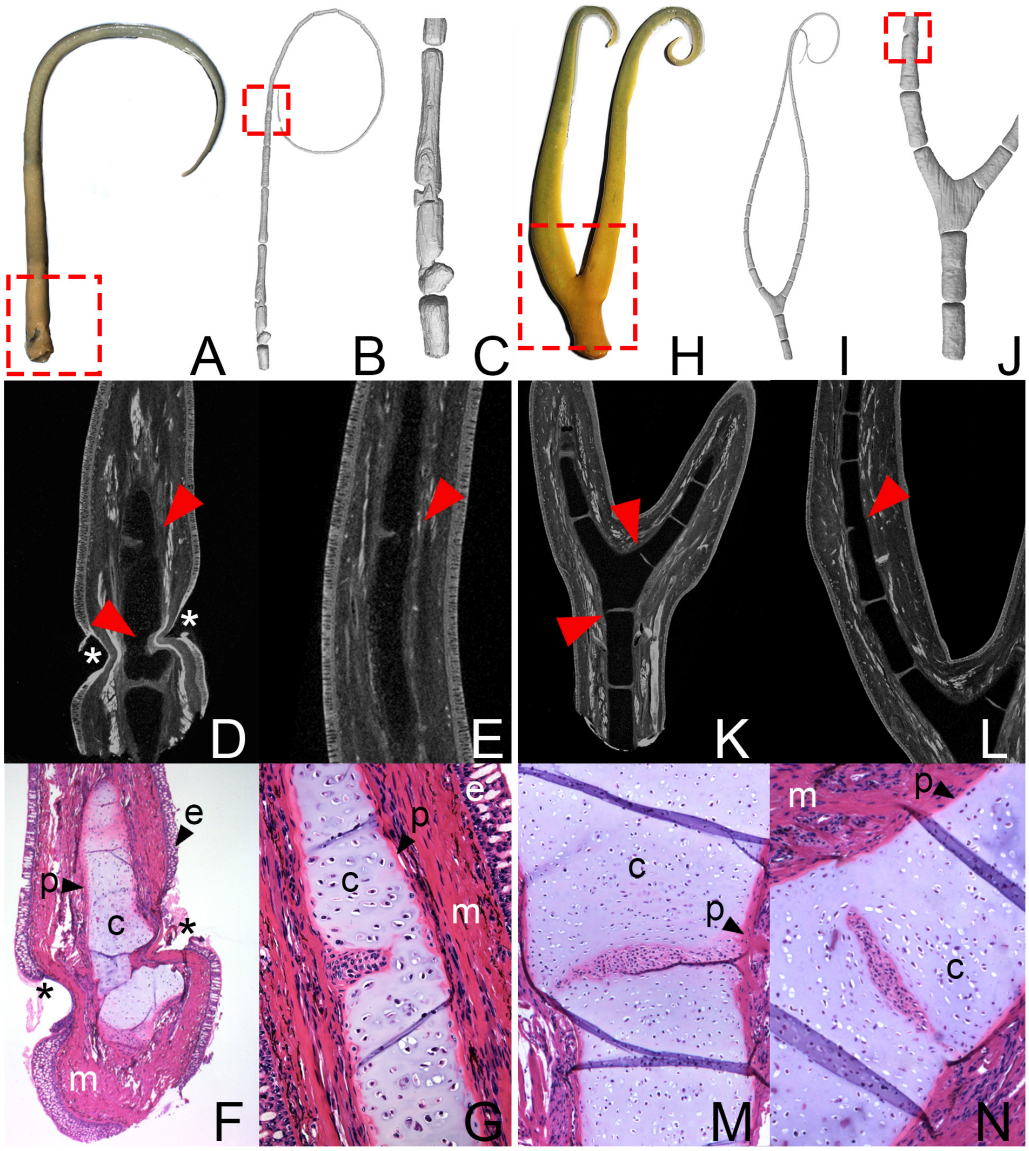


FIGURE 1 | (A–G) Pectoral fin of *Lepidosiren paradoxa* (ZMB_Pisces_37121). 3D reconstruction: **(A)** Exterior view with the suspected bite site (dashed box). 3D reconstruction: **(B)** of the entire fin, dashed box shows area of fused elements, and **(C)** detailed view of the proximal fin area. μCT scans: **(D)** of the constricted fin area and **(E)** fusion of the skeletal elements. Arrows indicate fused and constricted skeletal elements. Asterisks indicate area with missing epidermis. Histological serial section with Azan staining **(F)** of the constricted fin area and **(G)** fusion of the skeletal elements. **(H–N)** Bifurcated pectoral fin of *Lepidosiren paradoxa* (ZMB_Pisces_37121). **(H)** Exterior view with the suspected bite site (dashed box). 3D reconstruction: **(B)** of the entire fin and **(C)** detailed view of the proximal fin area, dashed box shows area of fused elements. μCT scans: **(D)** of the bifurcated element and **(E)** fusion of the skeletal elements. Arrows indicate fused skeletal elements. Histological serial section with Azan staining: **(F)** of the most proximal part of the bifurcated element and **(G)** fusion of the bifurcated element with the adjacent skeletal element. c, cartilage; e, epidermis; m, muscles; p, perichondrium.

The second fin of *Lepidosiren* (ZMB_Pisces_37121) shows an obvious anomaly that is already visible in gross observation. The fin divides at the proximal end and branches into two fin axes (**Figure 1H**, dashed box). Histology and μCT-scanning reveal that this bifurcation is caused by branching of a single proximal skeletal element (**Figures 1I–N**). Additionally, the bifurcated element displays partial amalgamation with the adjacent elements (**Figure 1K**, arrows; **Figures 1M,N**). Partial fusion is also visible between more distal elements (**Figure 1J**, dashed box; **Figure 1L**, arrow).

Protopterus (African Lungfish)

Protopterus fins have an overall similar structure to those of *Lepidosiren*, except for the filamentous structures on the pelvic fins of males, which are completely absent in *Protopterus*.

Six lungfish individuals of three *Protopterus* species with regeneration malformations were identified in the

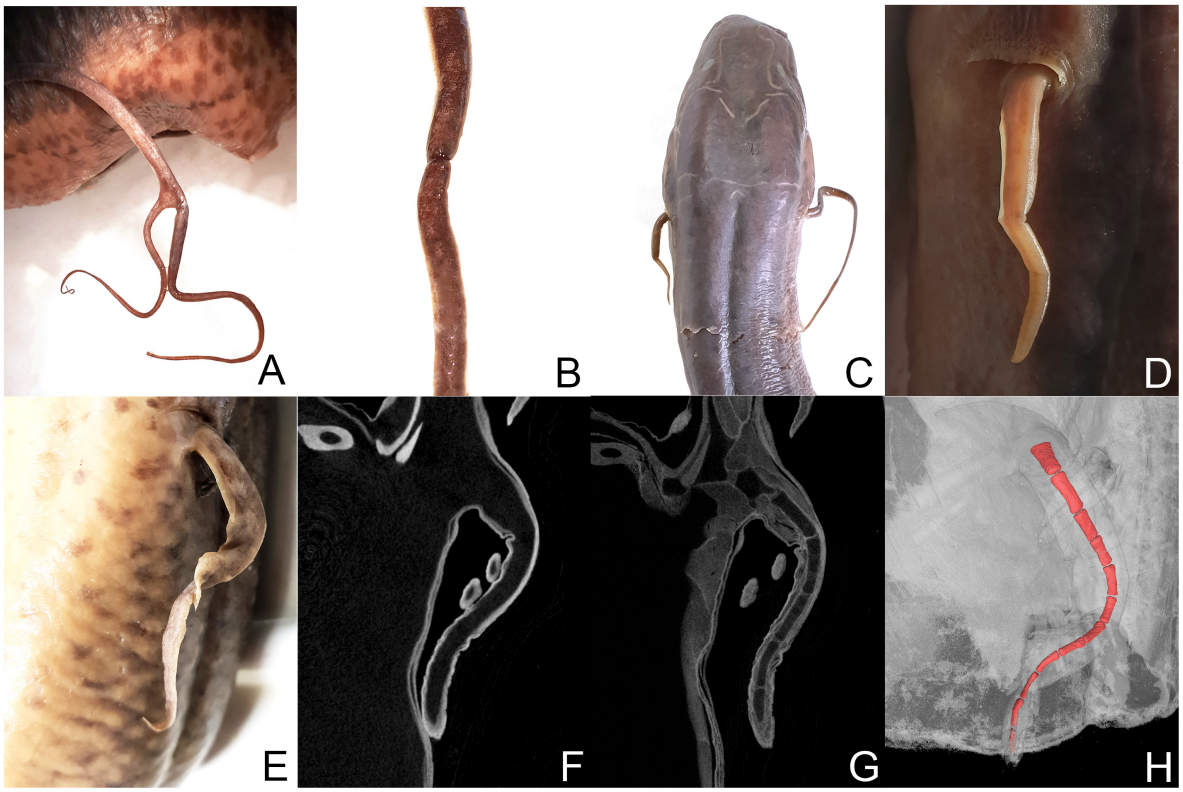


FIGURE 2 | *Protopterus aethiopicus* (RBINS 148): **(A)** bifurcation of the left pectoral fin and **(B)** constriction of the right pectoral fin. *Protopterus dolloi* (RBINS 8112): **(C)** paired pectoral fins. **(D)** Close-up of the shorten fin. *Protopterus annectens brieni* (RMCA_Vert_P.165214-165235): **(E)** left pectoral fin. **(F)** μCT scan without tissue staining. **(G)** μCT scan after staining with iodine. **(H)** 3D modeling of the fin.

collections of the Royal Belgian Institute of Natural Science and the Royal Museum for Central Africa (RBINS 148, RBINS 8112, RMCA_Vert_1973.015.P.0001, RMCA_Vert_1991.024.P.0001-0002, RMCA_Vert_P.124855-124859, RMCA_Vert_P.165214-165235). Anomalies were identified in both pectoral and pelvic fins. Evident characteristics are bifurcations, as well as constrictions and bilaterally asymmetrical, foreshortened fins (**Figures 2**, **3**).

One specimen of the species *Protopterus aethiopicus* (RBINS 148) exhibits bifurcation of the left pectoral fin and a constriction of the right pectoral fin, but was only available for external observation (**Figures 2A,B**). One individual of the species *Protopterus dolloi* (RBINS 8112) with paired fins of unequal length was investigated by micro CT (**Figures 2C,D**). However, permission for tissue staining was not granted for this specimen and hence the resolution was insufficient to reconstruct the fin skeleton in 3D. The strong differences in the visibility of structures in unstained and stained material are shown in the comparison of the CT scans of pectoral fins of *Protopterus annectens brieni* (RMCA_Vert_P.165214-165235) before and after staining with I_2KI and PTA (**Figures 2E–G**). The latter specimen shows a relatively short, strangely shaped fin morphology as well as incisions in the skin of the fin. The strange shape may be a preservational relic from being squeezed into a jar for an extended period. However, despite the unusual outer morphology, no skeletal abnormalities were identified in the 3D reconstruction (**Figure 2H**). Therefore, the regeneration process may have proceeded normally in this fin, but was not yet fully completed at the time the animal was collected.

Four fins of *Protopterus annectens* that showed bifurcations in external morphology, were investigated by μCT-scanning. The scans revealed that bifurcation do not all follow the same anatomical pattern but instead can be produced by different branching patterns involving the structures of the fin skeleton (**Figure 3**). In the left pelvic fin of RMCA_Vert_1991.024.P.0001-0002, one proximal element forms the base point for two more distal elements, which continue distally in building two separate fin radials (**Figures 3A–C**). In the left pelvic fin of *Protopterus annectens annectens* RMCA_Vert_1973.015.P.0001, the bifurcation is built by two elements, whereas an additional short fin element attaches laterally at the lower end of a significantly elongated element of the metapterygial axis and thus forms an additional lateral fin radial (**Figures 3D–F**). In this specimen the fins remain relatively short distal to the bifurcation. Whether this condition was caused by failed regeneration or rather in completed regeneration, cannot be resolved by the CT data.

A third branching pattern can be observed in the in the right pectoral fin of *Protopterus annectens annectens* RMCA_Vert_1973.015.P.0001. In this fin, a single fin element

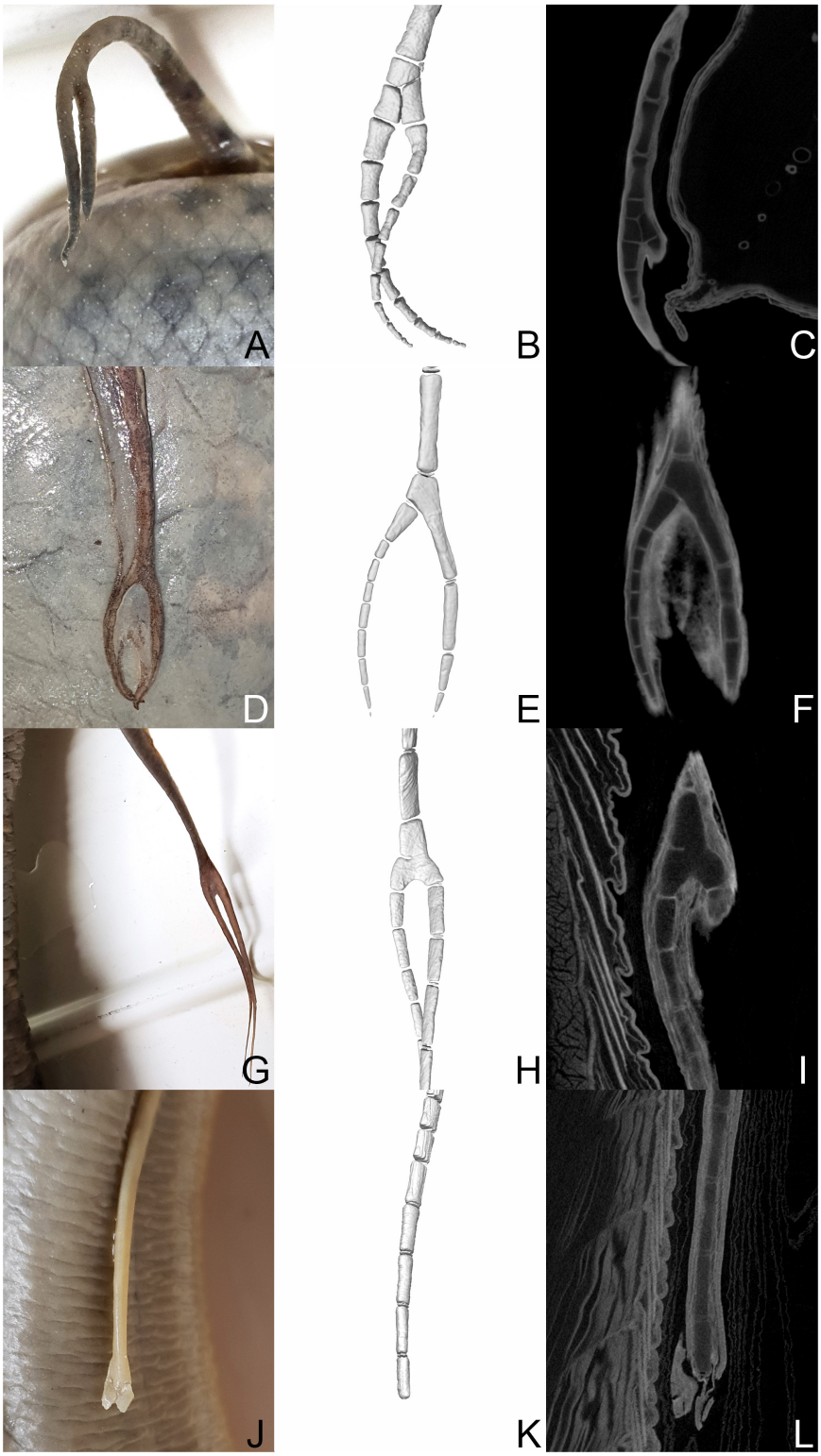


FIGURE 3 | *Protopterus annectens spec*. (RMCA_Vert_1991.024.P.0001-0002): **(A–C)** left pelvic fin. *Protopterus annectens annectens* (RMCA_Vert_1973.015.P.0001): **(D–F)** left pelvic fin, **(G–I)** right pectoral fin. *Protopterus annectens annectens* (RMCA_Vert_P.124855-124859): **(J–L)** Right pectoral fin. **(B,E,H,K)** 3D reconstructions of the areas of bifurcation. **(C,F,I,L)** μCT scannings of the areas of bifurcation.

bifurcates distally and forms the attachment point for two further fin radial elements. The 3D reconstruction of the branching point suggests that this type of bifurcation was caused by incomplete segmentation of several elements (**Figure 3G**). Yet another fin regenerate morphology is visible in the right pectoral fin of *Protopterus annectens annectens* (RMCA_Vert_P.124855-124859) (**Figures 3J–L**). It shows a short fin, which externally shows signs of bifurcation in the soft tissue, but the internal anatomy of the cartilaginous fin skeleton still shows a single row of fin elements. The distal end of the fin, which is most likely the site of a bite injury, contains no skeletal elements. This fin morphology can most likely be explained with incomplete regeneration at the time of death of the animal or the regeneration process has failed for some reason. Fusions of several adjacent axis elements distal to the presumed amputation plane, as observed in *Lepidosiren* fins (**Figure 1**), were not observed in the investigated regenerates of *Protopterus*.

Neoceratodus forsteri (Australian Lungfish)

The third genus of extant lungfish is *Neoceratodus* with only a single species, the Australian lungfish *Neoceratodus fosteri*. In contrast to the other extant lungfish genera, the fin morphology of *Neoceratodus* resembles most closely that of its fossil relatives in having strong, fleshy fins, with a much more complex skeletal anatomy (**Figures 4A,B**). The fin consists of a large element, mesomere 1, which articulates with the shoulder girdle and does not carry any radials. A series of further mesomeres articulate distally to mesomere 1 and form the medial axis of the fin with preaxial and postaxial radials articulating to the mesomeres. Although regenerative capacities have been assumed for *Neoceratodus*, to our knowledge there has thus far not been a published report as to whether and how well *Neoceratodus* is able to regenerate their fins. However, the following results of detailed studies of the fin in μCT scans and histology indicate that regeneration processes are taking place.

Figures 4C–N show some examples of pectoral and pelvic fins of living individuals of *Neoceratodus forsteri* with obvious fin abnormalities after regeneration following repeated biting. Shortened, misshapen and stunted fins can clearly be recognized. Some of these anomalies look very severe. The reason for the severity of the abnormalities lies most likely in repeated and multiple biting of the fins. We cannot state with certainty whether the regeneration of these fins has been completed, or whether the fins are still in the process of regeneration. Notably, observation and documentation of these *Neoceratodus* individuals and their fins over a period of 2 years (provided in **Supplementary Data**) did not show any noticeable changes in the shape of these fins.

In addition to investigations of the living animals, *Neoceratodus* specimen ZMB_Pisces_33693 from the collection of the Museum für Naturkunde Berlin showing obvious fin anomalies was provided for destructive sampling (**Figure 5**). In particular, both pectoral fins displayed strongly deformed morphology upon external observation (**Figures 5A,E**). Both fins are shortened, malformed and do not show the original leaf-like fin shape. The pelvic fins did not display clear external signs for regeneration, yet the right fin is slightly shorter than the left, indicating a possible earlier regeneration event (**Figures 5I,M**).

The results of the CT scans and 3D modeling clearly showed that in both the left and right pectoral fin the central axis is strongly foreshortened as compared to the normal fin anatomy (**Figures 5B,C,F,G**). Moreover, preaxial and postaxial fin radials are asymmetric, some radials show distal branching while other radials are missing altogether. In the left pectoral fin, the skeletal abnormalities begin at the level of the second mesomere (**Figures 5B–D**). The preaxial lateral radials attached to mesomere 2 is strongly shortened and consists of only one skeletal element instead of at least three as in the normal fin anatomy. Further distally, the skeletal anatomy of the fin is severely altered. The fourth and fifth mesomeres are completely deformed. The skeletal elements in this area are partially shifted in the transverse plane so that they overlap (**Figure 5D**). Furthermore, a postaxial radial shows clear branching (**Figure 5B**). Finally, the most distal end of the fin, which normally tapers peripherally to a thin thread, is missing entirely.

In the right pectoral fin, the pathologies start more distally than in the left fin at the height of the fifth mesomere, indicating a more distal bite wound. The anomaly in skeletal anatomy is overall less severe than in the left fin. The most noticeable anomalies are the missing, thread-like distal fin tip and bifurcating pre- and postaxial lateral radials (**Figures 5F,H**). One pre-axial lateral radial starting at the fifth mesomere even shows a double branch, which is extraordinarily wide.

μCT-scanning as well as clearing and double staining of the left pelvis fin revealed a bent distal tip of the main axis which is caused by multiple deformed axial elements at the distal end. At the level of the fifth mesomere, bifurcation of an element of the pre-axial lateral radial is visible (**Figures 5J–L**).

Branching in postaxial radial was also identified in the right pelvic fin, where also one preaxial lateral radial is fused with the fourth mesomere (**Figure 5N**). In addition, amalgamation of adjacent axial segments along the central axis is present in three positions (**Figure 5O**), as is particularly well visible in the histological serial sections (**Figure 5P**).

DISCUSSION

Regenerative Abilities of Lungfish

Earlier work has shown that the various extant lungfish genera are able to regenerate both tails and fins. Some studies have carried out controlled amputation experiments in the laboratory (Conant, 1970; Nogueira et al., 2016; Verissimo et al., 2020), while others reported numerous regenerated fins in natural populations (Nogueira et al., 2016). Most studies, however, are based on the South American lungfish, *Lepidosiren*, and the African lungfish, *Protopterus*. These genera are characterized by their thread-like fin structure and a lack of pre- and post-axial radial elements. To our knowledge, in contrast, nothing is known on the regenerative abilities of fins in the Australian lungfish, *Neoceratodus forsteri*. However, *Neoceratodus* is of particular interest for regeneration research, because among the

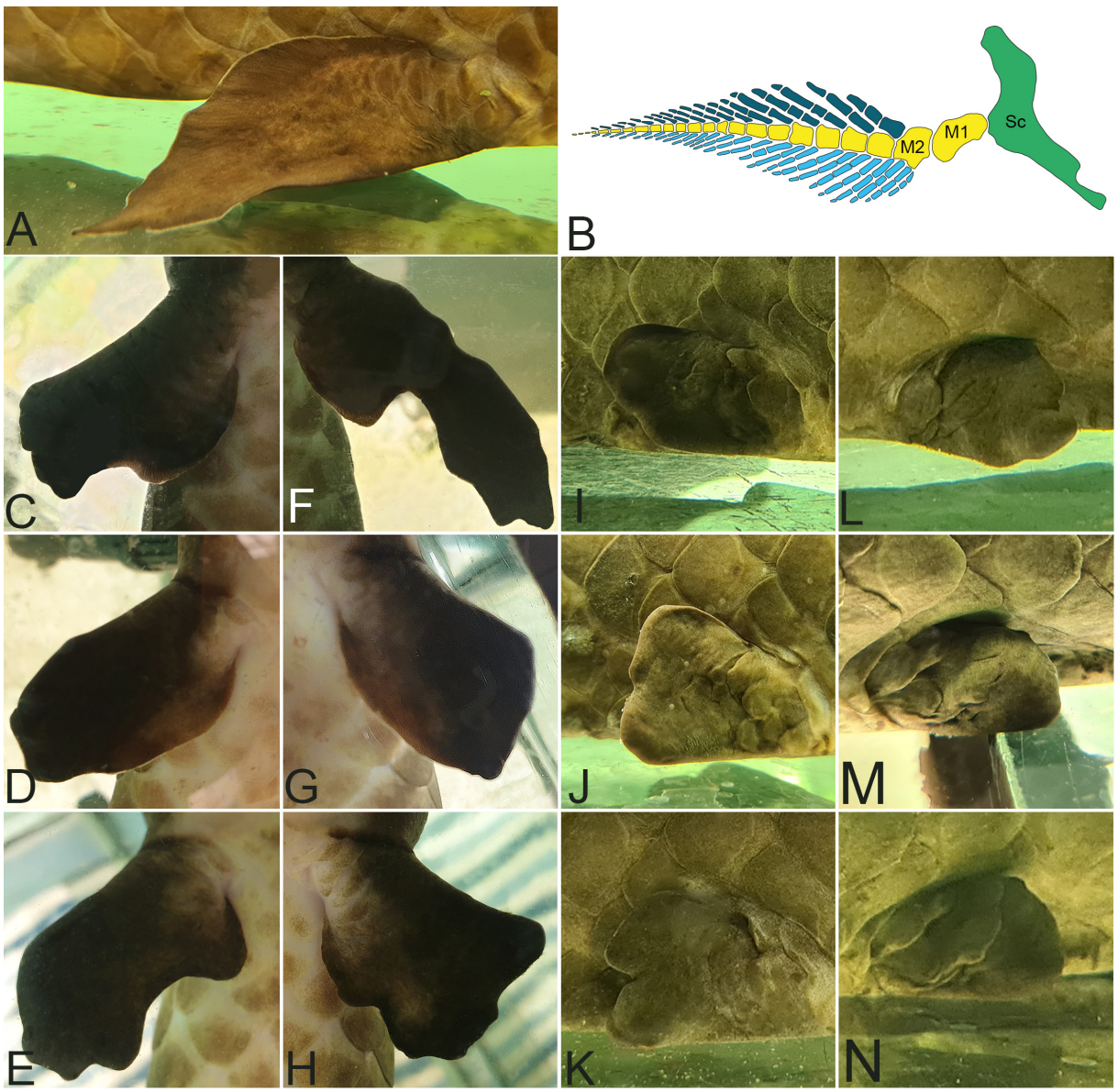


FIGURE 4 | *Neoceratodus fosteri*: **(A)** leaf-like shape of a not regenerated pelvic fin **(B)** Model of a Skeleton anatomy of a not regenerated pectoral lungfish fin. Sc, scapula in green; M, Mesomere in yellow. Preaxial radials in dark blue. Postaxial radial in light blue. **(C–H)** Left and right pectoral fins with deformations in ventral view. **(I–N)** Left and right pelvic fins with deformations in lateral view.

modern taxa its fin anatomy resembles most closely the ancestral condition of lungfish fin anatomy, which is quite a bit more complex than that of the other two genera. The phylogenetic position of lungfish as the closest living relatives of tetrapods also makes them crucial taxa for investigations on the evolution of the regenerative program allowing for fin and tail regeneration in sarcopterygians (Verissimo et al., 2020).

While not in every single case it can be excluded that fin anomalies were caused by developmental defects, our study demonstrates that all five studied lungfish species are very likely able to regenerate fins after natural bite injuries, including the Australian lungfish. Strong indicators for ongoing regenerative processes in *Neoceratodus* in contrast to malformations caused by severe bite injuries, are distally branching radials, abnormal numbers of radials attached to respective mesomeres and deformed mesomeres, which strongly deviate from the original anatomy.

In an evolutionary context, these findings suggest the ability to regenerate body appendages is plesiomorphic for modern lungfish genera, which is in line with molecular studies that indicate a deep evolutionary origin of appendage regeneration (Darnet et al., 2019; Verissimo et al., 2020).

Therein, the regeneration of the missing parts of the fin occurs to various degrees, from partial to near complete regrowth,

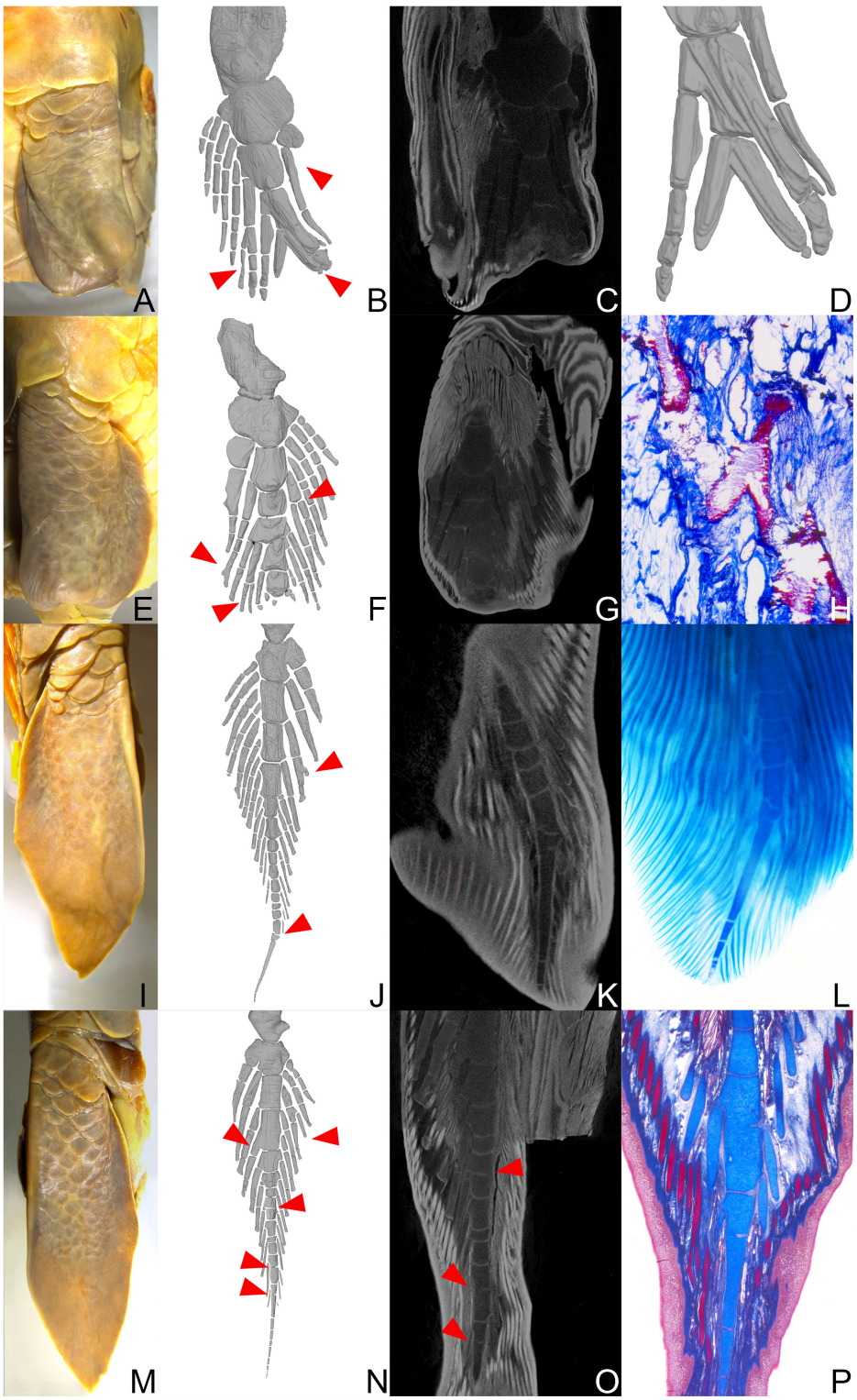


FIGURE 5 | *Neoceratodus forsteri* (ZMB_Pisces_33693): **(A)** Left pectoral fin. **(E)** Right pectoral fin. **(I)** Left pelvic fin. **(M)** Right pelvic fin. All fins in ventral view. **(B,F,J,N)** 3D reconstructions of the entire fins. **(C,G,K,O)** μCT scannings. **(D)** Detailed 3D reconstruction of the distal fin tip. **(H,P)** Histological serial sections, stained with Alcian blue. **(L)** Clearing and double staining of the distal fin tip. Red arrows indicate striking malformations of the fin skeleton.

with lighter and more severe pathologies. The factors are very likely depending on the living conditions and the occurrence of repeated biting, but differences between the overall regenerative capacities of different lungfish taxa can also not be ruled out based on our data.

Regeneration Investigations Under Controlled Laboratory Conditions vs. in the Wild

The frequency of regenerated fins found in specimens in natural history collections as well as in the living specimens in animal husbandry and in the wild (Nogueira et al., 2016) strongly suggest that fin regeneration and the occurrence of associated pathologies are widespread phenomena. Since lungfish are very territorial animals, damage to a fin or loss of a fin as a result of conspecifics biting happens frequently in both in captivity and the wild. Conant (1973) observed a rate of about 20% of bite injuries among captive African lungfish and Nogueira et al. (2016) report a similar rate of almost 19% of externally visible fin pathologies in wild-caught South American lungfish. Mlewa and Green (2004), on the other hand, reported a much lower rate of pathologies in the fins and tails of African lungfish *Protopterus athiopicus* (approx. 4%) during their investigations in the wild. In their study they also mention developmental abnormalities, but it remains unclear if these may indeed not represent cases of failed regeneration as well.

Moreover, all of the above-mentioned studies refer to the obvious, externally visible pathologies. Here we showed that fins with a seemingly normal external morphology indeed show anatomical abnormalities when investigated by histology and/or Ct scanning methods. Hence, the actual frequency of bite injuries and regenerated body appendages in lungfish populations is difficult to determine by gross observation alone and may indeed be much higher, especially when also taking into consideration the instances where regeneration proceeds normally and results in an anatomically normal fin. Moreover, environmental factors such as population density, food availability, age distribution, and season certainly have a strong influence on aggression and bite frequency and hence regeneration frequency in all lungfish taxa. Animals housed in natural history collections or caught in the wild for a different research purpose often lack the metadata that would allow for a better assessment of the impact of these parameters on regeneration frequency and the numerical distribution of pathologies. Therefore, there are several points that limit the interpretation of the results based on this material. In retrospect, it cannot be determined exactly whether limbs were completely severed or only partially. Especially in case of the very compact fins of the Australian lungfish, it seems likely that often only parts of the fin are lost to conspecific biting rather than a whole fin. It remains unknown whether regeneration in lungfish fins is hampered by, proceeds equally well or rather proceeds better if only a part of the fin is severed from a fin as compared to the loss of a complete fin. Observations have shown that some injuries are challenging for axolotls and not all types of wounds trigger a regeneration process. For example, lateral limb wounds and larger gabs of certain dimension in long bones do not heal properly or even show no evidence for regeneration at the injury site at all (Roy and Lévesque, 2006; Hutchison et al., 2007; Lee and Gardiner, 2012; Vieira et al., 2019). These findings indicate that the mechanisms underlying regeneration are different from those of regular bone healing. It is also possible that fins are injured repeatedly, and follow-up injuries can occur after regeneration is complete, during an ongoing regeneration process or during the important phase of blastema formation. In fact, a study by Bryant et al. (2017b) on regenerative abilities in axolotl limbs has revealed a state of persistent wound healing reaction generated by multiple repeated amputations at the same site, which in turn inhibits successful regeneration of the missing limb part. Therefore it can be assumed that the more often a fin is damaged, the higher the probability that it will develop anomalies during regeneration. Furthermore, recurring bite injuries cause renewed disruption of tissue structures and hence positional information needed for the proper replacement of missing body parts. This is also indicated by the fact that anomalies in skeletal limb and fin structure do not always only occur directly at the amputation level or location of bite injury, but sometimes also in more distal regions (Bothe et al., 2021), which can hamper identification of the exact plane of amputation.

Another aspect that remains unresolved by the data at hand is the age of the animal at the time when the fin was injured and the regeneration process started. It is unknown whether the regenerative abilities or qualities vary with age in lungfish, but this is well documented in frogs and salamanders. Frogs are only able to regenerate limbs in the tadpole stage, but once metamorphosis is completed, this ability is absent in postmetamorphic individuals (Dent, 1962). Salamanders, on the other hand, are able to regenerate appendages throughout their entire lifespan (Zeleny, 1909). However, the speed and quality of regeneration seem to decrease with increasing age (Vieira et al., 2020; Bothe et al., 2021). Coincidently with this, pathologies in the regenerated axolotl occur more frequently in older animals than in young larvae (Bothe et al., 2021). Furthermore, it is usually unknown whether the regeneration process was completed or stopped when the animal died. Shortened fins, for example, could either indicate a failed or halted regeneration in the living lungfish or be the result of incomplete regeneration, because the animal died before the process could be completed.

Finally, it cannot be completely ruled out that the identified fin anomalies have not already resulted from faulty fin development.

Quality of Regeneration and Types of Anomalies

Despite the limitation of the data outlined above, it is very important to examine regeneration after bite injuries in lungfish and to compare it to controlled amputation attempts in the laboratory. Studies on salamanders indicate that pathologies and anomalies occur predominantly after bite injuries caused either by conspecifics or predators (Bothe et al., 2021). Hence, these pathologies are not random oddities, but are a common feature of the regeneration process and understanding the

forms and causes of pathological regenerates is essential for understanding the underlying processes and evolutionary context of regeneration. In bites, tissue damage is much more severe than in the clean cuts of targeted amputations. The fin is torn, ruptures and is squeezed leading to frayed and disrupted tissue at the wound site. This in turn seems to often have a negative impact on wound healing, blastema development, and subsequent replacement of body parts. Multiple studies of cell identity during regeneration in the axolotl have discovered that cells at the injury site hold positional information in relation to one another along the proximodistal and anteroposterior limb axes, which are responsible for a successful rebuilding of limb structures with respect to growth and pattern formation (French et al., 1976; Bryant et al., 1981; Gardiner et al., 1995; Torok et al., 1998; Echeverri and Tanaka, 2005; Mercader et al., 2005; Roensch et al., 2013). Therefore, it appears logical that, the more severe the damage to the tissue, the more chaotic the tissue arrangement in the wound area is and the more often regenerative pathologies occur as a result to faulty positional information and pattern formation. Anomalies resulting from imperfect fin regeneration occur frequently and were visible by exterior and interior observation in all specimens studied. Some pathologies were less severe, others significantly changed the original anatomical structure of the respective lungfish fin. They include constrictions of various types of tissues, fusion of skeletal elements, distal branching and additional or less skeletal elements relative to the normal fin anatomy. The most basic element of the axillary radial (mesomere 1) articulated with the endoskeletal shoulder girdle or pelvic girdle and is rarely affected by pathologies. This is likely due to its position close to the body wall, which makes it less likely to be affected by bite injuries than more distal parts of the fin. No significant differences were registered in the type of pathology between pelvic and pectoral fins.

The data also hints at the possibility that there may be a connection between anatomical complexity and frequency and severity of regenerative pathologies. The fins of *Protopterus* and *Lepidosiren* have a less complex skeletal anatomy than the fins of *Neoceratodus* or a salamander limb. In *Protopterus* and *Lepidosiren* fin regeneration entails replacement of a thread-like fin without lateral radials and an overall rather simple anatomical structure, which therefore may be less prone to regenerative pathologies than the more complex structures of a *Neoceratodus* fin or a tetrapod limb. In accordance with this hypothesis, pathologies are less pronounced and less obvious externally, in *Protopterus* and *Lepidosiren* fins than in *Neoceratodus*. Moreover, the available data for *Neoceratodus* indicates that the more distal the injury, the less complex is the anatomical structures that has to be replaced by regeneration and the fewer pathologies occur. However, a potential connection between structure complexity and frequency of pathologies in regenerates will have to be tested in a rigorous experimental framework in order to be conclusively demonstrated or dismissed.

In any case, overall all lungfish fin anomalies follow a very similar patterns and structure and includes, failed segmentation, merging of elements, reduced or increased number of segments, bifurcation of elements, and constrictions of the various tissues and are likewise very similar to pathologies observed in regenerated salamander limbs (Dearlove and Dresden, 1976; Stock and Bryant, 1981; Bryant and Gardiner, 2016; Soto-Rojas et al., 2017; Bothe et al., 2021).

Biological Importance of Perfect Regeneration

The relative frequency with which more or less severe pathologies occur during appendage regeneration raises the question of how important the quality or anatomical perfection of the regenerated appendages is in a biological context, i.e., for the fitness of the animal.

The original function of the structures plays an important role in this context, especially with respect to paired appendages such as fins and limbs. Ecology and habitat may influence how strong the impact of a severely malformed limb regenerate is for a given individual or taxon, as e.g., the limbs are less important for effective locomotion in an aquatic axolotl that can propel through the water effectively with its tail, than for a highly terrestrial plethodontid salamander inhabiting steep rock surfaces. Likewise, a lungfish fin may be less physically strained in deep water locomotion as compared to the limb of a terrestrial salamander, but nevertheless fulfills important functions, e.g., the Australian lungfish uses its strong, fleshy fins as support for the tail when swimming forward in ascending movements, to maneuver in shallow water and to support on the substrate when eating (Dean, 1906; Kemp, 1986). Lungfish fins can also be used in a tetrapod-like fashion in *Neoceratodus* (Dean, 1912) which was also demonstrated for the African lungfish *Protopterus annectens*, despite its reduced fin anatomy (King et al., 2011). King et al. (2011) showed that pectoral fins of *Protopterus* are used to lift the body of the substrate for terapod-like walking and bounding movements in aquatic environments. Therefore, it is not easy to assess how strongly severe pathologies in regenerated appendages may impact the fitness of individual lungfish in natural environments. Therein, some types of pathologies such as bifurcation and fusion of skeletal elements are unlikely to have a major negative impact on movements such as swimming or supporting the body, whereas truncated or mutilated fins could severely restrict the usability of the fin for maneuvering and pushing off the ground. With this disability, these individuals could become victims of new bite attacks and predation more easily or have a disadvantage in the competition for food.

Comparison to Pathologies Occurring in Salamander Limb Regeneration

While axolotls are well known for their outstanding regenerative abilities, axolotl limbs often show a wide variety of limb and digit anomalies after bite injuries (Thompson et al., 2014; Bothe et al., 2021) that are very similar to those seen in lungfish fin regeneration. Common abnormalities after imperfect regeneration are for example syndactyly (fusion of two or more digits), ectrodactyly (split limb), brachydactyly (short digits), and limbs with additional or missing digits (Dearlove and Dresden, 1976; Young, 1977; Stock and Bryant, 1981; Bryant and Gardiner, 2016; Soto-Rojas et al., 2017; Bothe et al.,

2021). Thompson et al. (2014) investigated the probability of regenerating a proper limb after bite injuries by conspecifics among larvae and adult axolotl in a laboratory setting. The rate of bite injuries among the larvae was very high with a value of 80%. After regeneration, more than half of the larvae exhibited pathologies on at least one limb, including variant digit numbers, fused digits, and digits growing from atypical anatomical positions. However, not only after conspecific biting, but also after controlled amputations with clean surgical cuts, regenerated limbs often did not regenerate perfectly (Bothe et al., 2021). Malformations occurring after surgical amputations are usually less severe and not immediately noticeable in external observation. Frequently occurring anomalies are partial or full constriction of the perichondrium, intercellular space in the cartilage matrix, narrowing of radius and ulna, reduced numbers of mesopodial bones caused by fusions and shorter digits with a reduced number of phalangeal elements.

The ability to regenerate is extremely widespread in the salamander clade, and it is therefore not surprising that regeneration pathologies have been reported not only in the axolotl, but also in other salamander species, including the Eastern newt, *Notophthalmus viridescens* (Dearlove and Dresden, 1976), and the red−backed salamander, *Plethodon cinereus* (Dinsmore and Hanken, 1986). Overall, the frequency and patterns of anomalies following regeneration are very similar in the investigated salamander taxa and all species of modern lungfish and are suggestive of shared processes governing appendage regeneration in all these taxa.

Origin of Body Appendage Regeneration

The high regenerative capacities of some organisms, but the lack thereof in others has fascinated researchers for centuries and has led to discussions on the evolution of regeneration and the reasons for its emergence and loss in various animal lineages (e.g., Bely, 2010; Bely and Nyberg, 2010; Nogueira et al., 2016; Amaral and Schneider, 2018). Among extant tetrapods, only salamanders are capable of regenerating limbs (in addition to other body parts such as tails, lenses, and parts of inner organs) throughout their entire lifespan and with near perfection. Because of its uniqueness among extant tetrapods, it was frequently suggested that this ability arose independently in the evolutionary lineage of salamanders (Garza-Garcia et al., 2010; Brockes and Gates, 2014). This assumption was supported by the discovery of salamander lineage-specific genes (LSGs) which were shown to be involved in the limb regeneration process (Kumar et al., 2007; Looso et al., 2012, 2013; Brockes and Gates, 2014). However, the fossil record showed that high regenerative capacities as seen in modern salamanders are indeed not salamander specific, but salamander-like regeneration of limbs and tails was already present in the temnospondyl- and lepospondyl lineage of anamniote tetrapods some 300 million years ago (Fröbisch et al., 2014, 2015).

The fossil data was complemented by morphological and molecular studied, which further supported an ancient origin of epigenetic regeneration in vertebrates. Nogueira et al. (2016) compared the molecular program of appendage regeneration in axolotl and the South American lungfish, *Lepidosiren paradoxa*, and found extensive similarities in the molecular program deployed during appendage regeneration in both taxa. They also reported stark morphological similarities in the regeneration process, including in the formation of a wound epithelium, histolysis, dedifferentiation, subsequent blastema proliferation, and repatterning of missing structures. Similarly, great similarities mechanisms in the molecular program governing tail regeneration in salamanders and lungfish were found by Verissimo et al. (2020) lending further support for a shared molecular regeneration program in sarcopterygians.

Outside of Sarcopterygii, fin regeneration including the endoskeleton was demonstrated for the basal actinopterygian *Polypterus* by Cuervo et al. (2012) leading to the suggestion that appendage regeneration be plesiomorphic for Osteichthyes (bony vertebrates), which, however, seemed to stand in contrast to the notion that teleost fish are capable of regenerating dermal fin radials but not of regeneration of their fin endoskeleton. In a broad approach Darnet et al. (2019) combined fin regeneration assays and comparative RNA-sequencing analysis of *Polypterus* and axolotl blastemas revealing a shared regeneration-specific genetic program in the basal actinopterygian and salamanders. Moreover, Darnet et al. (2019) were able to show through fin endoskeleton amputation experiments, that further non-teleost actinopterygians, namely the American paddlefish (*Polyodon*) and the spottet gar (*Lepisosteus*), as well as three teleost species were capable of full fin regeneration after endochondral amputation (Darnet et al., 2019).

The similarity in frequency and patterns of fin regeneration pathologies between all three extant lungfish genera and salamander limbs demonstrated here lend further support for the similarity of the underlying processes in and limitations to appendage regeneration in these groups.

Taken together, the morphological, paleontological, and molecular data strongly suggests that the capacity for full appendage regeneration is a plesiomorphic feature for all sarcopterygians (lobe-finned fish including four limbed vertebrates), which was lost at least once, in the amniote lineage for reasons yet unknown.

CONCLUSION AND OUTLOOK

The findings of this study showed that modern lungfish are valuable and promising model organisms for body appendage regeneration and can provide important evolutionary and developmental insights into the mechanisms governing vertebrate regeneration. Currently, there is a lack of data with respect to initial lungfish fin development and the ecological and biological factors influencing regeneration in lungfish, such as age dependency of regenerative capacities and regeneration after partial vs. whole fin loss. These are in part based on the elaborate conditions for animal housing and breeding, restricting access to embryos and larvae as well as controlled conditions for adult animals. Despite its enormous size, the Australian lungfish genome has recently been published (Meyer et al., 2021) and in the future, developing the lungfish into a model organism holds great potential for studies on regeneration and evolution.

The results of this study have shown that it can be difficult to recognize regenerated body parts by external observation alone and that a more detailed analysis of the anatomy and severity of anomalies can only be achieved through histological observations or CT scanning. The advancement of new imagining and molecular technologies allow for an inclusion non-model organisms in studies on regeneration, which can contribute significant new data on patterns and processes in regeneration and ultimately to the development applications in human medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because collections material was used. No animals were killed for this study.

AUTHOR CONTRIBUTIONS

VB, IS, and NF designed the study, discussed the data, and wrote the manuscript. VB compiled and analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

The project was funded by the DFG Grant (FR 2647/8-1) to NF.

ACKNOWLEDGMENTS

We would like to thank Peter Bartsch, Edda Aßel, Olivier Pauwels, Emmanuel Vreven, and Miguël Parrent for access to the material; Kristin Mahlow for performing the CT scans and Dr. Christian Mitgutsch for his support in the laboratory.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.784828/full#supplementary-material

Supplementary Figure 1 | Pectoral fins of three lungfish, *Neoceratodus forsteri*. **(A–F)** Fins with obvious signs of fin regeneration. **(G–L)** Same fins after two years with no significant changes in shape.

Supplementary Table 1 | Histological staining protocol for Heidenhain's Azan.

Supplementary Table 2 | Histological staining protocol for Alcian blue/nuclear fast red.

REFERENCES

Alvarado, A. S. (2000). Regeneration in the metazoans: why does it happen? *Bioessays* 22:578590. doi: 10.1002/(SICI)1521-1878(200006)22:6<578::AID-BIES11>3.0.CO;2-#

Amaral, D. B., and Schneider, I. (2018). Fins into limbs: recent insights from Sarcopterygian fish. *Genesis* 56:e23052. doi: 10.1002/dvg.23052

Amemiya, C. T., Alföldi, J., Lee, A. P., Fan, S., Philippe, H., MacCallum, I., et al. (2013). The African coelacanth genome provides insights into tetrapod evolution. *Nature* 496, 311–316.

Bely, A. E. (2010). Evolutionary loss of animal regeneration: pattern and process. *Integr. Comp. Biol.* 50, 515–527. doi: 10.1093/icb/icq118

Bely, A. E., and Nyberg, K. G. (2010). Evolution of animal regeneration: re-emergence of a field. *Trends Ecol. Evol.* 25, 161–170. doi: 10.1016/j.tree.2009.08.005

Bischoff, T. L. W. (1840). *Lepidosiren Paradoxa: Anatomisch Untersucht und Beschrieben*. Leipzig: Verlag von Leopold Voss.

Biscotti, M. A., Gerdol, M., Canapa, A., Forconi, M., Olmo, E., Pallavicini, A., et al. (2016). The lungfish transcriptome: a glimpse into molecular evolution events at the transition from water to land. *Sci. Rep.* 6:21571. doi: 10.1038/srep21571

Boisvert, C. A., Joss, J. M., and Ahlberg, P. E. (2013). Comparative pelvic development of the axolotl (*Ambystoma mexicanum*) and the Australian lungfish (*Neoceratodus forsteri*): conservation and innovation across the fish-tetrapod transition. *EvoDevo* 4:3.

Bothe, V., Mahlow, K., and Fröbisch, N. B. (2021). A histological study of normal and pathological limb regeneration in the Mexican axolotl *Ambystoma mexicanum*. *J. Exp. Zool. Part B Mol. Dev. Evol.* 336, 116–128.

Braus, H. (1900). *Die Muskeln und Nerven der Ceratodusflosse*. Jena: G. Fischer.

Brockes, J. P., and Gates, P. B. (2014). Mechanisms underlying vertebrate limb regeneration: lessons from the salamander. *Biochem. Soc. Trans.* 42, 625–630.

Bryant, D. M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M. B., Payzin-Dogru, D., et al. (2017a). A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell Rep.* 18, 762–776.

Bryant, D. M., Sousounis, K., Payzin-Dogru, D., Bryant, S., Sandoval, A. G. W., Fernandez, J. M., et al. (2017b). Identification of regenerative roadblocks via repeat deployment of limb regeneration in axolotls. *NPJ Regen. Med.* 2:30.

Bryant, S. V., and Gardiner, D. M. (2016). The relationship between growth and pattern formation. *Regeneration* 3, 103–122.

Bryant, S. V., French, V., and Bryant, P. J. (1981). Distal regeneration and symmetry. *Science* 212, 993–1002. doi: 10.1126/science.212.4498.993

Chang, M. M., and Yu, X. (1984). Structure and phyogenetic significance of *Diabolepis speratus* gen. et sp. nov., a new dipnoan-like form from the Lower Devonian of eastern Yunnan, China. *Proc. Linn. Soc. N. S. W.* 107, 171–184.

Conant, E. B. (1970). Regeneration in the African lungfish, *Protopterus*. I. Gross aspects. *J. Exp. Zool.* 174, 15–31. doi: 10.1002/jez.1401740103

Conant, E. B. (1973). Regeneration in the African lungfish, *Protopterus*. III. Regeneration during fasting and estivation. *Biol. Bull.* 144, 248–261. doi: 10.2307/1540006

Cuervo, R., Hernández-Martínez, R., Chimal-Monroy, J., Merchant-Larios, H., and Covarrubias, L. (2012). Full regeneration of the tribasal *Polypterus* fin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3838–3843. doi: 10.1073/pnas.1006619109

Curry-Lindahl, K. (1956). On the ecology, feeding behaviour and territoriality of the African lungfish, *Protopterus aethiopicus* Heckel. *Arkiv Zool.* 9, 479–497.

Darnet, S., Dragalzew, A. C., Amaral, D. B., Sousa, J. F., Thompson, A. W., Cass, A. N., et al. (2019). Deep evolutionary origin of limb and fin regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 116, 15106–15115. doi: 10.1073/pnas.1900475116

Dean, B. (1906). "Notes on the living specimens of the Australian lungfish, *Ceratodus forsteri*, in the Zoological Society's collection," in *Proceedings of the Zoological Society of London*, Vol. 76, (Oxford: Blackwell Publishing Ltd), 168–178. doi: 10.1111/j.1469-7998.1906.tb08428.x

Dean, B. (1912). Additional notes on the living specimens of the Australian Lungfish (*Ceratodus forsteri*) in the collection of the Zoological Society of London. *Proc. Zool. Soc. Lond.* 82, 607–612. doi: 10.1111/j.1469-7998.1912.tb07541.x

Dearlove, G. E., and Dresden, M. H. (1976). Regenerative abnormalities in *Notophthalmus viridescens* induced by repeated amputations. *J. Exp. Zool.* 196, 251–261. doi: 10.1002/jez.1401960212

Dent, J. N. (1962). Limb regeneration in larvae and metamorphosing individuals of the South African clawed toad. *J. Morphol.* 110, 61–77. doi: 10.1002/jmor.1051100105

Department of the Environment, Water, Heritage and the Arts (2009). *Neoceratodus forsteri – Australian lungfish" (Online)*. Canberra, ACT: Department of the Environment, Water, Heritage and the Arts.

Dinsmore, C. E., and Hanken, J. (1986). Native variant limb skeletal patterns in the red-backed salamander, *Plethodon cinereus*, are not regenerated. *J. Morphol.* 190, 191–200. doi: 10.1002/jmor.1051900204

Echeverri, K., and Tanaka, E. M. (2005). Proximodistal patterning during limb regeneration. *Dev. Biol.* 279, 391–401. doi: 10.1016/j.ydbio.2004.12.029

Ericsson, R., Joss, J., and Olsson, L. (2010). "Early head development in the Australian lungfish, *Neoceratodus forsteri*," in *The Biology of Lungfishes*, eds J. M. Jorgensen and J. Joss (Boca Raton, FL: CRC Press), 165–184.

French, V., Bryant, P. J., and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* 193, 969–981.

Fröbisch, N. B., Bickelmann, C., and Witzmann, F. (2014). Early evolution of limb regeneration in tetrapods: evidence from a 300-million-year-old amphibian. *Proc. R. Soc. B Biol. Sci.* 281:20141550. doi: 10.1098/rspb.2014.1550

Fröbisch, N. B., Bickelmann, C., Olori, J. C., and Witzmann, F. (2015). Deep-time evolution of regeneration and preaxial polarity in tetrapod limb development. *Nature* 527, 231–234. doi: 10.1038/nature15397

Gardiner, D. M., Blumberg, B., Komine, Y., and Bryant, S. V. (1995). Regulation of HoxA expression in developing and regenerating axolotl limbs. *Development* 121, 1731–1741. doi: 10.1242/dev.121.6.1731

Garza-Garcia, A. A., Driscoll, P. C., and Brockes, J. P. (2010). Evidence for the local evolution of mechanisms underlying limb regeneration in salamanders. *Integr. Comp. Biol.* 50, 528–535. doi: 10.1093/icb/icq022

Girvan, J. E., Olson, W. M., and Hall, B. K. (2002). Hind-limb regeneration in the dwarf African clawed frog, *Hymenochirus boettgeri* (Anura: pipidae). *J. Herpetol.* 36, 537–543.

Hutchison, C., Pilote, M., and Roy, S. (2007). The axolotl limb: a model for bone development, regeneration and fracture healing. *Bone* 40, 45–56. doi: 10.1016/j.bone.2006.07.005

Irisarri, I., and Meyer, A. (2016). The identification of the closest living relative (s) of tetrapods: phylogenomic lessons for resolving short ancient internodes. *Syst. Biol.* 65, 1057–1075. doi: 10.1093/sysbio/syw057

Johanson, Z., Joss, J., Boisvert, C. A., Ericsson, R., Sutija, M., and Ahlberg, P. E. (2007). Fish fingers: digit homologues in Sarcopterygian fish fins. *J. Exp. Zool. Part B Mol. Dev. Evol.* 308, 757–768. doi: 10.1002/jez.b.21197

Jorgensen, J. M., and Joss, J. (Eds.) (2016). *The Biology of Lungfishes*. Boca Raton, FL: CRC Press.

Kardong, K. V. (1997). *Vertebrates: Comparative Anatomy, Function, Evolution*. Houston, TX: Heinle and Heinle Publishers.

Kemp, A. (1986). The biology of the Australian lungfish, *Neoceratodus forsteri* (Krefft 1870). *J. Morphol.* 190, 181–198.

Kind, P. K. (2002). *Movement Patterns and Habitat Use in the Queensland Lungfish Neoceratodus forsteri (Krefft 1870)*. Ph.D. thesis. St Lucia, QLD: The University of Queensland.

King, H. M., Shubin, N. H., Coates, M. I., and Hale, M. E. (2011). Behavioral evidence for the evolution of walking and bounding before terrestriality in sarcopterygian fishes. *Proc. Natl. Acad. Sci. U.S.A.* 108, 21146–21151. doi: 10.1073/pnas.1118669109

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., et al. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* 460, 60–65. doi: 10.1038/nature08152

Kumar, A., Godwin, J. W., Gates, P. B., Garza-Garcia, A. A., and Brockes, J. P. (2007). Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. *Science* 318, 772–777. doi: 10.1126/science.1147710

Lee, J., and Gardiner, D. M. (2012). Regeneration of limb joints in the axolotl (*Ambystoma mexicanum*). *PLoS One* 7:e5061. doi: 10.1371/journal.pone.0050615

Lenhoff, H. M., and Lenhoff, S. G. (1991). "Abraham Trembley and the origins of research on regeneration in animals," in *A History of Regeneration Research. Milestones in the Evolution of a Science*, ed. C. E. Dinsmore (Cambridge: Cambridge University Press), 47–66. doi: 10.3389/fgene.2021.670695

Lima, S. Q., Costa, C. M., Amemiya, C. T., and Schneider, I. (2017). Morphological and molecular analyses of an anatomical novelty: the pelvic fin filaments of the South American lungfish. *J. Exp. Zool. Part B Mol. Dev. Evol.* 328, 97–105. doi: 10.1002/jez.b.22711

Londono, R., Sun, A. X., Tuan, R. S., and Lozito, T. P. (2018). Tissue repair and epimorphic regeneration: an overview. *Curr. Pathobiol. Rep.* 6, 61–69.

Looso, M., Michel, C. S., Konzer, A., Bruckskotten, M., Borchardt, T., Krüger, M., et al. (2012). Spiked-in pulsed in vivo labeling identifies a new member of the CCN family in regenerating newt hearts. *J. Proteome Res.* 11, 4693–4704. doi: 10.1021/pr300521p

Looso, M., Preussner, J., Sousounis, K., Bruckskotten, M., Michel, C. S., Lignelli, E., et al. (2013). A de novo assembly of the newt transcriptome combined with proteomic validation identifies new protein families expressed during tissue regeneration. *Genome Biol.* 14:R16. doi: 10.1186/gb-2013-14-2-r16

McCusker, C., Bryant, S. V., and Gardiner, D. M. (2015). The axolotl limb blastema: cellular and molecular mechanisms driving blastema formation and limb regeneration in tetrapods. *Regeneration* 2, 54–71. doi: 10.1002/reg2.32

Mercader, N., Tanaka, E. M., and Torres, M. (2005). Proximodistal identity during vertebrate limb regeneration is regulated by Meis homeodomain proteins. *Development* 132, 4131–4142. doi: 10.1242/dev.01976

Meyer, A., Schloissnig, S., Franchini, P., Du, K., Woltering, J. M., Irisarri, I., et al. (2021). Giant lungfish genome elucidates the conquest of land by vertebrates. *Nature* 590, 284–289. doi: 10.1038/s41586-021-03198-8

Mlewa, C. M., and Green, J. M. (2004). Biology of the marbled lungfish, *Protopterus aethiopicus* Heckel, in Lake Baringo, Kenya. *Afr. J. Ecol.* 42, 338–345. doi: 10.1111/j.1365-2028.2004.00536.x

Morgan, T. H. (1898). Experimental studies of the regeneration of *Planaria maculata*. *Dev. Genes Evol.* 7, 364–397. doi: 10.1007/bf02161491

Nelson, J. S., Grande, T. C., and Wilson, M. V. (2016). *Fishes of the World*. Hoboken, NJ: John Wiley & Sons.

Nogueira, A. F., Costa, C. M., Lorena, J., Moreira, R. N., Frota-Lima, G. N., Furtado, C., et al. (2016). Tetrapod limb and sarcopterygian fin regeneration share a core genetic programme. *Nat. Commun.* 7:13364. doi: 10.1038/ncomms13364

Ovchinnikov, D. (2009). Alcian blue/alizarin red staining of cartilage and bone in mouse. *Cold Spring Harb. Protoc.* 2009:pdb-prot5170.

Reaumur, R.-A. F. D. (1712). Sur les diverses reproductions qui se fontdans les Ecrivisses, les Omars, les Crabes, etc. et entre Autressur celles de leurs jambes et de Leurs ecailles. *Mem. R. Sci.* 12, 226–245.

Reilly, S. M., and Lauder, G. V. (1990). The evolution of tetrapod feeding behavior: kinematic homologies in prey transport. *Evolution* 44, 1542–1557. doi: 10.1111/j.1558-5646.1990.tb03845.x

Roensch, K., Tazaki, A., Chara, O., and Tanaka, E. M. (2013). Progressive specification rather than intercalation of segments during limb regeneration. *Science* 342, 1375–1379. doi: 10.1126/science.1241796

Romer, A. S., Frick, H., and Starck, D. (1959). *Vergleichende Anatomie der Wirbeltiere*. Berlin: Parey.

Roy, S., and Lévesque, M. (2006). Limb regeneration in axolotl: is it superhealing? *Sci. World J.* 6, 12–25. doi: 10.1100/tsw.2006.324

Soto-Rojas, C., Suazo-Ortuño, I., Montoya Laos, J. A., and Alvarado-Díaz, J. (2017). Habitat quality affects the incidence of morphological abnormalities in the endangered salamander *Ambystoma ordinarium*. *PLoS One* 12:e0183573. doi: 10.1371/journal.pone.0183573

Stock, G. B., and Bryant, S. V. (1981). Studies of digit regeneration and their implications for theories of development and evolution of vertebrate limbs. *J. Exp. Zool.* 216, 423–433. doi: 10.1002/jez.1402160311

Thompson, S., Muzinic, L., Muzinic, C., Niemiller, M. L., and Voss, S. R. (2014). Probability of regenerating a normal limb after bite injury in the Mexican axolotl (*Ambystoma mexicanum*). *Regeneration* 1, 27–32. doi: 10.1002/reg2.17

Torok, M. A., Gardiner, D. M., Shubin, N. H., and Bryant, S. V. (1998). Expression of HoxD genes in developing and regenerating axolotl limbs. *Dev. Biol.* 200, 225–233. doi: 10.1006/dbio.1998.8956

Traquair, R. H. (1871). On the restoration of the tail in *Protopterus annectens*. *Rep. Brit. Ass. Adv. Sci.* 1871:143.

Tsonis, P. A. (2000). Regeneration in vertebrates. *Dev. Biol.* 221, 273–284.

Verissimo, K. M., Perez, L. N., Dragalzew, A. C., Senevirathne, G., Darnet, S., Barroso Mendes, W. R., et al. (2020). Salamander-like tail regeneration in the West African lungfish. *Proc. R. Soc. B* 287:20192939. doi: 10.1098/rspb.2019.2939

Vieira, W. A., Wells, K. M., and McCusker, C. D. (2020). Advancements to the axolotl model for regeneration and aging. *Gerontology* 66, 212–222. doi: 10.1159/000504294

Vieira, W. A., Wells, K. M., Raymond, M. J., De Souza, L., Garcia, E., and McCusker, C. D. (2019). FGF, BMP, and RA signaling are sufficient for the induction of complete limb regeneration from non-regenerating wounds on *Ambystoma mexicanum* limbs. *Dev. Biol.* 451, 146–157. doi: 10.1016/j.ydbio.2019.04.008

Young, H. E. (1977). Anomalies of limb regeneration in the adult salamander, *Ambystoma annulatum*. *J. Arkansas Acad. Sci.* 31, 110–111.

Zeleny, C. (1909). The relation between degree of injury and rate of regeneration—additional observations and general discussion. *J. Exp. Zool.* 7, 513–561. doi: 10.1002/jez.1400070306

Ziermann, J. M., Clement, A. M., Ericsson, R., and Olsson, L. (2018). Cephalic muscle development in the Australian lungfish, *Neoceratodus forsteri*. *J. Morphol.* 279, 494–516. doi: 10.1002/jmor.20784

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 23 December 2021
doi: 10.3389/fevo.2021.786037

Common Environmental Pollutants Negatively Affect Development and Regeneration in the Sea Anemone *Nematostella vectensis* Holobiont

Sylvia Klein[1†], Victoria Frazier[2], Timothy Readdean[1], Emily Lucas[1], Erica P. Diaz-Jimenez[1], Mitchell Sogin[2], Emil S. Ruff[2,3] and Karen Echeverri[1]**

[1] Eugene Bell Center for Regenerative Biology and Tissue Engineering, Marine Biological Laboratory, Woods Hole, MA, United States, [2] Bay Paul Center, Marine Biological Laboratory, Woods Hole, MA, United States, [3] The Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA, United States

OPEN ACCESS

Edited by:
Igor Schneider,
Federal University of Pará, Brazil

Reviewed by:
Adam Michael Reitzel,
The University of North Carolina at Charlotte, United States
Michel Vervoort,
UMR 7592 Institut Jacques Monod (IJM), France

***Correspondence:**
Emil S. Ruff
eruff@mbl.edu
Karen Echeverri
kecheverri@mbl.edu

†Present address:
Sylvia Klein,
University of Minnesota, Minneapolis, MN, United States

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

Received: 29 September 2021
Accepted: 15 November 2021
Published: 23 December 2021

Citation:
Klein S, Frazier V, Readdean T, Lucas E, Diaz-Jimenez EP, Sogin M, Ruff ES and Echeverri K (2021) Common Environmental Pollutants Negatively Affect Development and Regeneration in the Sea Anemone Nematostella vectensis Holobiont. Front. Ecol. Evol. 9:786037. doi: 10.3389/fevo.2021.786037

The anthozoan sea anemone *Nematostella vectensis* belongs to the phylum of cnidarians which also includes jellyfish and corals. Nematostella are native to United States East Coast marsh lands, where they constantly adapt to changes in salinity, temperature, oxygen concentration and pH. Its natural ability to continually acclimate to changing environments coupled with its genetic tractability render *Nematostella* a powerful model organism in which to study the effects of common pollutants on the natural development of these animals. Potassium nitrate, commonly used in fertilizers, and Phthalates, a component of plastics are frequent environmental stressors found in coastal and marsh waters. Here we present data showing how early exposure to these pollutants lead to dramatic defects in development of the embryos and eventual mortality possibly due to defects in feeding ability. Additionally, we examined the microbiome of the animals and identified shifts in the microbial community that correlated with the type of water that was used to grow the animals, and with their exposure to pollutants.

Keywords: ***Nematostella*, growth, microbiome, stressors, development**

INTRODUCTION

Nematostella vectensis is a sea anemone that belongs to the class *Anthozoa* in the phylum *Cnidaria*. This species inhabits marsh habitats on the East Coast of the United States, where they constantly adapt to changes in salinity, temperature, oxygen concentration, and pH (Darling et al., 2005; Reitzel et al., 2013; Elran et al., 2014; Tarrant et al., 2018). Studies of embryonic development in Nematostella have provided new insights into how tissue layers differentiate in diploblastic animals (Wikramanayake et al., 2003; Kraus and Technau, 2006; Röttinger et al., 2012; Schwaiger et al., 2014; Amiel et al., 2017; Steinmetz et al., 2017; Wijesena et al., 2017; Kirillova et al., 2018; Technau, 2020). Like most cnidarians, Nematostella have unique specialized cells called cnidocytes which facilitate capture of prey and serve as inherent defense mechanism (Marlow et al., 2012; Babonis and Martindale, 2014; Babonis et al., 2016; Sebe-Pedros et al., 2018). The ease of culturing in laboratory conditions combined with genetic tractability render Nematostella a valuable system for investigating the evolution and molecular mechanisms of specialized cell types. More recently

Nematostella has attracted attention in regenerative biology because of to their genetic tractability, rapid regeneration time and ability to easily compare development and regeneration (Trevino et al., 2011; Passamaneck and Martindale, 2012; Bossert et al., 2013; DuBuc et al., 2014; Amiel et al., 2015; Layden et al., 2016; Schaffer et al., 2016; Bossert and Thomsen, 2017; Warner et al., 2018; Amiel et al., 2021; Amiel and Röttinger, 2021; van der Burg and Prentis, 2021).

Many studies on cnidarians have shown that their genetic complexity and microbiome diversity rivals that of humans despite their having diverged from a common metazoan ancestor more than 1 billion years ago (Daniel et al., 1999; Darling et al., 2005; Fraune and Bosch, 2010; Fraune et al., 2010; Essock-Burns et al., 2020). The microbiome of several species of Nematostella from diverse geographical location has been mapped and it has been clearly shown that there is a distinct correlation between differences in the biogeography and microbiome (Mortzfeld et al., 2016). Several studies have shown the mutualistic association between host and microbes that lead to optimal fitness of the host (Thompson et al., 2014; Heath-Heckman et al., 2016; Rook et al., 2017; Essock-Burns et al., 2020; Bosch and McFall-Ngai, 2021). Species which live in coastal areas, especially marshes, exhibit residual plasticity in their physiology in response to continuous exposure to changing temperature and salinity. Agricultural and industrial pollutants have profound effects on marine ecosystems, however, our limited ability to make accurate predictions about the response, stability and resilience of the affected ecosystems and their inhabitants reflects our general lack of understanding of the complex interplay between genetic and environmental factors that influence acclimation and adaptation to environmental stressors.

This study focused on the environmental contaminants phthalates, specifically phthalic acid esters (PAEs) and nitrate, because they are common pollutants of salt marsh ecosystems in developed areas. PAEs are used in plasticizers and are found in a variety of plastic products, which can subsequently leach PAEs into the environment from landfills and sewage (Hu et al., 2021). Nitrate is a common pollutant in coastal ecosystems derived from agriculture and wastewater (McClelland and Valiela, 1998). We used both pollutants at concentrations between 1–20 μM, a range that is realistic for both compounds reflecting concentrations found in coastal and estuarine ecosystems (Gugliandolo et al., 2020; Valiela et al., 2021). Previous studies have demonstrated the detrimental effects of phthalates on the growth and development of a variety of vertebrates and invertebrates, including zebrafish, humans, and *Daphnia sp.* (Philippat et al., 2012; Kinch et al., 2016; Jergensen et al., 2019; Qian et al., 2020). Although this pollutant is prevalent in the *Nematostella vectensis* habitat, little is known about its effects on cnidarian growth and development. Similarly, elevated concentrations of nitrate are known to be toxic to many fish and invertebrates and are predicted to have similar detrimental effects on *Nematostella vectensis* development (Camargo et al., 2005).

Investigations that focus on altered gene expression patterns have commonly described adaptation to environmental shifts. Dysbiosis of an organism's microbiome can also substantially influence the phenotype of an organism. The microbiome plays an important role in different aspects of an organism's life cycle ranging from embryological development to nutrition, immune response, and development of disease (Zheng et al., 2020). Changes in the microbiome can correlate with numerous short-, mid- and long-term changes in the host, some of which promote adaption to new environmental conditions (Pita et al., 2018). Until recently, gene expression, epigenetics and microbiomes have all been studied separately and little is known about their interactions in terms of marine organisms and environmental pollution. In this study, we take advantage of the amenability of sea anemone *Nematostella vectensis* to culturing in the lab and use this organism to study more closely the effect of early exposure to environmental pollutants on its embryonic development and associated microbiome. We hypothesized that exposure to elevated levels of phthalates and nitrate would lead to increased relative abundances of taxa capable of metabolizing these environmental stressors.

MATERIALS AND METHODS

Animal Care

We maintained adult *Nematostella vectensis* at 17 to 20°C in a flow through aquatic system or in Pyrex glass bowls kept in the dark in 15 parts per thousand (ppt) instant ocean, referred to here as "Nematostella water." We fed adult animals 48-h old artemia, three times a week. The water quality of the system was monitored weekly. Animals kept in bowls were cleaned a few hours after feeding. Spawning of animals was induced by exposure to light and increase in temperature to 23–25°C. We collected embryos immediately after spawning, usually around 12 h after light exposure.

Environmental Stressor Experiments

Freshly laid embryos were transferred to multi-well dishes. Embryos were incubated in Nematostella water containing 1, 10, or 20 μM of potassium nitrate (KNO_3) (Sigma P8291) or containing 1, 10, or 20 μM of dioctyl phthalate (Sigma D201154). Control animals were incubated in multi-well plates in Nematostella water at 17–20°C. Solutions on all animals were changed every 3 days. We fed the developing animals' rotifers or 24-hour old artemia, starting at the four-tentacle stage. In experiments testing the seawater of the local Sippewissett Salt Marsh, we diluted the seawater to 15 ppt with deionized water to obtain the same salinity as the standard Nematostella water the animals are usually maintained in. The environmental stressors were added directly into the diluted seawater and control embryos were incubated in the diluted 15 ppt Sippewissett seawater.

When animals reached the four tentacle stage they were relaxed in 7.4% v/v $MgCl_2$ and then fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Animals were imaged on a Zeiss Discovery V8 Stereo microscope. From these images, tentacle number and pharynx length were quantified using the measure function in Fiji and analyzed using Prism GraphPad.

Regeneration Experiments

Regeneration experiments were performed on adult animals at least 12 months of age. Animals were relaxed in 7.4% v/v $MgCl_2$ for about 15 min. Individual animals were transferred to a 60 mm × 15 mm plastic petri dish (Fisher) using a glass pipette. Using a sterile no. 10 disposable scalpel (World Precision Instruments) animals were amputated at the bottom of the pharynx. The animals were then isolated into separate wells of 12 well cell culture plates. The treatments used were 15 ppt instant ocean water; 1 μM KNO_3, 10 μM KNO_3, 20 μM KNO_3; 1, 10, and 20 μM dioctyl phthalate. In all experiments, solutions were changed every 3 days. The animals were allowed to regenerate for 14 days then relaxed in 7.4% $MgCl_2$ and fixed in 4% PFA overnight and stored at 4°C. Animals were imaged on a Zeiss Discovery V8 Stereo microscope.

Cnidocyte Staining

Cnidocyte staining was carried out according to Wolenski et al. (2013). Animals were relaxed in 7.4% v/v $MgCl_2$ and then fixed overnight in 4% PFA plus 10 mM EDTA and washed 3 × 5 min in Tris-EDTA wash buffer (10 mM Tris, 10 mM EDTA, 10 mM NaCl, pH 7.6). The animals were incubated in 200 μg/ml DAPI diluted in Tris-EDTA buffer for 30 min at room temperature followed by rinsing 3 × 5 min in Tris-EDTA buffer. Animals were imaged on a Leica DMI6000B inverted microscope.

DNA Extraction

For the microbiome analyses we used animals from cultures investigating the effects of pollutants on embryonic growth and development. We used embryos spawned from mixed populations of animals for each biological replicate, embryos were collected 10 days post fertilization for DNA extraction. We flash froze each cohort of about 200 animals per condition prior to extraction, at 10 days post fertilization the control animals had reached the 4 tentacle stage. Three replicas of each condition were separately frozen, each replica was a different well. Brief centrifugation at 6000 rpm pelleted the suspended *Nematostella sp.* embryos before transfer of 50 μl solutions to bead beating tubes for DNA extraction using the PowerLyzer PowerSoil DNA Isolation kit (Qiagen, Hilden, Germany). Two parallel DNA extraction batches for each sample included randomized samples, environmental controls, and two extraction controls per batch.

Amplicon Library Preparation and Sequencing

Triplicate PCR reactions for the V4-V5 region of the 16S rRNA gene employed fusion primers which consisted of Illumina-specific adaptors for sequencing, indexes and barcodes for multiplexing samples, and the primer set 515F (5′-CCAGCAGCYGCGGTAAN-3′) and 926R (8:1:1 mixture of 5′-CCGTCAATTCNTTTRAGT-3′, 5′-CCGTCAATTTCTTTGAGT-3′, 5′-CCGTCTATTCCTTTGANT-3′). No-template negative controls were prepared for each sample by moving 25 μl of the 125 μl reaction to a separate well prior to final assembly of the sequencing reaction. The remaining 100 μl was split into triplicate 33 μl reactions following addition of 6 μl template DNA. Thermocycler conditions for amplification included initial denaturation at 94°C for 3 min, 30 cycles of denaturing at 94°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 1 min, and a final extension of 72°C for 10 min. Visualization on a TapeStation 4200 using D1000 ScreenTapes and D1000 DNA ladder (Agilent Technologies, California, United States) confirmed amplification. Treatment with AMPure XP magnetic beads (Agencourt, Beckmann-Coulter, United States) according to the manufacturer's instructions, purified and concentrated the amplicons libraries. Pooled amplicon libraries at equivalent amounts of DNA (determined on the TapeStation 4200) ensured equal coverage across samples during sequencing. 515F/926R primers may also amplify host 18S rRNA genes yielding fragments of ∼760 bp length. We thus size selected the target 16S amplicon within a size range of 425 and 625 bp with a BluePippin instrument using a 1.5% agarose cassette and R2 marker (Sage Science, Massachusetts, United States). The multiplexed amplicon pools were then sequenced on an Illumina MiSeq instrument using a V3 600 cycle kit according to the manufacturer's protocol (Illumina, California, United States). Samples, accession numbers and associated contextual data are listed in **Supplementary Table 1**.

16S rRNA Gene Amplicon-Based Community Analyses

Raw sequences were analyzed using DADA2 (Callahan et al., 2016) following the DADA2 Pipeline Tutorial v1.16[1]. In brief, forward and reverse reads were quality-trimmed to 275 bp and 205 bp, respectively, and primer sequences (17 bp forward, 21 bp reverse) were removed. Reads with more than two expected errors were discarded, paired reads were merged, and chimeric sequences were removed. Species level taxonomy was assigned with the silva_nr_v138_train_set and silva_species_assignment_v138 based on the Silva small subunit reference database SSURef v138 [release date: 16-Dec-2019 (Quast et al., 2013)]. After quality control and removal of blanks and controls we obtained 42 bacterial amplicon datasets comprising a total of 6.59×10^7 sequence reads belonging to 2024 unique ASVs. Each sample had on average $1.46 \pm 0.41 \times 10^5$ reads (average ± standard deviation) and 352 ± 139 unique ASVs (**Supplementary Table 1**). The ASV-by-sample table was used to determine the number of observed ASV, absolute singletons, relative singletons, relative abundance, and composition. Alpha diversity (richness, Shannon entropy, Inverse Simpson Diversity and Chao1 estimated richness) was calculated from the ASV-by-sample table using a subsampling of 87327 randomly chosen sequences to account for unequal sampling effort (**Supplementary Table 1**). Differences in diversity between conditions were tested using the Wilcoxon signed rank-test (*ggsignif*) as implemented in *ggplot2* (Wickham, 2009). Bray-Curtis dissimilarities (Bray and Curtis, 1957) between all samples were calculated and used for two-dimensional non-metric multidimensional scaling (NMDS) ordinations with 20 random starts (Kruskal, 1964). All analyses were carried out

[1]https://benjjneb.github.io/dada2/tutorial.html

with VisuaR v02[2] a publicly available workflow based on the R statistical environment, custom R scripts and several R packages including *vegan* (Oksanen et al., 2012) and *ggplot2*.

RESULTS

Early Exposure to Common Environmental Stressors Inhibits Embryonic Growth

We have investigated the effect of common environmental stressors on the development and regeneration of *Nematostella vectensis*. Nematostella have a relatively rapid development, reaching a young independent feeding stage with four tentacles in about 1 week (Genikhovich and Technau, 2009; Marlow et al., 2009; Layden et al., 2016). Embryos were collected directly after spawning and between 200–300 embryos were placed in different concentrations of the common pollutants, dioctyl phthalate, which is derived from plastics, or potassium nitrate (KNO_3), a common component of widely used fertilizers, both of which are often found in coastal waters. Two weeks post exposure the overall body length of the animal was measured from the tip of the pharynx to the foot of the animals (**Figure 1A**). Embryos exposed to phthalates or nitrites exhibited a gross difference in overall body size as compared to the control animals (**Figures 1A–D**). Increasing the concentration of stressors did not correlate with a decrease in body size, and similar defects in body size were observed in increased concentration (**Figures 1B,C**). However, exposure to higher concentrations, 20 μM and above led to possible toxicity and high morbidity. When the morphology of the animals was carefully examined, the animals generally appeared to develop all the expected visible structures. However, defects were noted first in the tentacles. Animals treated with phthalates all had fewer tentacles and the tentacles that did grow were uneven in length and number (**Figures 2**, **3**). We noted other defects in which some animals had bifurcated tentacles, while others had tentacles that permanently curled at the end (**Figure 1**). The Nematostella pharynx, where the food is taken in did not show a significant difference in size in low concentration of phthalates, which contrasts with a significant decrease in the overall length of the pharynx at 10 μM concentrations (**Figure 2C**). Finally, developing animals exposed to low and high concentrations of phthalates exhibited significantly shorter mesenteries compared to control animals (**Figure 2D**).

The same quantifications were carried out on animals exposed to KNO_3 during early development. In these animals, we observed the same decrease in overall tentacle length and decrease in tentacle number (**Figures 3A,B**). Interestingly, embryos exposed to KNO_3 did not have any significant difference in the length of their pharynx in comparison to controls (**Figure 3C**). However, these animals had much smaller mesenteries in comparison to control animals that were growing in KNO_3 concentrations greater than 10 μM but not at lower concentrations. This observation suggests that growth of the mesenteries tolerates low levels of KNO_3 (**Figure 3D**). The dramatic difference in overall size of the animals, and in most cases aberrant development of mesenteries and tentacles, prompted questions about the impact of tested environmental stressors on specialized cell types. Cnidocytes are an ectodermal derived cell types used for defense, prey capture and environmental sensing. Overall, we found that animals exposed to Phthalates or KNO_3 have cnidocytes on the ectodermal layer but fewer than in controls (**Figures 4A–C**). We also examined if earlier in development cnidocytes develop was effected, we carried out in situs on embryos 72 h post fertilization using the gene minicollagen which is expressed in all developing cnidocytes (Babonis and Martindale, 2017) and found decreased expression in phthalate of nitrite treated embryos (**Supplementary Figure 1**), suggesting that that these pollutants may affect early differentiation of these specialized cell types. We more closely examined the cnidocytes on the tentacles, which the animals use to capture their food and found far fewer cnidocytes on the tentacles of animals exposed to the environmental stressors (E, F). In particular, animals incubated in KNO_3 had very few cnidocytes of the normal elongated shape, suggesting that these pollutants may affect differentiation of these specialized cell types.

Since Nematostella has the robust ability to regenerate (Amiel et al., 2015; Schaffer et al., 2016; Warner et al., 2018), we examined whether the presence of these environmental stressors influences regrowth of the tentacles. Adult animals were amputated through the bottom of the pharynx and then incubated in the presence of 20 μM Phthalate or KNO_3 for 2 weeks. At the end of the time period, the animals were relaxed and imaged. Animals exposed to phthalates regenerated the pharynx and partial tentacles. In all animals' defects in tentacle regeneration were observed, in most cases different numbers and lengths of tentacles were regenerated (**Figure 5B**) and often the tentacles were fused or bifurcated (data not shown). Animals exposed to KNO_3 mainly failed to regrow the tentacles or in some cases 1 tentacle regrew, even in lower concentrations of KNO_3 the oral portion of the animal failed to regrow (**Figure 5C**). We also examined if the cnidocytes are regenerated on the limited tentacles that are regenerated. Like in embryonic development, we observed a decrease in number of cnidocytes per tentacle in comparison to the control animal (**Figures 5D–F**), and the cnidocytes in animals exposed to the stressors were shorter and less elongated than in control animals.

Taken together, these data suggest that early exposure to two common environmental stressors has a major impact on developmental growth, possibly due to a failure of the animals to feed due to lack of normal tentacles and decreased numbers of cnidocytes which are used to capture their food. Additionally, we observed that exposure of adult Nematostella to these pollutants after amputation leads either to complete failure of tentacle regeneration or results in major defects in the number and length of the tentacles and of the cnidocytes regenerated.

Effect of Pollutants on the Nematostella Microbiome

To determine whether early exposure to environmental pollutants not only causes developmental defects but also leads

[2]https://github.com/EmilRuff/VisuaR

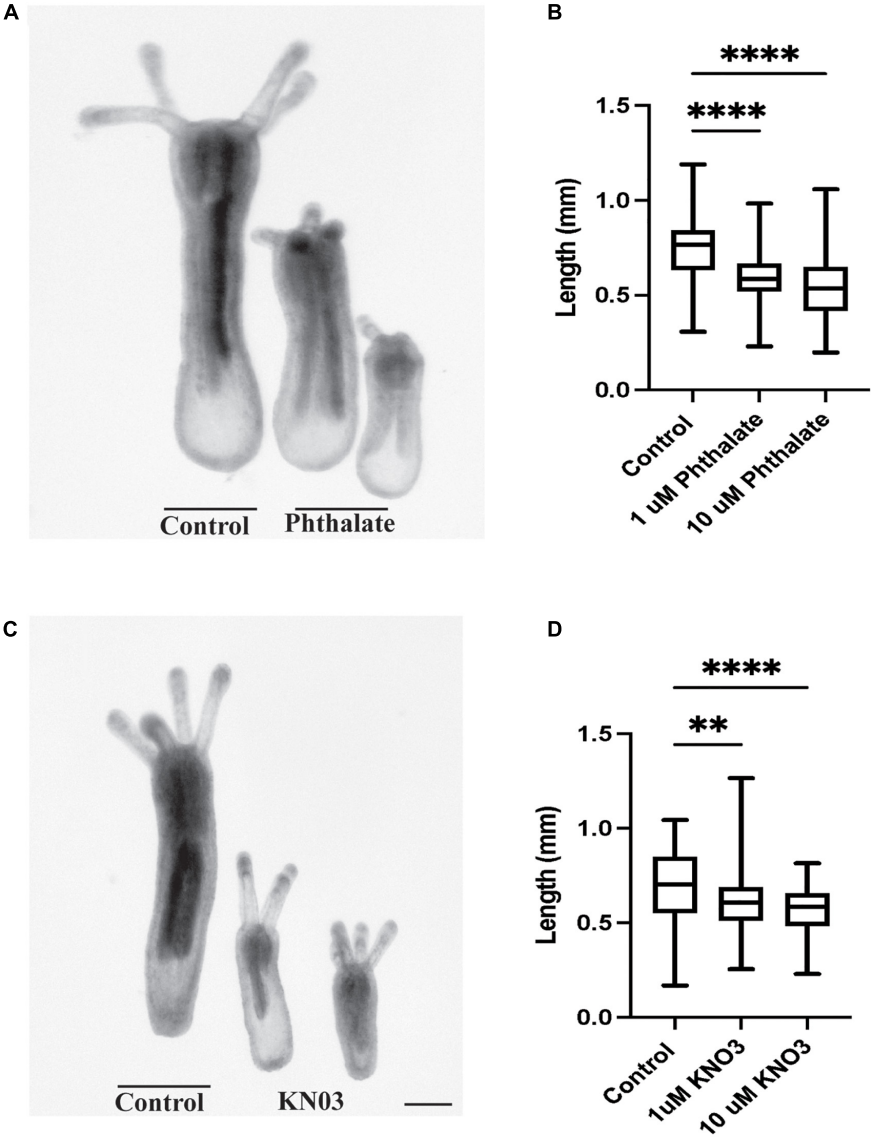


FIGURE 1 | Environmental Stressors Lead to Growth defects in Nematostella embryos. **(A)** Nematostella embryos exposed to phthalate are significantly shorter when compared to control siblings. **(B)** Increasing concentration of phthalates does not cause greater defects in overall length of the developing animals (control $n = 149$, 1 μM: $n = 162$, 10 μM $n = 161$). **(C)** Exposure to Potassium nitrate also causes defects in overall embryonic growth in comparison to control. **(D)** Defects in embryo size do not scale with increasing doses of KNO_3 (control $n = 90$, 1 μM: $n = 78$, 10 μM $n = 65$). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, N.S. is not significant. Scale bar = 100 μM.

to changes in the microbiome, we employed 16S rRNA gene sequencing to investigate the microbiome of Nematostella embryos after a 10-day exposure to different concentrations of KNO_3 or phthalates. In addition, we tested each pollutant concentration on animals that were grown in either instant ocean medium or natural seawater, to test if a potential effect of the pollutants is similar under different environmental regimes. The exposure to pollutants caused shifts in the microbial community structure and composition. Within a set of experiments, e.g., exposing the animals to different concentrations of KNO_3, these shifts were minor regarding the richness and evenness of the microbiomes (**Figure 6**), independent of the medium the animals were grown in. This suggests that the exposure to increasing pollutant concentrations did not change the richness of the animal-associated microbiome. Although no significant differences in alpha diversity were observed between pollutant treatments and their controls, alpha diversity was significantly different between incubation media (**Figure 6**). Animals that were grown in instant ocean artificial seawater had a significantly lower diversity than those grown in seawater from the nearby Sippewissett salt marsh, a native habitat of Nematostella (**Supplementary Figure 2** NMDS) which is rich in natural seawater microbiota. Yet the overall lack of significant changes in microbial richness and evenness with exposure to different

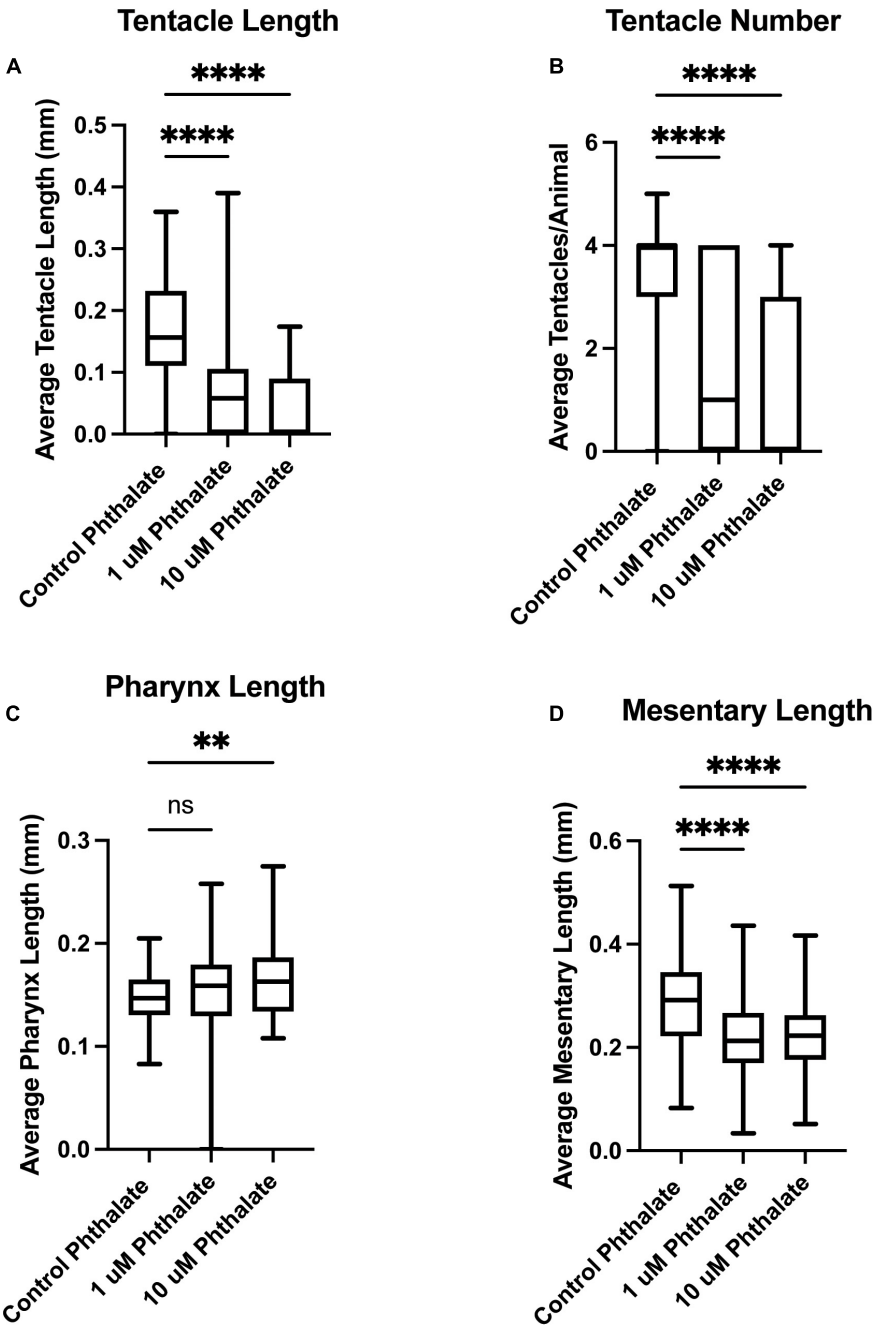


FIGURE 2 | Early exposure to phthalates leads to defects in tissue development. Two weeks post fertilization Nematostella have grown at least four tentacles and are independently feeding. Animals exposed to 1 or 10 μM phthalate have much shorter tentacles **(A)** and developed fewer tentacles when compared to control animals **(B)**. The length of pharynx was also measured but no significant difference was found in overall length in comparison to sibling control animals at 1 μM but at 10 μM the pharynx was significantly shorter **(C)**. The overall length of the mesenteries was also found to be shorter in animals exposed to phthalates **(D)**. (control $n = 76$, 1 μM $n = 113$, 10 μM $n = 82$) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, N.S. is not significant.

concentrations of KNO_3 or different concentrations of phthalates was similar in both media. Despite minor differences in alpha diversity, we saw substantial change in the community structure. This trend was independent of the basal medium used to incubate the animals. The communities that have been exposed to the two different pollutants KNO_3 and phthalates were well separated in an NMDS ordination (**Figure 7**). Especially in the case of nitrate, it seems as if there was a clear pattern of increasing community dissimilarity with increasing concentration. The clear separation based on pollutants and concentration is similar in animals that were raised in the two different sources of salt water, despite the overall large differences caused by the two types of salt water used

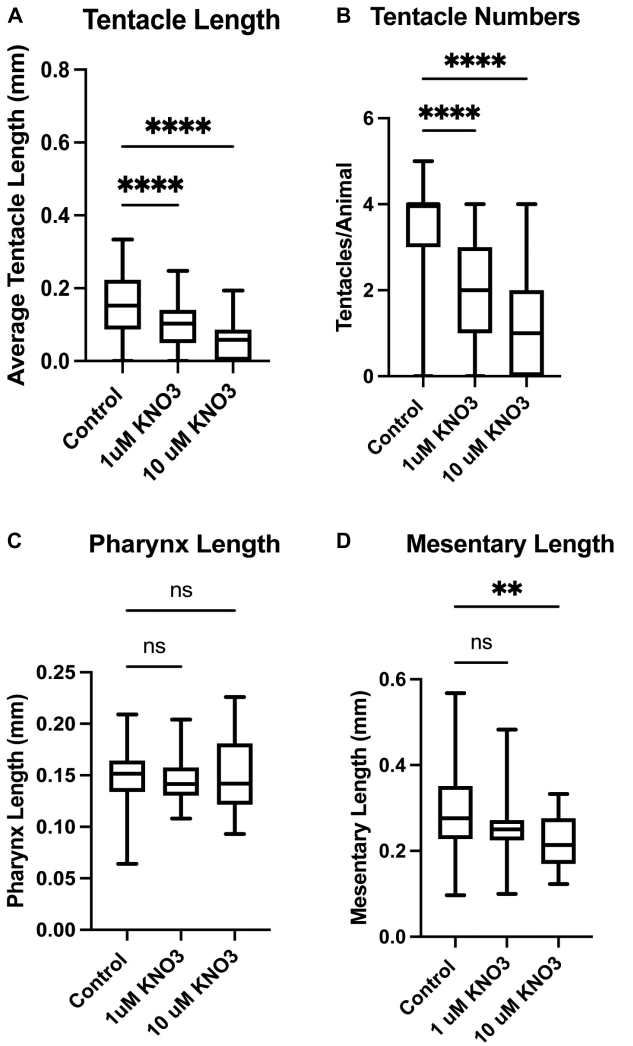


FIGURE 3 | Exposure to Potassium Nitrate leads to defects in tissue development. Animals exposed to 1 or 10 μM KNO_3 have much shorter tentacles **(A)** and developed fewer tentacles when compared to control animals **(B)**. The length of pharynx was also measured but no significant difference was found in overall length in comparison to sibling control animals **(C)**. The overall length of the mesenteries was also found to be shorter in animals exposed to phthalates **(D)**. Control $n = 72$, 1 μM $n = 66$, 10 μM $n = 68$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, N.S. is not significant.

for growth of the embryos (**Supplementary Figure 2** NMDS). The three biological replicates generally cluster tightly in the NMDS, indicating that each pollutant and each concentration caused very similar deterministic community shifts.

The relative sequence abundance of an uncultured population within the genus *Flavobacterium* was elevated in the KNO_3 treatments compared to the unamended controls and phthalates treatments, and relative abundance of this group increased with increasing nitrate concentrations (**Figure 8**). In contrast, a population affiliating with the genus *Mariniflexile*, which was abundant in the unamended conditions, decreased at higher KNO_3 concentrations. The animals that were grown in natural seawater, and were thus exposed to a diverse marine microbiome, were colonized by very different genera than those cultured in instant ocean. Here, the KNO_3 and phthalates exposed animals showed a very different microbiome. In the KNO_3 condition, the most sequence abundant population belonged to *Pseudomonas* and the phthalate cultures showed high sequence abundances of an unknown genus within the family *Saprospiraceae* (**Figure 7**). Overall, the majority of reads in any given condition belonged to organisms within the *Bacteroidia* (marked with an asterisk in **Figure 8**), including six of the seven most abundant lineages on species level, which accounted for more than 50% of the reads on average per sample.

In summary, our results indicate that embryonic exposure to two common environmental pollutants leads to severe defects in embryonic development. In addition, we found that the source of the water in which embryos are grown influences the complexity of an animal's microbiome.

DISCUSSION

Early Exposure to Environmental Stressors Has a Detrimental Effect on Nematostella Embryonic Development

Nematostella vectensis undergo rapid development when cultured in lab conditions, fertilized embryos emerge from the egg mass at around 48 h post fertilization and quickly developed into ciliated planula, with an apical cilium by 3 days. The free-swimming planula progressively changes shape, becoming more elongated and by 5–7 days have 4 tentacle buds (Hand and Uhlinger, 1992; Layden et al., 2016). We investigated the effect of early exposure to two common environmental stressors found in marsh waters on the early development of Nematostella. Phthalates and potassium nitrate were used as environmental stressors in this experiment because they are some of the most frequently found toxins in heavily populated marsh areas. These impurities in the water come mainly from plastics, which leach phthalic acid esters from plasticizers, and from freshwater run-off containing nitrates from fertilizers into the march areas. We observed very significant overall defects in the size of the embryos by two-week post-fertilization, all embryos incubated even in low concentrations of the pollutants were overall much shorter than control embryos (**Figure 1**). Furthermore, we observed clear defects in the number and length of the tentacles and in size of the pharynx and mesenteries (**Figures 2**, **3**). We also looked more closely at the composition of the tentacles, Nematostella have an ectodermal derived specialized cell type known as the cnidocyte which it uses as a defense mechanism and to capture its food. In all cases we found a reduction in number of the cnidocytes especially in the tentacles (**Figure 4**). The lack of cnidocytes especially on the tentacles is suggestive of an inability of the animals to capture their food. When the first tentacles are observed around 7 days post-fertilization, we started to feed the animals rotifers, as we noted that at this timepoint no significant size difference was observed between animal, however by 10 days we could already see clear size

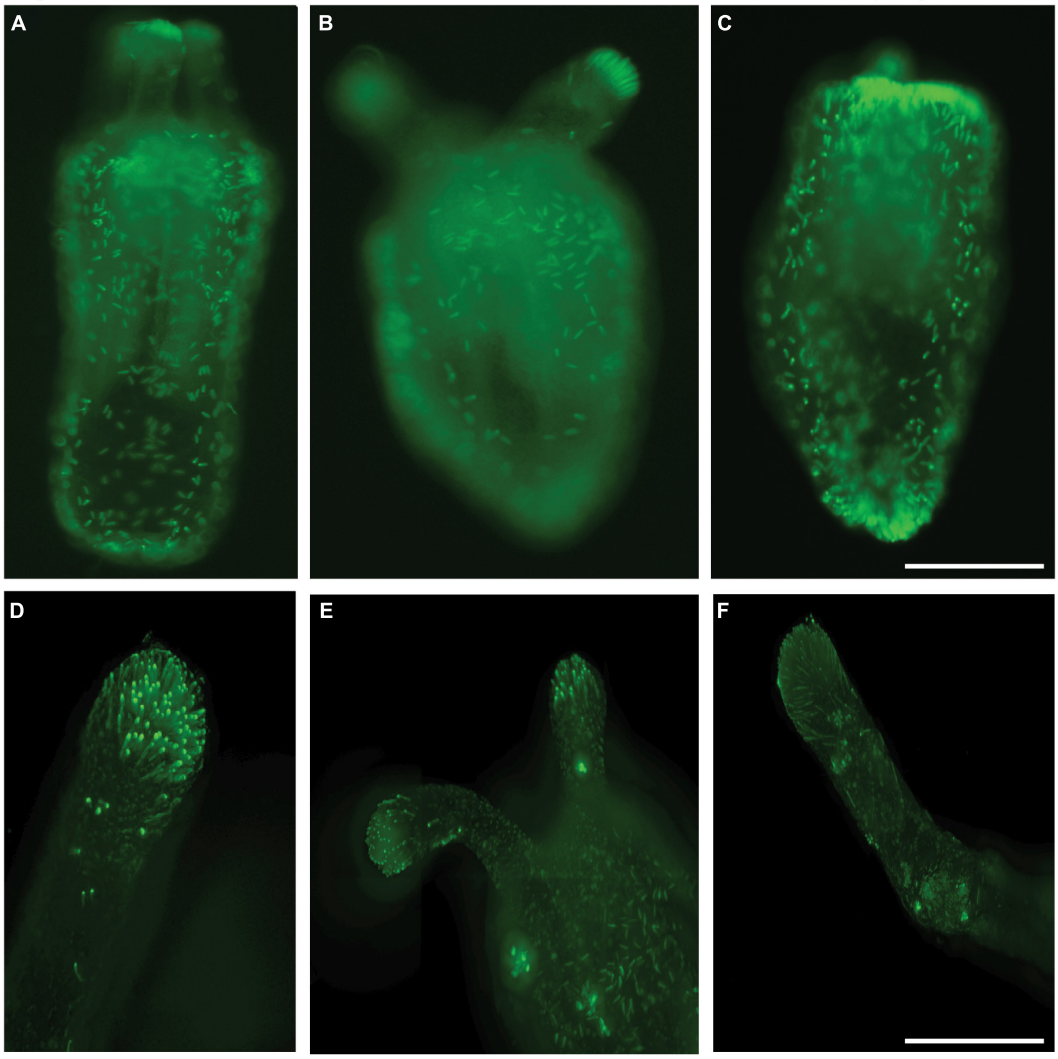


FIGURE 4 | Common environmental stressors cause defects in cnidocytes. **(A)** DAPI staining showing cnidocytes all over the outer body wall **(A)** and on the tentacle **(D)**. Embryos exposed to phthalates appear to have fewer cnidocytes all over the body **(B)**, especially on the tentacles **(D)**. Similarly, embryos exposed to KNO_3 have slightly less cnidocyte on the body **(C)**. Higher magnification images of the tentacles show fewer cnidocytes **(D,F)**. **(A–C)**, 10 × Scale bar = 500 μm **(D–E)**, 20 ×, Scale bar = 50 μm.

difference. Additionally, when we observed feeding behavior under the microscope, we could see the animals in the pollutants had no rotifers or fewer rotifers in their abdomen. Work of Ikmi et al. (2020) has identified that tentacle growth and increase in number occurs in a feeding dependent manner, this would suggest that the failure in growth we see in these stressed embryos is partially due to an inability to obtain enough food to drive tentacle growth. Far fewer cnidocytes, the specialized cell type that the animals use to capture its food were seen in the animals exposed to the environmental pollutants (**Figure 4**). We used a high concentration of DAPI staining method that label the poly-Y-glutamate in the matrix of mature cnidocytes to identify these cells (Szczepanek et al., 2002; Babonis and Martindale, 2014; Babonis and Martindale, 2017), our images suggest a lack of mature cnidocytes, however from this data we cannot distinguish whether or not this is due to apoptosis of these mature cells due to exposure to the toxins or if there is a defect in the early specification and differentiation of these cells types. We also tested the impact of these environmental pollutants on the adult animal's ability to regenerate its tentacles. Here we again saw strong phenotypes with a failure to regenerate the correct number and length of tentacles (phthalates) or in many cases exposure to KNO_3 resulted in a lack of regeneration or 1 or 2 tiny tenacles. Imaging of the specialized cnidocytes on the regenerated tentacles again showed a lack of these specialized cell types in the regenerates in comparison to controls (**Figure 5**). The magnitude of the defects in adult regeneration suggests in the case of exposure to KNO_3 a failure to deploy the "regeneration program,"

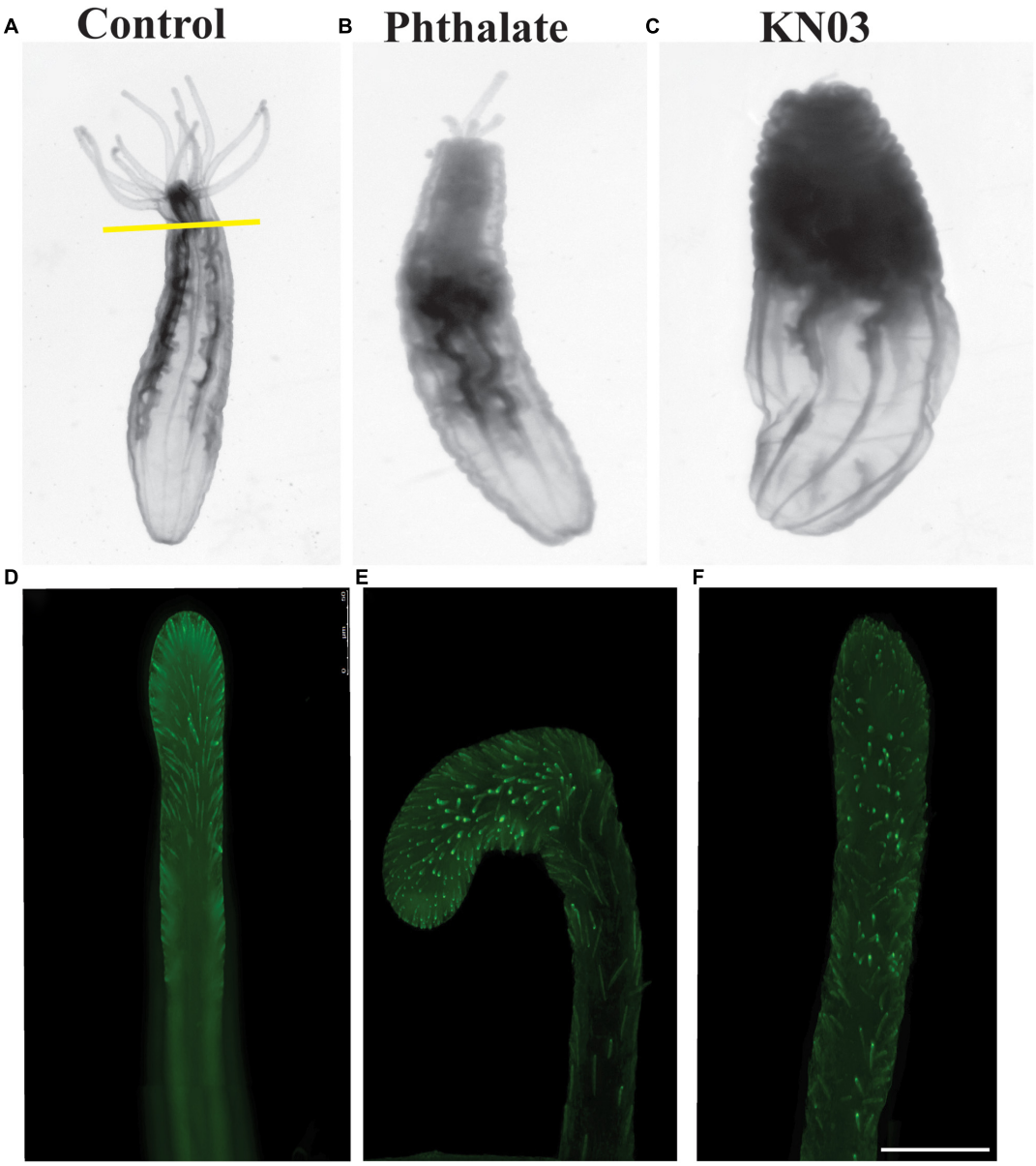


FIGURE 5 | Common environmental stressors inhibit oral regeneration. Nematostella can regenerate throughout life. Adult animals were amputated through the pharynx and assessed for completion of oral regeneration 2 weeks post injury. **(A)** Control animals regenerated all tentacles (n = 85). **(B)** Animals exposed to phthalates at 20 μM failed to regenerate tentacles of the correct length (n = 90). In contrast animals exposed to KNO_3 during regeneration mainly failed to regenerate tentacles, occasionally one tiny tentacle was regenerated **(C)** (n = 95). Staining of cnidocytes revealed that control animals fully regenerate the cnidocytes within 2 weeks **(D)**, while animals exposed to phthalates or KNO_3 have very few cnidocytes on the limited tentacles that are regenerated **(E,F)**. **(D–F)** Scale bar = 50 μm.

while the phthalate phenotype suggests more a fault in the execution of the "regeneration program" leading to incomplete regeneration and differentiation of the required amount of tissue and differentiated cell types.

Interestingly, other studies of the effect of phthalates on development in several species including zebrafish and frogs has also identified defects in body growth and spinal defects (Philippat et al., 2012; Kinch et al., 2016; Jergensen et al., 2019; Qian et al., 2020) similar to what we see here with the marine invertebrate Nematostella, suggesting a very common negative side effect of exposure to phthalates during embryonic development is slower body growth and defects in cells of the ectodermal lineage. Similar defects were seen when embryos were incubated in potassium nitrate and this has been observed in other species like newts, frogs and zebrafish (Fan and Steinberg, 1996; Ortiz et al., 2004; Orton et al., 2006; Ortiz-Santaliestra et al., 2007; Ortiz-Santaliestra and Sparling, 2007; Kinch et al., 2016; Conlin et al., 2018). Additionally in these species negative impacts on the endocrine system and on fertility have been documented (Fan and Steinberg, 1996; Fisher, 2004;

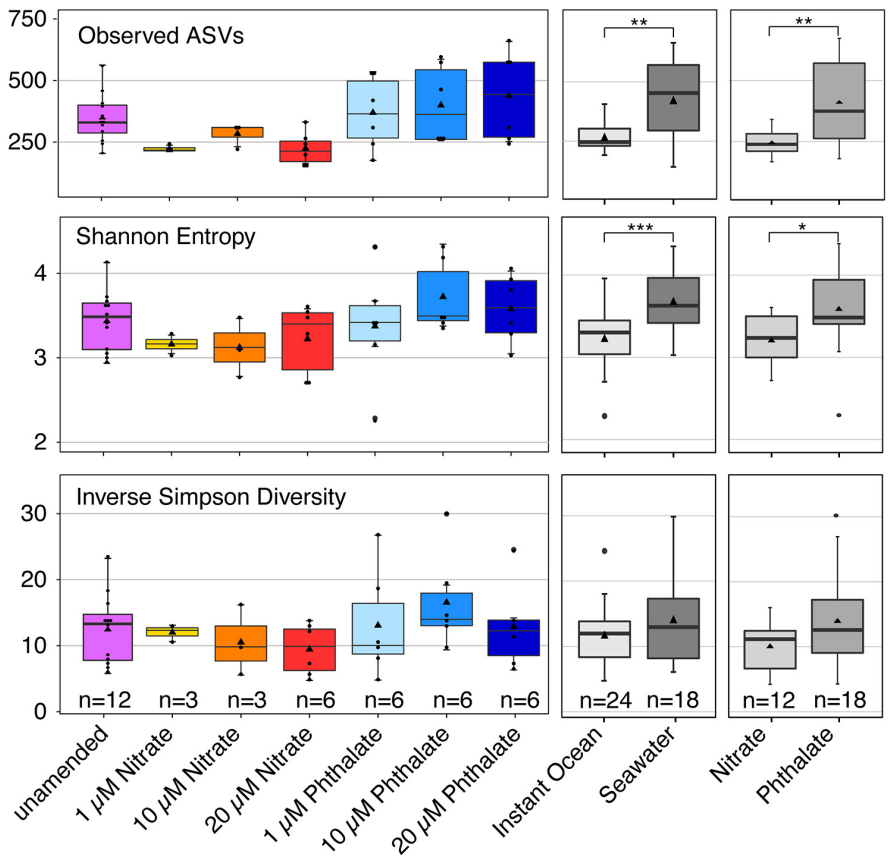


FIGURE 6 | Alpha diversity indices of different groupings of samples. Richness is shown as the number of observed bacterial amplicon sequence variants (ASV). Evenness is represented by the Inverse Simpson Diversity, Shannon entropy takes into account both richness and evenness. The number (n) of included samples per group is shown.

Orton et al., 2006; Krishnamurthy and Smith, 2011; Jannat et al., 2014; Conlin et al., 2018; Lv et al., 2020; Sharma et al., 2020; Tang et al., 2020).

The Composition of the Nematostella Microbiome Changes With Exposure to Nitrate or Phthalates

We also explored how these environmental pollutants may affect the host's microbiome. Prior reports describe shifts in the microbiome that might serve as indicators for changes in host health in marine organisms, including corals (Glasl et al., 2016) and vertebrates (Sehnal et al., 2021). In the case of Nematostella previous research has shown that the host microbiome is affected by changes in temperature and light conditions (Leach et al., 2019). Here, we studied potential connections between the Nematostella microbiome and host during exposure to environmental pollutants. In the samples of animals that were grown in Instant Ocean artificial seawater without pollutants we found high sequence abundances of populations affiliating with the genera *Tenacibaculum*, *Flavobacterium* and *Mariniflexile* (**Figure 8**). In the datasets from animals grown in unamended seawater *Pseudomonas* and *Saprospiraceae* were most abundant, indicating that the medium that was used to culture the embryos had a large effect on which microbiota colonized the animals, supporting previous studies showing substantial variability of the Nematostella microbiome with environment, season and biogeography (Har et al., 2015; Mortzfeld et al., 2016). It was also shown that stochastic community assembly processes can play a major role and result in different host-associated microbiomes independent of the traits of the host or the microbiota (Douglas, 2019). Such stochastic assembly processes during colonization may explain that not all microbiomes of animals grown in unamended seawater had a similar community structure after 10 days of incubation. The differences between these controls indicate that substantial variation exists in the microbiomes of groups of embryos grown separately, and that the separation of embryos into different wells early on may drive changes in the development of an organism's microbiome and lead to different community trajectories.

Despite the phylogenetic differences on genus-level, the microbiome of all conditions featured sequence abundant populations affiliating with the phylum *Bacteroidia*, which were shown to be of particular importance in the microbiome at early stages of the animals' development (Mortzfeld et al., 2016). The different genera that were enriched in the microbiome under

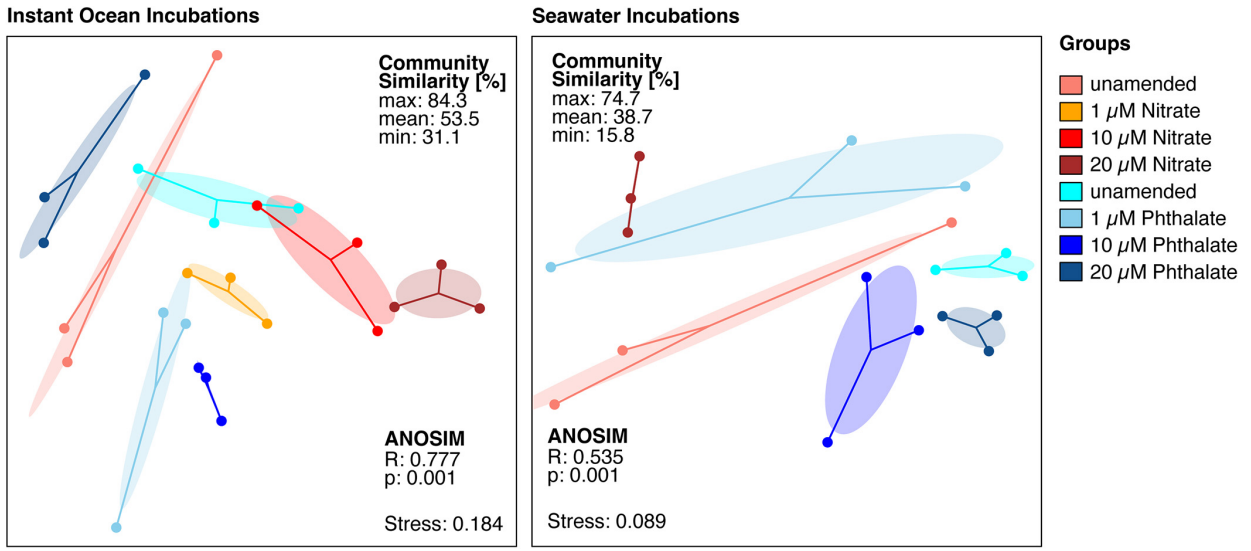


FIGURE 7 | Non-metric multidimensional scaling ordination based on a distance matrix of all available amplicon sequence variants (ASVs). The microbial communities exposed to different pollutants and concentrations are significantly different from each other. Each dot represents the total bacterial community of a sample, the closer the dots are the more similar are the underlying communities. The dots of each condition are connected to the weighted average mean of within group distances (centroid), ellipses represent one standard deviation of the centroid. Ellipses that do not overlap generally show substantial differences between groups.

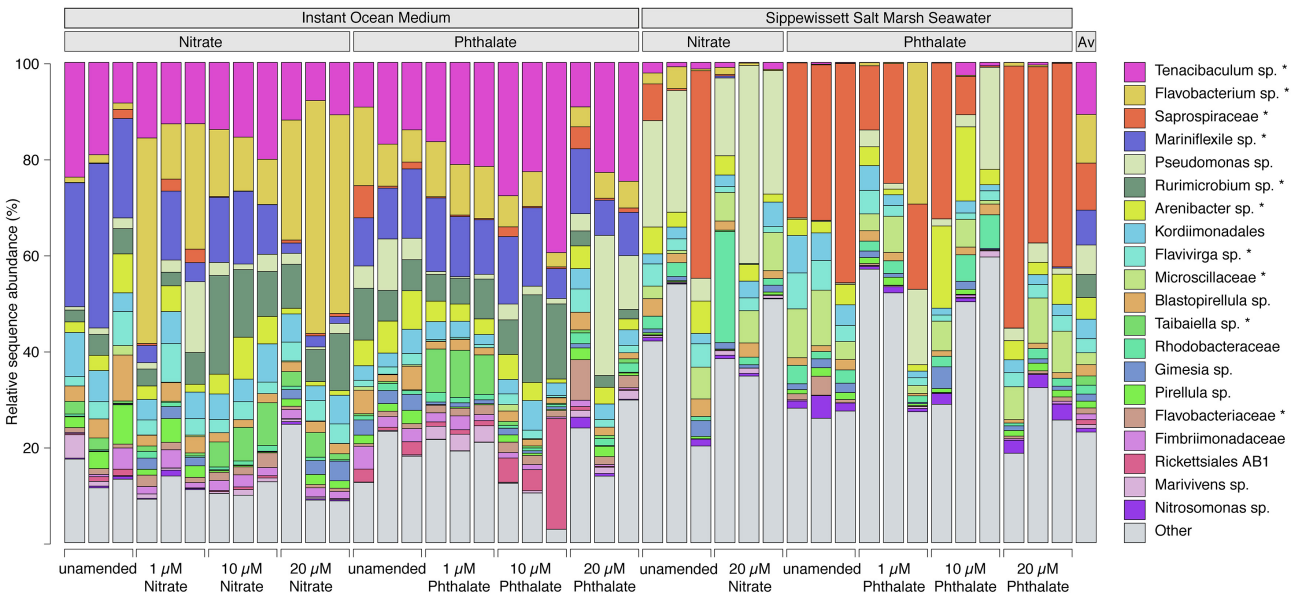


FIGURE 8 | Bacterial community composition of populations at species level. Each bar represents one sample and shows the 20 species-level lineages with the highest relative sequence abundance averaged across all datasets. All other lineages are summed up as "Other." The average composition across all samples is shown in the last column. An unclassified population within the genus Flavobacterium (yellow) substantially increases with increasing nitrate concentrations. "Asterisk" denotes lineages that belong to the phylum *Bacteroidia*. Lineages that were not classified to genus level represent species in unclassified families (e.g., *Saprospiraceae* – red) or unclassified orders (e.g., *Kordiimonadales* – sky blue).

certain conditions often belonged to the same family within the *Bacteroidia* and may thus be functionally redundant indicating that microbial function played a role in microbiome assembly. Overall, the composition of the Nematostella microbiome was similar to that reported in previous studies where Bacteroidia and Proteobacteria sequences represented the most abundant taxa (Har et al., 2015; Mortzfeld et al., 2016; Baldassarre et al., 2021). We found Spirochetes in low abundance in the host microbiomes as well, which agrees with a previous study showing that Spirochetes colonize the capitulum (Bonacolta et al., 2021).

When comparing the unamended animal microbiomes to those exposed to different concentrations of pollutants, we observed that the detrimental effects caused by each pollutant on the animals' development apparently did not have a similar impact on the animal-associated microbiome. We did not find significant changes in the richness and evenness of the microbial communities when comparing animals that were grown without pollutant with those grown under different concentrations of each pollutant. The slightly higher variability in microbial richness especially between the phthalate treatments as compared to the unamended cultures (**Figure 6**) may be due to increased stress, as stressors can impact alpha diversity (Rocca et al., 2019). The effect of pollutants apparently manifested in shifts of community structure (**Figure 7**) and composition (**Figure 8**) rather than richness. These shifts indicate that taxa in the *N. vectensis* microbiome were replaced rather than completely eliminated. In contrast to certain unamended cultures that featured relatively strong differences in community composition, potentially caused by stochastic processes during colonization, deterministic processes may have played an important role in the pollutant treated animals., In most cases the microbiomes of the different treatments formed well separated clusters in the NMDS ordination with low beta diversity within replicates of a given treatment. Such patterns can be caused by stressors that select for certain taxa and increase their abundance leading to deterministic community changes (Zaneveld et al., 2017).

The high relative sequence abundance of *Tenacibaculum sp.* across the Instant Ocean artificial seawater incubations, but not in those using Sippewissett salt marsh water growth medium, is likely due to initial differences in microbial community composition between the growth media. However, Tenacibaculum spp. are capable of thriving on polysaccharides and proteins (Pérez-Pascual et al., 2017) and many are pathogenic or associated with diseased fish and anemones (Wang et al., 2008) which might explain the slight increase in relative sequence abundance for this organism in the 10 μM phthalate treatment when the health of Nematostella was impaired. *Flavobacterium* increased in relative abundance and a member of the genus *Mariniflexile* decreased in relative abundance with increasing nitrate concentrations. All three genera belong to the family *Flavobacteriaceae* and are known to include marine species that can degrade polysaccharides (Barbeyron et al., 2008; Nedashkovskaya et al., 2014), yet only the genus *Flavobacterium* contains organisms that can reduce nitrate (Nupur et al., 2013). It is likely that they have similar niches concerning carbon sources but can differently utilize nitrate. For example, nitrate-reducing *Flavobacterium columnare* are associated with disease in fish experiencing environmental stress (Abdelhamed et al., 2021). In this situation, nitrate can be used by opportunist pathogenic Flavobacterium sp. as an alternative electron acceptor in oxygen-limited microenvironments, such as in biofilms or during infection of tissue (Abdelhamed et al., 2021).

The phthalate exposed microbiomes in the Sippewissett salt marsh seawater cultures were enriched with a lineage affiliating with Saprospiraceae. These organisms also affiliate with Bacteroidetes and are not only related to the most abundant clades in the instant ocean cultures but have a similar metabolic capabilities degrading complex organic matter such as polysaccharides (McIlroy and Nielsen, 2014). It is thus very likely that functionally redundant, yet taxonomically different clades were recruited from the communities of the initial culture medium. Organisms belonging to the genus *Pseudomonas* were present all animal microbiomes (**Figure 8**), yet sporadically appeared in higher relative sequence abundance in animals cultivated in Sippewissett salt marsh seawater. This genus is known to contain organisms able to degrade phthalates (Vamsee-Krishna and Phale, 2008), however, the highest relative sequence abundances of this organism were found mainly in KNO_3 - treated samples and unamended controls, and the observed pattern does not indicate that the presence of phthalate or KNO_3 selected for *Pseudomonas* in either growth media. The activity of the microbiome determines potential physiological feedbacks between the microbiome and host and is thus an important factor for the examination of holobiont health. Due to the limitations of taxonomy-based analyses future studies would benefit from analyses of the functional capabilities and activity of the microbiome to understand feedbacks between the microbiome and host health.

In this study, the *Nematostella vectensis* microbiome was significantly influenced by the growth medium (**Supplementary Figure 2** NMDS). The reported deterministic changes in the microbial community structure caused by the pollutants can therefore be easily missed. From the findings of this study, we conclude that the detrimental effects of pollutants on the development of marine invertebrates are not always mirrored to the same degree in the animals' microbiome. However, as the embryos were exposed to the pollutants for 10 days only this may not be enough time to result in a substantial change in the microbiome. Moreover, the source of seawater, biogeography and the environmental variability of Nematostella itself (Darling et al., 2004) can apparently have large effects on the outcome of such cultivation experiments potentially masking the underlying positive or negative trends.

In summary, our results demonstrate that common pollutants found in salt marshes adversely affect the development of Nematostella embryos, ultimately leading to death. This is an important finding as globally populations of Nematostella are decreasing and as their natural habitats is the marsh lands, they are very susceptible to pollution. This study looks mainly at the effect on embryos, in the future it will be interesting to determine if exposure of adult animals to these pollutants also causes changes in the microbiome and ultimate fitness of the offspring.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene sequence datasets are publicly archived at NCBI under BioProject PRJNA767880.

AUTHOR CONTRIBUTIONS

SK carried out development and regeneration assays, took all measurements and prepared the graphs using PRISM. TR, ED-J, and EL contributed to the testing of stressors on

embryos and regenerating animals. VF carried out the DNA isolation and library preparation and sequencing. MS helped design and oversee the microbial sequencing. ER analyzed and visualized nucleic acid sequence data. KE and ER conceived the project, oversaw the design and execution of the experiments and wrote the manuscript. All authors read and contributed to the manuscript.

FUNDING

This work was funded by a Pilot Program award to ER and KE from the Microbiome Center at the University of Chicago. The microbiome sequencing was funded by a grant from the McDonnell Initiative to ER. KE was supported by a grant from NICHD R01 HD092451, start-up funds from the MBL and funding from the Owens Family Foundation. ER was supported by start-up funds from the MBL and MLS receives support from the Unger G. Vetlesen Foundation.

ACKNOWLEDGMENTS

We thank Sherlynette Pérez Castro and Keith Sabin who supervised high school students Melissa Ham and Alley Rivera to first work on the effect of environmental stressors on Nematostella regeneration as a science fair project which led to the conception of this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.786037/full#supplementary-material

REFERENCES

Abdelhamed, H., Nho, S. W., Karsi, A., and Lawrence, M. L. (2021). The role of denitrification genes in anaerobic growth and virulence of Flavobacterium columnare. *J. Appl. Microbiol.* 130, 1062–1074. doi: 10.1111/jam.14855

Amiel, A. R., Johnston, H., Chock, T., Dahlin, P., Iglesias, M., Layden, M., et al. (2017). A bipolar role of the transcription factor ERG for cnidarian germ layer formation and apical domain patterning. *Dev. Biol.* 430, 346–361. doi: 10.1016/j.ydbio.2017.08.015

Amiel, A. R., Johnston, H. T., Nedoncelle, K., Warner, J. F., Ferreira, S., and Rottinger, E. (2015). Characterization of Morphological and Cellular Events Underlying Oral Regeneration in the Sea Anemone, Nematostella vectensis. *Int. J. Mol. Sci.* 16, 28449–28471. doi: 10.3390/ijms161226100

Amiel, A. R., Michel, V., Carvalho, J. E., Shkreli, M., Petit, C., and Röttinger, E. (2021). [The sea anemone Nematostella vectensis, an emerging model for biomedical research: mechano-sensitivity, extreme regeneration and longevity]. *Med. Sci.* 37, 167–177. doi: 10.1051/medsci/2020282

Amiel, A. R., and Röttinger, E. (2021). Experimental Tools to Study Regeneration in the Sea Anemone Nematostella vectensis. *Methods Mol. Biol.* 2219, 69–80. doi: 10.1007/978-1-0716-0974-3_4

Babonis, L. S., and Martindale, M. Q. (2014). Old cell, new trick? Cnidocytes as a model for the evolution of novelty. *Integr. Comp. Biol.* 54, 714–722. doi: 10.1093/icb/icu027

Babonis, L. S., and Martindale, M. Q. (2017). PaxA, but not PaxC, is required for cnidocyte development in the sea anemone Nematostella vectensis. *Evodevo* 8:14. doi: 10.1186/s13227-017-0077-7

Babonis, L. S., Martindale, M. Q., and Ryan, J. F. (2016). Do novel genes drive morphological novelty? An investigation of the nematosomes in the sea anemone Nematostella vectensis. *BMC Evol. Biol.* 16:114. doi: 10.1186/s12862-016-0683-3

Baldassarre, L., Levy, S., Bar-Shalom, R., Steindler, L., Lotan, T., and Fraune, S. (2021). Contribution of Maternal and Paternal Transmission to Bacterial Colonization in Nematostella vectensis. *Front. Microbiol.* 12:726795. doi: 10.3389/fmicb.2021.726795

Barbeyron, T., L'haridon, S., Michel, G., and Czjzek, M. (2008). Mariniflexile fucanivorans sp. nov., a marine member of the Flavobacteriaceae that degrades sulphated fucans from brown algae. *Int. J. Syst. Evol. Microbiol.* 58, 2107–2113. doi: 10.1099/ijs.0.65674-0

Bonacolta, A. M., Connelly, M. T., Rosales, S. M., del Campo, J., and Traylor-Knowles, N. (2021). The starlet sea anemone, *Nematostella vectensis*, possesses body region-specific bacterial associations with spirochetes dominating the capitulum. *FEMS Microbiol. Lett.* 368:fnab002. doi: 10.1093/femsle/fnab002

Bosch, T. C. G., and McFall-Ngai, M. (2021). Animal development in the microbial world: re-thinking the conceptual framework. *Curr. Top. Dev. Biol.* 141, 399–427. doi: 10.1016/bs.ctdb.2020.11.007

Bossert, P., and Thomsen, G. H. (2017). Inducing Complete Polyp Regeneration from the Aboral Physa of the Starlet Sea Anemone Nematostella vectensis. *J. Vis. Exp.* 119:54626. doi: 10.3791/54626

Bossert, P. E., Dunn, M. P., and Thomsen, G. H. (2013). A staging system for the regeneration of a polyp from the aboral physa of the anthozoan Cnidarian Nematostella vectensis. *Dev. Dyn.* 242, 1320–1331. doi: 10.1002/dvdy.24021

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of Southern Wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Camargo, J. A., Alonso, A., and Salamanca, A. (2005). Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. *Chemosphere* 58, 1255–1267. doi: 10.1016/j.chemosphere.2004.10.044

Conlin, S. M., Tudor, M. S., Shim, J., Gosse, J. A., Neilson, A., and Hamlin, H. J. (2018). Elevated nitrate alters the metabolic activity of embryonic zebrafish. *Environ. Pollut.* 235, 180–185. doi: 10.1016/j.envpol.2017.12.069

Daniel, Y. C. W., Kumar, S., and Hedges, S. B. (1999). Divergence Time Estimates for the Early History of Animal Phyla and the Origin of Plants, Animals and Fungi. *Proc. Biol. Sci.* 266, 163–171. doi: 10.1098/rspb.1999.0617

Darling, J. A., Reitzel, A. M., and Finnerty, J. R. (2004). Regional population structure of a widely introduced estuarine invertebrate: nematostella vectensis Stephenson in New England. *Mol. Ecol.* 13, 2969–2981. doi: 10.1111/j.1365-294X.2004.02313.x

Darling, J. A., Reitzel, A. R., Burton, P. M., Mazza, M. E., Ryan, J. F., Sullivan, J. C., et al. (2005). Rising starlet: the starlet sea anemone, Nematostella vectensis. *Bioessays* 27, 211–221. doi: 10.1002/bies.20181

Douglas, A. E. (2019). Simple animal models for microbiome research. *Nat. Rev. Microbiol.* 17, 764–775. doi: 10.1038/s41579-019-0242-1

DuBuc, T. Q., Traylor-Knowles, N., and Martindale, M. Q. (2014). Initiating a regenerative response; cellular and molecular features of wound healing in the cnidarian Nematostella vectensis. *BMC Biol.* 12:24. doi: 10.1186/1741-7007-12-24

Elran, R., Raam, M., Kraus, R., Brekhman, V., Sher, N., Plaschkes, I., et al. (2014). Early and late response of Nematostella vectensis transcriptome to heavy metals. *Mol. Ecol.* 23, 4722–4736. doi: 10.1111/mec.12891

Essock-Burns, T., Bongrand, C., Goldman, W. E., Ruby, E. G., and McFall-Ngai, M. J. (2020). Interactions of Symbiotic Partners Drive the Development of a Complex Biogeography in the Squid-Vibrio Symbiosis. *mBio* 11, e00853–20. doi: 10.1128/mBio.00853-20

Fan, A. M., and Steinberg, V. E. (1996). Health implications of nitrate and nitrite in drinking water: an update on methemoglobinemia occurrence and reproductive and developmental toxicity. *Regul. Toxicol. Pharmacol.* 23, 35–43. doi: 10.1006/rtph.1996.0006

Fisher, J. S. (2004). Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. *Reproduction* 127, 305–315. doi: 10.1530/rep.1.00025

Fraune, S., Augustin, R., Anton-Erxleben, F., Wittlieb, J., Gelhaus, C., Klimovich, V. B., et al. (2010). In an early branching metazoan, bacterial colonization of the embryo is controlled by maternal antimicrobial peptides. *Proc. Natl. Acad. Sci. U. S. A.* 107, 18067–18072. doi: 10.1073/pnas.1008573107

Fraune, S., and Bosch, T. C. (2010). Why bacteria matter in animal development and evolution. *Bioessays* 32, 571–580. doi: 10.1002/bies.200900192

Genikhovich, G., and Technau, U. (2009). The starlet sea anemone Nematostella vectensis: an anthozoan model organism for studies in comparative genomics and functional evolutionary developmental biology. *Cold Spring Harb. Protoc.* 2009:pdb.emo129. doi: 10.1101/pdb.emo129

Glasl, B., Herndl, G. J., and Frade, P. R. (2016). The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance. *ISME J.* 10, 2280–2292. doi: 10.1038/ismej.2016.9

Gugliandolo, E., Licata, P., Crupi, R., Albergamo, A., Jebara, A., Lo Turco, V., et al. (2020). Plasticizers as Microplastics Tracers in Tunisian Marine Environment. *Front. Mar. Sci.* 7:589398. doi: 10.3389/fmars.2020.589398

Hand, C., and Uhlinger, K. R. (1992). The Culture, Sexual and Asexual Reproduction, and Growth of the Sea Anemone Nematostella vectensis. *Biol. Bull.* 182, 169–176. doi: 10.2307/1542110

Har, J. Y., Helbig, T., Lim, J. H., Fernando, S. C., Reitzel, A. M., Penn, K., et al. (2015). Microbial diversity and activity in the Nematostella vectensis holobiont: insights from 16S rRNA gene sequencing, isolate genomes, and a pilot-scale survey of gene expression. *Front. Microbiol.* 6:818. doi: 10.3389/fmicb.2015.00818

Heath-Heckman, E. A., Foster, J., Apicella, M. A., Goldman, W. E., and McFall-Ngai, M. (2016). Environmental cues and symbiont microbe-associated molecular patterns function in concert to drive the daily remodelling of the crypt-cell brush border of the Euprymna scolopes light organ. *Cell. Microbiol.* 18, 1642–1652. doi: 10.1111/cmi.12602

Hu, R., Zhao, H., Xu, X., Wang, Z., Yu, K., Shu, L., et al. (2021). Bacteria-driven phthalic acid ester biodegradation: current status and emerging opportunities. *Environ. Int.* 154:106560. doi: 10.1016/j.envint.2021.106560

Ikmi, A., Steenbergen, P. J., Anzo, M., Mcmullen, M. R., Stokkermans, A., Ellington, L. R., et al. (2020). Feeding-dependent tentacle development in the sea anemone Nematostella vectensis. *Nat. Commun.* 11:4399. doi: 10.1038/s41467-020-18133-0

Jannat, M., Fatimah, R., and Kishida, M. (2014). Nitrate (NO3(-)) and nitrite (NO2(-)) are endocrine disruptors to downregulate expression of tyrosine hydroxylase and motor behavior through conversion to nitric oxide in early development of zebrafish. *Biochem. Biophys. Res. Commun.* 452, 608–613. doi: 10.1016/j.bbrc.2014.08.114

Jergensen, T., Cusmano, D., and Roy, N. M. (2019). Di-butyl phthalate (DBP) induces craniofacial defects during embryonic development in zebrafish. *Ecotoxicology* 28, 995–1002. doi: 10.1007/s10646-019-02100-7

Kinch, C. D., Kurrasch, D. M., and Habibi, H. R. (2016). Adverse morphological development in embryonic zebrafish exposed to environmental concentrations of contaminants individually and in mixture. *Aquat. Toxicol.* 175, 286–298. doi: 10.1016/j.aquatox.2016.03.021

Kirillova, A., Genikhovich, G., Pukhlyakova, E., Demilly, A., Kraus, Y., and Technau, U. (2018). Germ-layer commitment and axis formation in sea anemone embryonic cell aggregates. *Proc. Natl. Acad. Sci. U. S. A.* 115, 1813–1818. doi: 10.1073/pnas.1711516115

Kraus, Y., and Technau, U. (2006). Gastrulation in the sea anemone Nematostella vectensis occurs by invagination and immigration: an ultrastructural study. *Dev. Genes Evol.* 216, 119–132. doi: 10.1007/s00427-005-0038-3

Krishnamurthy, S. V., and Smith, G. R. (2011). Combined effects of malathion and nitrate on early growth, abnormalities, and mortality of wood frog (*Rana sylvatica*) tadpoles. *Ecotoxicology* 20, 1361–1367. doi: 10.1007/s10646-011-0692-3

Kruskal, J. B. (1964). Nonmetric multidimensional scaling: a numerical method. *Psychometrika* 29, 115–129. doi: 10.1007/BF02289694

Layden, M. J., Rentzsch, F., and Rottinger, E. (2016). The rise of the starlet sea anemone Nematostella vectensis as a model system to investigate development and regeneration. *Wiley Interdiscip. Rev. Dev. Biol.* 5, 408–428. doi: 10.1002/wdev.222

Leach, W. B., Carrier, T. J., and Reitzel, A. M. (2019). Diel patterning in the bacterial community associated with the sea anemone Nematostella vectensis. *Ecol. Evol.* 9, 9935–9947. doi: 10.1002/ece3.5534

Lv, H., Liu, B., and Qin, Y. (2020). Isosorbide mononitrate promotes angiogenesis in embryonic development of zebrafish. *Genet. Mol. Biol.* 43:20190233. doi: 10.1590/1678-4685-gmb-2019-0233

Marlow, H., Roettinger, E., Boekhout, M., and Martindale, M. Q. (2012). Functional roles of Notch signaling in the cnidarian Nematostella vectensis. *Dev. Biol.* 362, 295–308. doi: 10.1016/j.ydbio.2011.11.012

Marlow, H. Q., Srivastava, M., Matus, D. Q., Rokhsar, D., and Martindale, M. Q. (2009). Anatomy and development of the nervous system of Nematostella vectensis, an anthozoan cnidarian. *Dev. Neurobiol.* 69, 235–254. doi: 10.1002/dneu.20698

McClelland, J. W., and Valiela, I. (1998). Linking nitrogen in estuarine producers to land-derived sources. *Limnol. Oceanogr.* 43, 577–585. doi: 10.4319/lo.1998.43.4.0577

McIlroy, S. J., and Nielsen, P. H. (2014). "The Family Saprospiraceae," in *The Prokaryotes: other Major Lineages of Bacteria and The Archaea*, eds E. Rosenberg, E. F. Delong, S. Lory, E. Stackebrandt, and F. Thompson (Berlin, Heidelberg: Springer Berlin Heidelberg).

Mortzfeld, B. M., Urbanski, S., Reitzel, A. M., Künzel, S., Technau, U., and Fraune, S. (2016). Response of bacterial colonization in Nematostella vectensis to development, environment and biogeography. *Environ. Microbiol.* 18, 1764–1781. doi: 10.1111/1462-2920.12926

Nedashkovskaya, O. I., Balabanova, L. A., Zhukova, N. V., Kim, S.-J., Bakunina, I. Y., and Rhee, S.-K. (2014). Flavobacterium ahnfeltiae sp. nov., a new marine polysaccharide-degrading bacterium isolated from a Pacific red alga. *Arch. Microbiol.* 196, 745–752. doi: 10.1007/s00203-014-1010-2

Nupur, Bhumika, V., Srinivas, T. N. R., and Kumar, P. A. (2013). Flavobacterium nitratireducens sp. nov., an amylolytic bacterium of the family Flavobacteriaceae isolated from coastal surface seawater. *Int. J. Syst. Evol. Microbiol.* 63, 2490–2496. doi: 10.1099/ijs.0.046524-0

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2012). *vegan: Community Ecology Package*. Available online at: https://cran.r-project.org/web/packages/vegan/index.html

Ortiz, M. E., Marco, A., Saiz, N., and Lizana, M. (2004). Impact of ammonium nitrate on growth and survival of six European amphibians. *Arch. Environ. Contam. Toxicol.* 47, 234–239. doi: 10.1007/s00244-004-2296-x

Ortiz-Santaliestra, M. E., Marco, A., Fernández-Benéitez, M. J., and Lizana, M. (2007). Effects of ammonium nitrate exposure and water acidification on the dwarf newt: the protective effect of oviposition behaviour on embryonic survival. *Aquat. Toxicol.* 85, 251–257. doi: 10.1016/j.aquatox.2007.09.008

Ortiz-Santaliestra, M. E., and Sparling, D. W. (2007). Alteration of larval development and metamorphosis by nitrate and perchlorate in southern leopard frogs (*Rana sphenocephala*). *Arch. Environ. Contam. Toxicol.* 53, 639–646. doi: 10.1007/s00244-006-0277-y

Orton, F., Carr, J. A., and Handy, R. D. (2006). Effects of nitrate and atrazine on larval development and sexual differentiation in the northern leopard frog Rana pipiens. *Environ. Toxicol. Chem.* 25, 65–71. doi: 10.1897/05-136R.1

Passamaneck, Y. J., and Martindale, M. Q. (2012). Cell proliferation is necessary for the regeneration of oral structures in the anthozoan cnidarian Nematostella vectensis. *BMC Dev. Biol.* 12:34. doi: 10.1186/1471-213X-12-34

Pérez-Pascual, D., Lunazzi, A., Magdelenat, G., Rouy, Z., Roulet, A., Lopez-Roques, C., et al. (2017). The Complete Genome Sequence of the Fish Pathogen Tenacibaculum maritimum Provides Insights into Virulence Mechanisms. *Front. Microbiol.* 8:1542. doi: 10.3389/fmicb.2017.01542

Philippat, C., Mortamais, M., Chevrier, C., Petit, C., Calafat, A. M., Ye, X., et al. (2012). Exposure to phthalates and phenols during pregnancy and offspring size at birth. *Environ. Health Perspect.* 120, 464–470. doi: 10.1289/ehp.1103634

Pita, L., Rix, L., Slaby, B. M., Franke, A., and Hentschel, U. (2018). The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome* 6:46. doi: 10.1186/s40168-018-0428-1

Qian, L., Liu, J., Lin, Z., Chen, X., Yuan, L., Shen, G., et al. (2020). Evaluation of the spinal effects of phthalates in a zebrafish embryo assay. *Chemosphere* 249:126144. doi: 10.1016/j.chemosphere.2020.126144

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Reitzel, A. M., Herrera, S., Layden, M. J., Martindale, M. Q., and Shank, T. M. (2013). Going where traditional markers have not gone before: utility of and promise for RAD sequencing in marine invertebrate phylogeography and population genomics. *Mol. Ecol.* 22, 2953–2970. doi: 10.1111/mec.12228

Rocca, J. D., Simonin, M., Blaszczak, J. R., Ernakovich, J. G., Gibbons, S. M., Midani, F. S., et al. (2019). The Microbiome Stress Project: toward a Global Meta-Analysis of Environmental Stressors and Their Effects on Microbial Communities. *Front. Microbiol.* 9:3272. doi: 10.3389/fmicb.2018.03272

Rook, G., Bäckhed, F., Levin, B. R., McFall-Ngai, M. J., and Mclean, A. R. (2017). Evolution, human-microbe interactions, and life history plasticity. *Lancet* 390, 521–530. doi: 10.1016/S0140-6736(17)30566-4

Röttinger, E., Dahlin, P., and Martindale, M. Q. (2012). A framework for the establishment of a cnidarian gene regulatory network for "endomesoderm" specification: the inputs of ß-catenin/TCF signaling. *PLoS Genet.* 8:e1003164. doi: 10.1371/journal.pgen.1003164

Schaffer, A. A., Bazarsky, M., Levy, K., Chalifa-Caspi, V., and Gat, U. (2016). A transcriptional time-course analysis of oral vs. aboral whole-body regeneration in the Sea anemone Nematostella vectensis. *BMC Genomics* 17:718. doi: 10.1186/s12864-016-3027-1

Schwaiger, M., Schönauer, A., Rendeiro, A. F., Pribitzer, C., Schauer, A., Gilles, A. F., et al. (2014). Evolutionary conservation of the eumetazoan gene regulatory landscape. *Genome Res.* 24, 639–650. doi: 10.1101/gr.162529.113

Sebe-Pedros, A., Saudemont, B., Chomsky, E., Plessier, F., Mailhe, M. P., Renno, J., et al. (2018). Cnidarian Cell Type Diversity and Regulation Revealed by Whole-Organism Single-Cell RNA-Seq. *Cell* 173, 1520–1534.e20. doi: 10.1016/j.cell.2018.05.019

Sehnal, L., Brammer-Robbins, E., Wormington, A. M., Blaha, L., Bisesi, J., Larkin, I., et al. (2021). Microbiome Composition and Function in Aquatic Vertebrates: small Organisms Making Big Impacts on Aquatic Animal Health. *Front. Microbiol.* 12:567408. doi: 10.3389/fmicb.2021.567408

Sharma, A., Mollier, J., Brocklesby, R. W. K., Caves, C., Jayasena, C. N., and Minhas, S. (2020). Endocrine-disrupting chemicals and male reproductive health. *Reprod. Med. Biol.* 19, 243–253. doi: 10.1002/rmb2.12326

Steinmetz, P. R. H., Aman, A., Kraus, J. E. M., and Technau, U. (2017). Gut-like ectodermal tissue in a sea anemone challenges germ layer homology. *Nat. Ecol. Evol.* 1, 1535–1542. doi: 10.1038/s41559-017-0285-5

Szczepanek, S., Cikala, M., and David, C. N. (2002). Poly-gamma-glutamate synthesis during formation of nematocyst capsules in Hydra. *J. Cell Sci.* 115, 745–751. doi: 10.1242/jcs.115.4.745

Tang, Z. R., Xu, X. L., Deng, S. L., Lian, Z. X., and Yu, K. (2020). Oestrogenic Endocrine Disruptors in the Placenta and the Fetus. *Int. J. Mol. Sci.* 21:1519. doi: 10.3390/ijms21041519

Tarrant, A. M., Payton, S. L., Reitzel, A. M., Porter, D. T., and Jenny, M. J. (2018). Ultraviolet radiation significantly enhances the molecular response to dispersant and sweet crude oil exposure in Nematostella vectensis. *Mar. Environ. Res.* 134, 96–108. doi: 10.1016/j.marenvres.2018.01.002

Technau, U. (2020). Gastrulation and germ layer formation in the sea anemone Nematostella vectensis and other cnidarians. *Mech. Dev.* 163:103628. doi: 10.1016/j.mod.2020.103628

Thompson, J. R., Rivera, H. E., Closek, C. J., and Medina, M. (2014). Microbes in the coral holobiont: partners through evolution, development, and ecological interactions. *Front. Cell. Infect. Microbiol.* 4:176. doi: 10.3389/fcimb.2014.00176

Trevino, M., Stefanik, D. J., Rodriguez, R., Harmon, S., and Burton, P. M. (2011). Induction of canonical Wnt signaling by alsterpaullone is sufficient for oral tissue fate during regeneration and embryogenesis in Nematostella vectensis. *Dev. Dyn.* 240, 2673–2679. doi: 10.1002/dvdy.22774

Valiela, I., Lloret, J., Chenoweth, K., Elmstrom, E., and Hanacek, D. (2021). Control of N Concentrations in Cape Cod Estuaries by Nitrogen Loads, Season, and Down-Estuary Transit: assessment by Conventional and Effect-Size Statistics. *Estuaries Coasts* 44, 1294–1309. doi: 10.1007/s12237-020-00869-z

Vamsee-Krishna, C., and Phale, P. S. (2008). Bacterial degradation of phthalate isomers and their esters. *Indian J. Microbiol.* 48, 19–34. doi: 10.1007/s12088-008-0003-8

van der Burg, C. A., and Prentis, P. J. (2021). The Tentacular Spectacular: evolution of Regeneration in Sea Anemones. *Genes* 12:1072. doi: 10.3390/genes12071072

Wang, J.-T., Chou, Y.-J., Chou, J.-H., Chen, C. A., and Chen, W.-M. (2008). Tenacibaculum aiptasiae sp. nov., isolated from a sea anemone Aiptasia pulchella. *Int. J. Syst. Evol. Microbiol.* 58, 761–766. doi: 10.1099/ijs.0.65437-0

Warner, J. F., Guerlais, V., Amiel, A. R., Johnston, H., Nedoncelle, K., and Rottinger, E. (2018). NvERTx: a gene expression database to compare embryogenesis and regeneration in the sea anemone Nematostella vectensis. *Development* 145:dev162867. doi: 10.1242/dev.162867

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag New York. Available online at: https://ggplot2-book.org/

Wijesena, N., Simmons, D. K., and Martindale, M. Q. (2017). Antagonistic BMP-cWNT signaling in the cnidarian Nematostella vectensis reveals insight into the evolution of mesoderm. *Proc. Natl. Acad. Sci. U. S. A.* 114, E5608–E5615. doi: 10.1073/pnas.1701607114

Wikramanayake, A. H., Hong, M., Lee, P. N., Pang, K., Byrum, C. A., Bince, J. M., et al. (2003). An ancient role for nuclear beta-catenin in the evolution of axial polarity and germ layer segregation. *Nature* 426, 446–450. doi: 10.1038/nature02113

Wolenski, F. S., Bradham, C. A., Finnerty, J. R., and Gilmore, T. D. (2013). NF-κB is required for cnidocyte development in the sea anemone *Nematostella vectensis*. *Dev. Biol.* 373, 205–215. doi: 10.1016/j.ydbio.2012.10.004

Zaneveld, J. R., Mcminds, R., and Vega Thurber, R. (2017). Stress and stability: applying the Anna Karenina principle to animal microbiomes. *Nat. Microbiol.* 2:17121. doi: 10.1038/nmicrobiol.2017.121

Zheng, D., Liwinski, T., and Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Res.* 30, 492–506. doi: 10.1038/s41422-020-0332-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cell and Developmental Biology

REVIEW
published: 26 January 2022
doi: 10.3389/fcell.2021.768783

The Role of the Microbiota in Regeneration-Associated Processes

*Lymarie M. Díaz-Díaz, Andrea Rodríguez-Villafañe and José E. García-Arrarás**

Department of Biology, University of Puerto Rico, Río Piedras Campus, San Juan, Puerto Rico

The microbiota, the set of microorganisms associated with a particular environment or host, has acquired a prominent role in the study of many physiological and developmental processes. Among these, is the relationship between the microbiota and regenerative processes in various organisms. Here we introduce the concept of the microbiota and its involvement in regeneration-related cellular events. We then review the role of the microbiota in regenerative models that extend from the repair of tissue layers to the regeneration of complete organs or animals. We highlight the role of the microbiota in the digestive tract, since it accounts for a significant percentage of an animal microbiota, and at the same time provides an outstanding system to study microbiota effects on regeneration. Lastly, while this review serves to highlight echinoderms, primarily holothuroids, as models for regeneration studies, it also provides multiple examples of microbiota-related interactions in other processes in different organisms.

Keywords: regeneration, echinoderm, development, symbiosis, microbiota, microbiome, sea cucumber

OPEN ACCESS

Edited by:
Igor Schneider,
Federal University of Pará, Brazil

Reviewed by:
Julia Ganz,
Michigan State University, United States
Suleyman Yildirim,
Istanbul Medipol University, Turkey
Brooke Weigel,
University of Washington, United States

****Correspondence:***
José E. García-Arrarás
jegarcia@hpcf.upr.edu

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 September 2021*
Accepted: *03 December 2021*
Published: *26 January 2022*

Citation:
Díaz-Díaz LM, Rodríguez-Villafañe A and García-Arrarás JE (2022) The Role of the Microbiota in Regeneration-Associated Processes. Front. Cell Dev. Biol. 9:768783. doi: 10.3389/fcell.2021.768783

INTRODUCTION

Microorganisms evolved billions of years before animals (reviewed in Knoll, 2003). It is now widely accepted that these microorganisms shaped the environment in which animals evolved (Szathmáry and Smith, 1995; Narbonne, 2005; Knoll, 2011). As a result, animals have conserved close associations with microorganisms, making the microbes an integral part of the animal's environment. In recent years our understanding of the relationship between animals and microorganisms has advanced greatly, thanks in part to new technologies, such as sequencing technologies and mass spectrometers. These advances have brought with them new or redefined terms to describe the participants and/or relationships (Turnbaugh et al., 2007). Terms such as "microbiota" to describe the microbial taxa composition that are found within a certain environment, and "microbiome" to describe the collective genome of such symbionts (Turnbaugh et al., 2007) are now commonly used, and will be part of the terminology used in this review. Naturally, the impacts of the microorganisms have been, for many centuries, associated with disease. However, during the last decades, many studies have shown hitherto unrecognized roles, such as, protecting against pathogens (Iacob et al., 2019), modulating host metabolism, digestion, and nutrition (Kellow et al., 2013; Vijay-Kumar et al., 2010; Turnbaugh et al., 2006; O'Hara and Shanahan, 2006; Turnbaugh et al., 2009; Long et al., 2017), and immune system response (Neish, 2009; Round and Mazmanian, 2009; Bäckhed and Crawford, 2010; Fraune and Bosch, 2010). For example, it is now well established that an altered gut microbial ecosystem impairs gut homeostasis and health. Accordingly, an imbalance (dysbiosis) in the gut microbial community has been associated to diseases such as obesity (Ley et al., 2006; Turnbaugh et al., 2008), malnutrition (Kau et al., 2011), atherosclerosis (Karlsson et al., 2012), and diabetes type 2 (Qin et al., 2012) demonstrating the importance of the gut microbiota composition.

TABLE 1 | Model systems used to decipher the associations between the microbiota and the intestinal regeneration in biomedical research.

Model system	Hallmarks of the model	Microbial association	Limitations of the model	References
Planarian	Display whole body regeneration	Pro- and anti- regenerative properties of *Pseudomonas* and *Aquitalea* sp in whole body regeneration. Apoptosis regulation	Intestinal regeneration cannot be separated from whole body regeneration	Arnold et al. (2016), Lee et al. (2018)
Fruit flies (*Drosophila melanogaster*)	Have the basic structure of the digestive system with simpler microbial communities. Ease of studying roles of the microbiome in the modulation of host signaling pathways and physiology	Microbial community modulates stress response and promotes stem cell proliferation and epithelial regeneration. Specifically, *Erwinia carotovora* was shown to help intestinal epithelial repair	Invertebrate/Protostome. Limited to intestinal epithelial homeostasis and renewal. It was suggested that *Drosophila* gut structure allows oxygen to circulate across the tract, which differs from vertebrates	Shin et al. (2011), Buchon et al. (2009), Chandler et al. (2011), Charroux and Royet (2012)
Zebrafish (*Danio rerio*)	Vertebrate model to study roles of the microbiome in the modulation of host signaling pathways and physiology	*Aeromonas veronii* and *Helicobacter pylori* facilitate epithelial cell proliferation. Microbiota was also shown to promote intestinal epithelial cell fate determination	Only the regeneration of the intestinal luminal epithelium has been studied	Bates et al. (2007), Cheesman et al. (2011), Neal et al. (2013), Rawls et al. (2004)
Rodents	Mammal models to study the gut microbiota in the intestine	The microbial community contributes to the modulation of intestinal epithelial cell proliferation, differentiation, and migration. Microbiota promotes tissue regeneration through induction of the immune system	Only the regeneration of the intestinal luminal epithelium has been studied	Hou et al. (2017), Thomas (2016), Sommer and Bäckhed (2013), Pellegatta et al. (2016), Abrams et al. (1963), Uribe et al. (1994)
Isolated cells/cell lines (mammal models)	Easy handling and maintenance	The microbial community contributes to the modulation of intestinal epithelial cell proliferation, differentiation, and migration. *Akkermansia muciniphila* and *Lactobacillus rhamnosus* are associated with epithelial wound healing	2D model of isolated cells, lacks the composition and integrity of the intestine	Alam and Neish (2018), Hooper et al. (2001), Pull et al. (2005), Rakoff-Nahoum et al. (2004), Lam et al. (2007), Alam et al. (2016), Swanson et al. (2011)
Organoids (mammal models)	Non-invasive methods to study the microbial community in mammals. Share the cellular and structural composition, as well as the self-renewal dynamics, of the intestinal epithelium	*Lactobacillus reuteri* protects the morphology of intestinal organoids and normal proliferation. Proliferation and differentiation occurred through a TLR4-dependent pathway triggered by bacterial-derived LPS	Reduced view of the digestive system, limited to cells from intestinal lineage	Lancaster and Knoblich (2014), Hou et al. (2017), Hou et al. (2018), Naito et al. (2017)
Sea cucumber *Holothuria glaberrima*	Deuterostome model. Has the basic structure of the digestive system with simpler microbial communities. Can regenerate the small and large intestine upon evisceration. The cellular events that control the regeneration have been well characterized	Antibiotics delayed the intestinal regeneration. Gram-positive bacteria (Firmicutes and Actinobacteria) may have a crucial role in the progression of their intestinal regeneration	Marine invertebrate ecosystem. Few studies characterizing the microbiota and their possible roles during the regeneration process	García-Arrarás et al. (1998), Quiñones et al. (2002), Mashanov et al. (2005), Candelaria et al. (2006), García-Arrarás et al. (2011), Mashanov and García-Arrarás (2011), García-Arrarás et al. (2018), Quispe-Parra et al. (2021), Pagán-Jiménez et al. 2019), Díaz-Díaz et al. (2021)

Genomic and molecular approaches, and the characterization of the microbiota role have allowed for new discoveries that extend beyond host health/disease issues (Weinstock, 2012). Recently, the microbiota has been associated with host development, including processes that were thought to be dependent on the host's genetic program, such as morphogenesis and organ development (Sommer and Bäckhed, 2013). Moreover, it has been proposed that the microbiota might play roles in behavior, reproduction, and even in degenerative diseases, among others (Wang et al., 2017).

The present review focuses on the relationship between the microbiota and the process of regeneration. This is a relatively new area of research that explores how the associated microbial taxa within a particular host might modulate the regeneration of a particular tissue, organ or even the whole-body of the host species. We include a summary of models that have been used to study the role of the gut microbiota during intestinal regeneration and associated processes (**Table 1**). Therefore, for the writing of this review, we screened for articles relevant to our topics in the search engine PubMed (pubmed.ncbi.nlm.nih.gov)

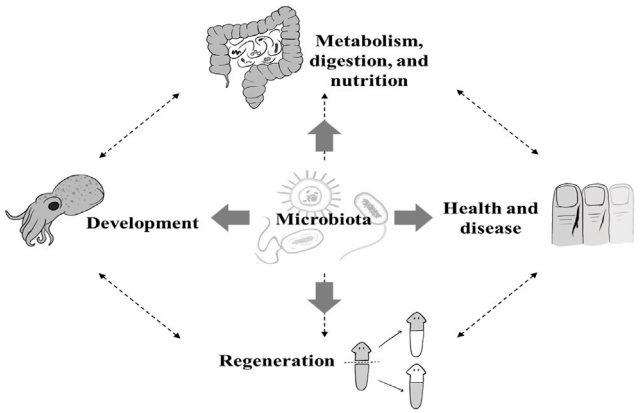


FIGURE 1 | *The influence of microbiota on host physiology.* This figure outlines the aim of this review where we describe the role of the microbial composition associated with an animal host. In this review we focus on the regeneration process. However, we incorporated studies that link the microbiota to the metabolism, digestion and nutrition, health, and development of animal hosts to point out the interconnection between all these processes (dashed arrows).

using keywords such as "microbiota", "microbiome" and "regeneration", among others, and included information considered pertinent. Specifically, we have highlighted research done in animals that belong to the phylum Echinodermata, a phylum known for extraordinary regeneration abilities such as partial or total re-growth of different appendages or internal organs (García-Arrarás and Dolmatov, 2010). In particular, many of them are able to regenerate their digestive tract, thus providing the venue to study the effect of the microbiota in one of the organs best known for microbiota-host associations. This review serves to present a group of echinoderms, the holothurians or sea cucumbers, as excellent models to study microbiome-host associations and their impact on regenerative processes.

Prior to delving into microbiome-regeneration studies, we begin by reviewing some findings from three regeneration-related fields where microbiome associations are important to the host. These are the association of the microbiota with: 1) the host metabolic/digestive processes, 2) embryonic developmental processes, and 3) wound healing (**Figure 1**). These three processes play important roles in regeneration and two of them (wound healing and embryonic development), share key mechanisms with regeneration, thus, the particular interest in singling them out.

Microbiota is Essential for Host Metabolism, Digestion, and Nutrition

From the roles ascribed to the microbiota, probably the best understood is their importance on host metabolism, which impacts their digestion and nutrition, by the assimilation of the digested food for the host physiological process. Multiple studies have shown the involvement of the mammalian gut microbiota in metabolic processes and energy homeostasis of host animals (O'Hara and Shanahan, 2006; Turnbaugh et al., 2006; Turnbaugh et al., 2009; Vijay-Kumar et al., 2010; Long et al., 2017). The gut microbiome was found to be crucial in processing non-digestible substrates that are necessary for host health maintenance and thus physiology (Gill et al., 2006). For example, the fermentation of dietary fibers and endogenous intestinal mucus, ensured by the intestinal microbiota, allows the growth of microorganisms that produce short-chain fatty acids (SCFAs) and gases (Wong et al., 2006). Acetate, the most abundant SCFA, is used in cholesterol metabolism and lipogenesis in the peripheral tissues (Frost et al., 2014). Butyrate, another major SCFA, is the main energy source for human luminal cells in the colon (De Vadder et al., 2014), and is key for generating a hypoxia state in epithelial cells, oxygen balance, and prevention of gut microbiota dysbiosis (Byndloss et al., 2017). The butyrate producer *Faecalibacterium prausnitzii*, one of the most represented bacteria in the intestine of healthy human adults, has exhibited anti-inflammatory effects in a colitis-mouse model (Miquel et al., 2013). Propionate, another dominant SCFA, regulates gluconeogenesis and satiety signaling through interaction with the gut fatty acid receptors in the liver (De Vadder et al., 2014). Another example of bacteria metabolites that can alter a host's physiology is polyhydroxybutyrate (PHB), which is a polyhydroxylkanoate that comprises the primary product of carbon assimilation from glucose and starch. Microorganisms retain PHB and metabolize it when other common energy sources are not available, principally when carbon concentration is higher than nitrogen's (Madison and Huisman, 1999; Jendrossek and Pfeiffer, 2014). Moreover, PHBs are used for host development both in fish and crustacean aquaculture (De Schryver et al., 2010; Nhan et al., 2010; Najdegerami et al., 2012).

The data shown above, focusing on a minor subset of gut bacterial products, clearly present the interdependence of the microbiota with its host highlighting how bacterial metabolites are not only essential for the host physiological processes but are also needed for the growth of other bacteria.

Microbiota Role in Development: Focus on Immune System and Organ Formation

Immune system development and activation- The actions of some symbionts go well beyond localized functions and are crucial for the overall development of the host. This provides a useful background for our discussion of microbiota effects on regeneration, specifically because of the links between embryological development and regeneration. Multiple studies from different organisms have demonstrated that the cellular and molecular mechanisms used in regenerative processes are similar, and in many cases identical, to those that take place during development (Arvizu et al., 2006; Yokoyama et al., 2008; Petersen and Reddien, 2009; Tu and Johnson, 2011; Tischer et al., 2013; Bryant et al., 2017; Reddy et al., 2019).

Therefore, the role of the microbiota during an organism's developmental history can lead to important insights on a possible role during regeneration processes in the same or closely related organisms. A classic example of the effect of

microbiota during embryonic or postnatal development is the development of the immune system in vertebrates (Round and Mazmanian, 2009; Bäckhed and Crawford, 2010; Fraune and Bosch, 2010). Studies have revealed that mutualistic or commensal microbe colonization are pivotal for the development, maturation, and activation of the immune system. Developmental effects of the microbiota in vertebrate species have usually been studied using germ-free models. In some of the key studies, germ-free reared animals presented deficient development of the immune system, including underdeveloped lymphatic organs (Falk et al., 1998; Macpherson and Harris, 2004; Bouskra et al., 2008), and defects in T cell regulation and B cells antibody production (Round and Mazmanian, 2009). In addition to the direct effects of these symbionts through the production of antimicrobial substances, immune response in germ-free animals lacked a priori instruction, induced by commensals (Hansen et al., 2014). This was confirmed with the propensity to infections when microbes were reintroduced to germ-free animals.

The study of Toll-like receptor (TLR) signaling in host-microbiome models has shown the mechanism by which the microbiota interacts with immune system activation and maturation (Akira and Takeda, 2004). This pathway is highly conserved in metazoans (Khalturin et al., 2004; Iwanaga and Lee, 2005; Roach et al., 2005; Satake and Sekiguchi, 2012; Nie et al., 2018), increasing the number of possible models in which to examine the relationship between microbiota and host immunity. The Toll pathway is activated by the binding of various microbe-associated molecular patterns (MAMPs) to the Toll-like receptors (TLRs) (Janssens and Beyaert, 2003; Kawai and Akira, 2010; Narayanan and Park, 2015; Wang et al., 2020). In some invertebrates, the pathway is activated indirectly, when the cytokine-like endogenous molecule Spätzle detects the microorganisms and activates the Toll receptors (Kawai and Akira, 2010). The activation of Toll pathway provokes the secretion of toxic molecules, such as antimicrobial peptides and reactive oxygen species (ROS) (Tzou et al., 2000; Ha, EM, et al., 2005).

Studies in mice have identified possible mediators of the microbiota-host immune response. These studies revealed that mice harbor specific Firmicutes, *Candidatus arthromitus* (Snel et al., 1995), that influence the innate immune system maturation (Suzuki et al., 2004; Gaboriau-Routhiau et al., 2009; Ivanov, II, et al., 2009). This suggests that Fusobacteria and Firmicutes may be important in the regulation of immune system development, immune-inflammatory response, and gut homeostasis. However, these filamentous bacteria have only been found in some infants younger than 3 years old (Yin et al., 2013), and a similar role in immune maturation in humans remains to be discovered. Moreover, recent studies have evidenced that metabolite generation, including SCFAs and adenosine triphosphate, influences the host's immunity (Atarashi et al., 2008; Furusawa et al., 2013).

Organ morphogenesis- That the microbiota is involved in the process of immune system development and maturation might be expected, since after all, one of the system's main functions involves the direct interaction of immune cells with the environmental bacteria. Other findings that associate the microbiota with an organism's development are somewhat more surprising. One such study is the symbiotic association between the marine bobtail squid *Euprymna scolopes* and bioluminescent *Vibrio fischeri*. This model has arguably played a pivotal role in advancing the field of host-microbe associations involved in developmental processes (Nyholm and McFall-Ngai, 2004). This model provides an interesting phenomenon where the host-microbiome interaction is crucial to the formation of an anatomical complex structure and at the same time is not associated with health/disease issues, as are most other cases involving the microbiota. In this system, during development, the squid forms a structure named "the light organ" which helps in the protection of the host from predators (Boettcher and Ruby, 1990; McFall-Ngai and Ruby, 1998; Jones and Nishiguchi, 2004). This organ is colonized by bacteria during the day, the photosynthetic bacterium camouflages the squid from predators at night, and then at dawn, the squid ejects the light organ bacteria into the ocean, a cycle that is repeated daily.

Researchers have described in detail the process of host colonization and bacterial interactions (Nyholm and McFall-Ngai, 2004). Newly hatched juveniles are born with fields of ciliated epithelia on the nascent squid rudimentary light organ (McFall-Ngai and Ruby EG, 1991). They acquire the bacteria from the ocean environment (Ruby and Lee, 1998). When the host is exposed to bacterial peptidoglycan, the epithelial cells produce mucus that promotes the aggregation of bacteria (Nyholm et al., 2000; Nyholm et al., 2002). The symbiont then moves in the mucus to the crypt spaces of the light organ and colonizes it. As a result, it triggers developmental changes of the squid light organ (Doino and McFall-Ngai, 1995). Some of these adaptations include constriction of the ducts that lead to the crypt space delimitation, suspension of mucus secretion, and a regression of the ciliated epithelium, which might prevent further colonization of environmental symbionts. Other changes include trafficking of hemocytes into the blood of the ciliated epithelium (Nyholm and McFall-Ngai, 2004) facilitating the retrogression of the ciliated epithelium (Koropatnick et al., 2007), and increasing the density of microvilli in crypt cells (Nyholm and McFall-Ngai, 2004) which increases the surface area of interaction between the bacteria and the crypt cells (Lamarcq and McFall-Ngai, 1998). In addition to morphological and mechanical adaptations, chemical changes also take place. For example, following colonization by *V. fischeri* during crypt metamorphosis, a decrease in nitric oxide (NO) production is observed (Davidson et al., 2004). All these events favor the *V. fischeri* selection and proliferation to ensure mature organ light formation and bioluminescence (Nyholm and McFall-Ngai, 2004).

Antibiotic induced *V. fischeri* clearance from the crypts produces some irreversible developmental changes, such as the permanent loss of the surface ciliated epithelium and the attenuation of NO in the ducts (Nyholm and McFall-Ngai, 2004). Mutant *V. fischeri* that are defective in producing luminescence because of a mutation in the luxA gene (Visick et al., 2000) or deletion of lux operon do not persist in the crypts

(Bose et al., 2008). Apart from not producing the required luminescence, these mutants cause developmental effects on the host, which fail to appropriately induce swelling of the crypt epithelial cells, hemocyte trafficking, and apoptosis of cells of the epithelial fields (McFall-Ngai et al., 2012). The mutant bacteria also have an altered expression of lipopolysaccharide (LPS) lipid A and peptidoglycan (PGN) tracheal cytotoxin (TCT) monomer. This correlates with observed changes in squids exposed to mutant bacteria that have a different expression of their LPS-binding proteins and peptidoglycan-recognition proteins. Thus, *V. fischeri*'s luminescence is somehow dependent on the expression of MAMPs and host pattern-recognition receptors to induce the immune system to cause the developmental changes in *E. scolopes* (McFall-Ngai et al., 2012).

Another interesting aspect of this symbiosis is the fact that *V. fischeri* that colonize the light organ are not eliminated by the immune system of *E. scolopes* (McFall-Ngai et al., 2012). It is thought that the recognition of *V. fischeri* molecules play a pivotal role in the selection of bacterial species by the immune system and therefore, the morphogenesis of the light organ of the host (Koropatnick et al., 2004; Troll et al., 2010).

Other developmental effects- Microbiota effects on embryonic development have been studied in other invertebrates (these are usually chosen because they generally have simpler microbial communities). The *Drosophila-Acetobacter* system has been a convenient model for understanding the genetic and functional roles of the microbiome in the modulation of host signaling pathways and physiology. Extensive studies in *Drosophila* and its symbiont *Acetobacter pomorum* showed that this gut bacteria impacts not only the metabolism of its hosts, but the growth, body size gain, and stem cellular activity (Shin et al., 2011). An *A. pomorum* mutant library has been used to decipher their beneficial role on host's developmental homeostasis. This has led to the finding that the periplasmic pyrroloquinoline quinone–dependent alcohol dehydrogenase (PQQ-ADH)–dependent oxidative respiratory chain of the *A. pomorum* interaction with the insulin/insulin-like growth factor (IGF)-1 signaling (IIS) of the host is necessary for the maintenance of the gut mutualism. However, the sole bacterial PQQ-ADH is insufficient to promote the *A. pomorum*–mediated effects on host physiology, suggesting that the host genetic program and gut bacteria regulate each other. Additional studies using multiple insect models have confirmed the role of the hindgut bacteria in various aspects of digestion and host development. These cases include digestive efficiency of soluble plant polysaccharides and growth rate in crickets (Kaufman and Klug, 1991), insect generation time, adult body weight gain, and methane production in cockroaches (Gijzen and Barugahare, 1992), cellulose breakdown and nitrogen fixation in beetles (Morales-Jiménez et al., 2009), and potential proteolytic activity in aphids (Wang and Zhang, 2015).

Many studies performed in germ-free mammals have shown that the intestinal microbiota influences the postnatal development of the gastrointestinal tract in these organisms. For example, in mice, successions in the microbiota composition during development were shown to lead to gastrointestinal maturation (Wagner et al., 2008; Reinhardt et al., 2009). The intestine of an adult mouse accommodates a sophisticated vascular network that originates from a system of vessels that form postnatally in small intestinal villi. The formation of this network occurs concurrent with the assembly of the microbiota. Comparative studies of the capillary networks of germ-free mice versus animals colonized (ex-germ-free) during or after gut development demonstrated abnormalities in the capillary network of adult germ-free mice (Stappenbeck et al., 2002). However, colonization either with conventionalized mice microbiota or with *Bacteroides thetaiotaomicron* restarted and completed the developmental program. Other studies, using germ-free transgenic mice lacking Paneth cells (which secrete antibacterial peptides that affect luminal microbial ecology) in the intestinal epithelium, showed that this angiogenesis was regulated by *B. thetaiotaomicron* colonization of the mucosal surface (Stappenbeck et al., 2002). In addition, the associated microbial community contributes to the modulation of intestinal epithelial cell proliferation, as evidenced by the scarcity of proliferating cells in the intestines of germ-free rodents (Abrams et al., 1963; Uribe et al., 1994) and zebrafish (Rawls et al., 2004).

Further information on the role of the microbiota on vertebrate developmental processes has been obtained using germ-free and gnotobiotic zebrafish (Milligan-Myhre et al., 2011). Both zebrafish and murine germ-free models presented significant differences in the intestinal morphology in comparison with conventional controls, including reduced cell division, decreased number of goblet cells and intestinal associated immune cells, and perturbed expression of genes involved in metabolism and innate immunity (Savage et al., 1981; Kandori et al., 1996; Cebra et al., 1998; Hooper et al., 2001; Rawls et al., 2004; Bates et al., 2007; Bouskra et al., 2008; Cheesman et al., 2011; Kanther et al., 2011).

A New Role for Microbiota as Regulator of Regenerative Processes

The role of the microbiota has been studied, quite extensively, in processes associated with wound healing. These processes are usually the initial steps in more complex regenerative events, and will be briefly reviewed here, prior to discussing the role of the microbiota in overall regeneration of tissues and organs.

Wound healing following injury - The first response after a trauma or injury to an organism is the wound healing cascade which ensures the repair of the wound and avoids the colonization or translocation of pathogens. This takes place prior to the reorganization of the injured tissue (Guo and Dipietro, 2010) and might involve the microbiota (Thomas, 2016; Maheswary et al., 2021) as shown by studies of human skin microbiota during wound healing processes. Many of the findings on the role of microbiota in wound healing were facilitated by studies of chronic wounds, such as diabetic foot ulcers and non-healing surgical wounds, which represent major healthcare problems. These studies provided valuable data on

how the microbiota can shape the process of wound healing and perhaps other processes related to regeneration.

Chronic wounds are caused by a disruption of the cutaneous wound healing process, preventing the restoration of the skin barrier. The main bacterial phyla identified in acute and chronic wounds are also found in healthy skin, however wounds are characterized by skin dysbiosis where their relative abundance differs significantly by wound type (Ammons et al., 2015; Loesche et al., 2017). *Pseudomonas* and *Staphylococcus* dominate in all types of chronic wounds (Dowd et al., 2008; James et al., 2008; Gardner et al., 2013; Wolcott et al., 2016; Gardiner et al., 2017), and usually are present in acute wounds created by blunt or penetrating trauma (Hannigan et al., 2014; Bartow-McKenney et al., 2018), burns (Hannigan et al., 2014; Liu et al., 2018), or atopic dermatitis (Seite et al., 2014). However, higher levels of anaerobic bacteria are present in chronic wounds and are commonly associated with worse prognosis (Loesche et al., 2017).

Moreover, pathogenic microorganisms are suspected of playing a substantial role in delayed wound healing. Hence, perturbations of microbial communities that are not promoting cutaneous wound healing may be beneficial. As shown by Loesche et al. the use of antibiotics to destabilize pathogenic wound microbiomes, resulted in faster wound healing (Loesche et al., 2017). In other studies, when probiotic bacteria were applied to a rodent wound, the bacterial load was decreased and tissue repair was promoted (Rodrigues et al., 2005; Valdez et al., 2005; Huseini et al., 2012). Similarly, wounded dermal tissues of mice showed improved proliferation of epidermal cells, vascularization, and re-epithelialization after inoculation with *Pseudomonas aeruginosa* strain PAO1 (Kanno et al., 2011). Also, in humans, topical application of probiotics exerted positive wound healing properties for chronic venous ulcers infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Peral et al., 2009). Consequently, microbial communities may be useful for the diagnosis of wound healing progresses (by predicting those wounds that will experience infectious complications). Hence, studies in skin microbiota provide an example of interactions between host and microbiomes with biomedical relevance to health issues.

In addition, the gut microbiota has been implicated in intestinal epithelial repair. This is highlighted by recent studies on intestinal wounds where gut microbiota enhanced epithelial wound repair (Alam and Neish, 2018). Specifically, intestinal commensal bacteria have been found to regulate the proliferation, migration, and survival of host epithelial cells, as well as promote barrier function and resolution of epithelial wounds (Hooper et al., 2001; Rakoff-Nahoum et al., 2004; Pull et al., 2005; Lam et al., 2007). One of these commensals is *Akkermansia muciniphila*, which is enriched in healing mucosal wounds and dominates the wound-mucosa-associated microbiota (Alam et al., 2016). When mice are treated with exogenous *A. muciniphila* to treat colonic mucosal wounds enhanced mucosal closure occurs. The bacterial treatment stimulates the mice intestinal cellular proliferation and enterocyte migration from the crypt apparently through the generation of ROS when the bacteria colonize the wounded area. The possibility that ROS might be the mediator in this phenomenon is strengthened by experiments with another gut commensal, *Lactobacillus rhamnosus*. This bacterium has also been associated with intestinal epithelium repair by experiments showing that the sole contact of intestinal epithelial cells (IECs) with *L. rhamnosus* strain GG (LGG) induces ROS accumulation, consequently stimulating cellular proliferation and migration (Swanson et al., 2011).

Metazoans have different regeneration capabilities. Since mammals are not well know for their regenerative potential, the roles of microbiota in the regeneration of tissues or organs have been focused on particular model organisms. Various species, well known for their regenerative responses, such as planarians, salamanders, and zebrafish have been used to study whether the microbiome can regulate the regeneration potential of their hosts or are directly involved in the regeneration process (**Figure 2**). Some of these roles will be discussed below.

Whole body regeneration in planarians- Two studies in the planaria *Schmidtea mediterranea* have shown that bacteria can influence whole body regeneration. In the first study, the microbiome of healthy planarians was characterized, revealing a high Bacteroidetes to Proteobacteria ratio (Arnold et al., 2016). Animal manipulations such as tissue amputations and changes in culturing conditions (which elicits a relative increase of Proteobacteria) and cultures with a strain of *Pseudomonas*, produced ectopic lesions and progressive tissue degeneration. Furthermore, infection with the *Pseudomonas* strain enhanced apoptosis, in contrast to what occurs in the absence of infection where regeneration represses apoptosis. To explain this phenomenon, Arnold et al. suggested that activation of an innate immunity signaling (TAK1/MKK/p38) pathway had an opposite role in host immunity versus normal regeneration. In a second study, a different group studied the impact of bacterial metabolites on the regeneration of planarians (Lee et al., 2018). They described the microbial community of *Dugesia japonica*, a close relative to *S. mediterranea,* and inoculated tail and head-amputated antibiotic-treated organisms with representative bacteria species. Lee and colleagues found that regeneration was compromised in animals inoculated with an indole producing bacteria, *Aquitalea* sp., and tail and head formation was delayed. To test whether the production of indole (which is formed from tryptophan by bacterial enzymatic action) was the causative agent, amputated trunks were incubated with *Aquitalea* sp. in tryptophan supplemented media. Animals exposed to both tryptophan and indole producing bacteria presented a delayed regeneration in comparison to controls. These experiments demonstrated a direct effect of an indole-producing bacteria on the regenerative properties of planarians.

Limb regeneration in salamanders- A possible association between bacteria and regeneration has also been observed in one of the best studied vertebrate regeneration models, the Mexican axolotl *Ambystoma mexicanum* (Demircan et al., 2019). This amphibian is capable of regenerating internal organs such as heart, brain, and lungs and external organs such as limbs, gills, and tail (Vieira et al., 2020). In Demircan and colleagues work, a 16S rRNA amplicon dataset was obtained from limbs at different days post amputation (dpa) and correlated with axolotl limb regeneration stages; the stages (0-, 1-, 4-, 7-, 30-, and 60- dpa) (Demircan et al., 2019). Although the study was

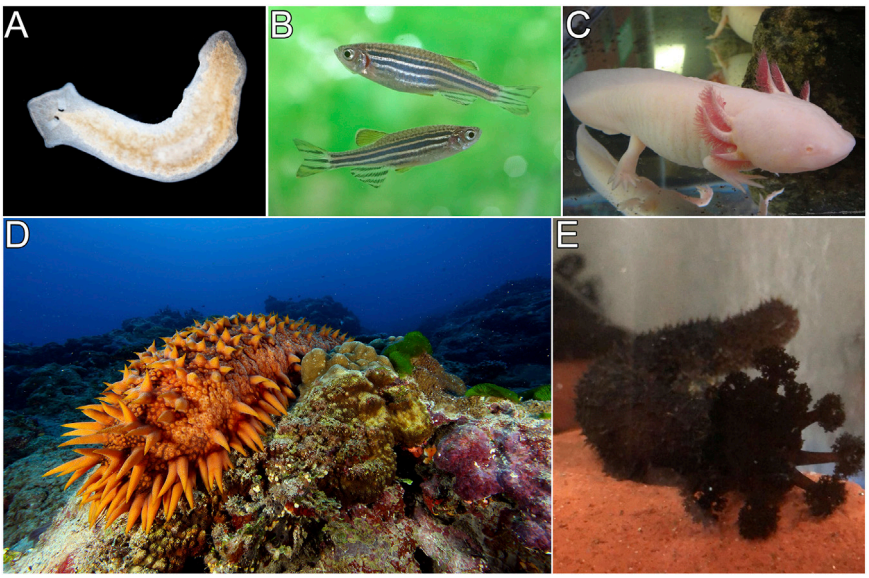


FIGURE 2 | *Models of regeneration.* This figure portrays organisms that are used as regeneration models: planaria **(A)**, zebrafish **(B)**, axolotl **(C)**, and two holothurian species, *Apostichopus japonicus* **(D)** and *Holothuria glaberrima* **(E)**.

purely correlative, it showed changes in the microbiota during regeneration, suggesting that certain bacterial groups might be associated with the regenerating tissues. At the phylum level, the bacterial communities in normal animals were dominated by Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Verrucomicrobia. In regenerating limbs, a temporal shift in bacterial composition was observed, which included differential phylum abundances at certain limb regeneration stages. Post-amputated groups had different microbial communities compared to aquarium control groups, since there was a shift from Firmicutes-enriched (controls) to Proteobacteria-enriched (regenerating) relative abundance. The significant differences observed between the water and the regenerating limb microbiotas suggested selective colonization of axolotl limb tissues and that substantial restructuring of bacterial communities occur in regenerating tissues. Moreover, a comparison of the microbial community demonstrated less variation in the relative abundance of bacterial communities between samples at the same stage of regeneration, and higher variation between groups at different stages. Also, they found differences between limb microbial communities among the regeneration phases: the 0- and 1- dpa samples, 4- and 7- dpa samples, and 30- and 60- dpa samples all differed between them in the measures of beta-diversity. That different bacterial communities were found at specific limb regeneration stages, such as wound healing, dedifferentiation, and re-development, could indicate that specific bacterial groups have specific roles in these processes.

Tissue layer (luminal epithelium regeneration) in vertebrates- Many investigators have studied the regeneration of the luminal epithelial layer (**Figure 3A**) of the vertebrate digestive tract (see Santos et al., 2018 for review). This tissue layer is continuously being formed as the cells undergo damage by the exposure to the digestive lumen content and the digestive process itself (Barker et al., 2007; Sailaja et al., 2016; Santos et al., 2018). In addition to the ongoing epithelial turnover to achieve gut homeostasis, this tissue can undergo regeneration if injured by exposure to factors such as toxins, radiation or others (Metcalfe et al., 2014; Beumer and Clevers, 2016). Homeostatic maintenance of the luminal epithelium is well understood and has been well described particularly in the mammalian intestine (Barker et al., 2007). The renovation of the layer is dependent on the intestinal stem cells (ISC) and their associated environment (ISC niche). These cells are found within the luminal crypts and give rise to the different cell types in the epithelium. The stem cells divide within the crypts and their progeny continue this division as they transit to the intestinal villi where they differentiate into the intestinal luminal epithelial phenotypes. As cells reach the tip of the intestinal villi, they are shed into the lumen, maintaining a continuous migration of cells from the crypts to the villi.

As response to injury, the ISC niche adapts to ensure epithelial regeneration beyond the homeostatic state (Beumer and Clevers, 2016). The epithelial restitution is achieved either by proliferation of active ISCs ($Lgr5^+$ ISCs) or by mature cells dedifferentiated to ISC. This regeneration of mucosal epithelia has been found to be modulated by the microbiota (Thomas, 2016; Hou et al., 2017). Also, the microbiota has been suggested to promote gut healing regeneration through induction of immune responses (Sommer and Bäckhed, 2013; Pellegatta et al., 2016; Thomas, 2016; Hou et al., 2017).

Various experiments demonstrate a similar role of microbiota on luminal epithelium regeneration in zebrafish. For one, in the developing zebrafish intestine, epithelial cell proliferation was shown to be facilitated by their symbiont bacteria, *Aeromonas veronii* (Cheesman et al., 2011). In other studies, the virulence factor CagA from *Helicobacter pylori* also promoted intestinal cell proliferation through Wnt pathway signaling (Neal et al., 2013).

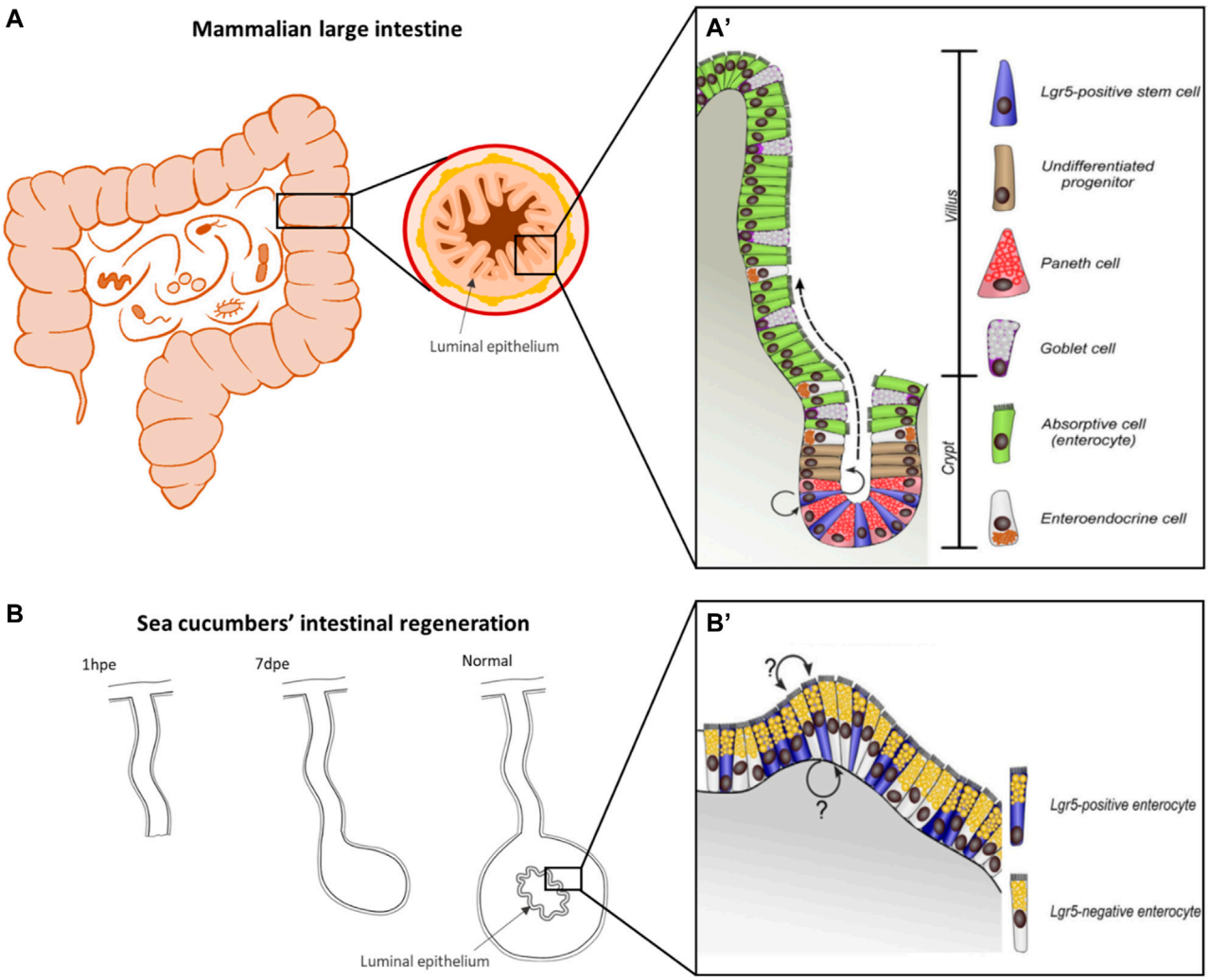


FIGURE 3 | *Comparison of mammalian and holothurian intestinal epithelium anatomy and renewal.* Representative organization of the luminal epithelium of mammal intestine **(A)** and the mucosal epithelium in the digestive tube of sea cucumbers **(B)**, highlighting the difference in cell renewal mechanisms. **(A)** In mammals, Lgr5-positive intestinal stem cells are localized at the bottom of the crypt, which self-renew and produce dividing transit-amplifying progenitors (arrows), which gradually migrate apically and form the villus (dashed arrow), where are localized the specialized cells. Paneth cells (which appear to be unique in mammals) are the only differentiated cell type that remains in the stem cell niche. **(B)** In the digestive epithelium of echinoderms, the spatial organization of mammals is not present, instead Lgr5-positive cells are interspersed among Lgr5-negative or differentiated cells, but the lineage of these cells is not well understood. **(A′,B′)** were retrieved from Mashanov et al., 2014 and modified by LD-D, **(A,B)** are drawings by the authors of this article (AR-V and LD-D, respectively) for the purposes of this comparison.

Lastly, microbiota was also shown to promote intestinal epithelial cell fate determination via the Notch-MyD88 signaling (Bates et al., 2007; Cheesman et al., 2011).

Additional model systems, mainly *in vitro* models comprising cell cultures, tissue explants, and organoids, have been developed to decipher the microbiota's influence on the homeostasis and regeneration of mammalian intestines. Among these, organoids have been used to understand the effects that the commensal microbiota, or a particular microorganism, might have on intestinal epithelium homeostasis (Peck et al., 2017; Blutt et al., 2019). Organoids are three-dimensional tissue structures obtained from stem cells in culture, that are differentiated into multiple organ-specific cell types. Thus, cells in these structures acquire some of the organ or tissue organization and functions (Lancaster and Knoblich, 2014). Small intestinal organoids share the cell and structural composition of the small intestinal epithelium, as well as the self-renewal dynamics. (Sato et al., 2009; Sato et al., 2011). Using organoids, it was shown that live *Lactobacillus reuteri* protected the morphology of intestinal organoids and normal proliferation (Hou et al., 2017; Hou et al., 2018). The protection of the intestinal barrier and activation of intestinal epithelial proliferation seemed to control intestinal inflammation.

A possible mechanism for the bacterial effect was described in a recent work showing that the ISC expresses nucleotide binding oligomerization domain-containing protein 2 (NOD2). This protein interacts with a peptidoglycan motif expressed on most bacterial organisms, suggesting a putative pathway for

communication between the microbiome and the ISC niche (Nigro et al., 2014). Treatment of organoids with ligands for NOD2 resulted in an increase in their number and size, indicating that these ligands induce epithelial proliferation. Additional support that bacterial species have a role in the ISC niche comes from studies in mice, where a crypt-specific microbiome has been associated with homeostatic proliferation. This finding led to organoid studies showing that modulation of the colonic epithelial balance between proliferation and differentiation occurred through a TLR4-dependent pathway triggered by bacterial-derived LPS (Naito et al., 2017). Other work showed that colonic crypts from mice devoid of microbiota lose their regenerative capacity, as assessed by the ability to form organoids (Zaborin et al., 2017). There, the regenerative capacity was recovered by fecal microbiota transplantation that restored the crypt microbial communities. Furthermore, in recent studies, lactate derived from bacteria was shown to mediate small intestinal epithelial proliferation through stimulation of the stem cells in murine organoid cultures (Lee et al., 2018), suggesting there may be specific bacteria-derived factors that interact with the host cells to modulate the ISC response. These findings provide strong evidence for a microbiome role in homeostasis of the ISC niche.

Although the day-to-day regeneration of the luminal epithelium has been well studied and has provided important information, as described above, there is a "catch" to these studies that must be addressed. This regeneration is considered to be homeostatic, meaning that it is an ongoing replacement of the lost cells and whose mechanism is deeply embedded within the physiology of the organ in order to maintain its function. Many researchers differentiate this type of regeneration from the one that takes place following injury to the organ or tissue. Available data support the notion that the mechanisms by which homeostatic regeneration takes place differ from the regeneration that follows injury (Beumer and Clevers, 2016). In this respect, the data shown above relates to the microbiota role in homeostatic regeneration and might not apply to the regeneration of the luminal epithelium under injury or to massive loss due to other manipulations.

In an attempt to understand the ongoing interactions within the digestive tract, invertebrates have been used as simplified organisms. The understanding of the impact of gut microbiota on host physiology has been limited, due to restricted in-depth integrated genetic analysis of both the microbes and the host. In this respect, the study of insect non-binary, yet simpler bacterial communities than mammals, is noteworthy. Intestinal bacterial communities of insects have been widely studied, and the amenability of *Drosophila melanogaster*, allowed its implementation to study animal symbioses. Numerous studies have shown that *Drosophila*'s bacterial communities are simpler than mammals; hundreds of species are present in humans (Qin, et al., 2010), while the adult *Drosophila* midgut symbiotic commensal community is composed of 5–20 different microbial species (Corby-Harris et al., 2007; Cox and Gilmore, 2007; Ren et al., 2007; Ryu et al., 2008; Chandler et al., 2011; Wong et al., 2011). Among them, the families of Acetobacteraceae, Lactobacillales, and Enterobacteriaceae are the most prevalent microbes identified in the *Drosophila* gut microbiota (Ryu et al., 2008; Roh et al., 2008; Chandler, et al., 2011). The simplicity of their microbial communities have made them attractive models for host-microbe studies. Thus, the microbiota effect on intestinal epithelial renewal was studied in *Drosophila*. A crosstalk between the gut and its microbial community was demonstrated to modulate stress response and promote stem cell proliferation and epithelial regeneration (Buchon et al., 2009). Specifically, the pathogenic bacterium *Erwinia carotovora* was shown to be important to undergo intestinal epithelial repairs. This result supports the influence of gut microbiota in epithelial healing, as seen in mammal models. However, unlike vertebrate gut, in *Drosophila*, the intestinal microbiota is composed of either aerotolerant or obligate aerobes, suggesting that oxygen is able to circulate across the *Drosophila* gut (Chandler et al., 2011; Charroux and Royet, 2012). This provides a limitation when comparing the essential compartmentalization that drives the complex ecosystem in humans and non-human vertebrate bodies.

Leading Studies in Echinoderm Microbial Community

Microbiota has also been associated in echinoderms with other processes important for regeneration such as metabolism and growth. In an early study focused on another echinoderm group, brittle stars, it was suggested that subcuticular and intestinal bacteria could metabolize dissolved organic matter and use it as a significant carbon source (Fielman et al., 1991; Hoskins et al., 2003). These products from microorganisms such as *Pseudoalteromonas atlantica* are proposed to be important for echinoderm physiology, including regeneration processes. The link between microbiota and nutrient availability has also been studied in sea stars, where the need for symbionts' assistance to ingest structurally complex polysaccharides or require detoxification of dietary products has been suggested (Douglas, 2009). These organic compounds produced by symbionts are potentially used as energy to promote growth and regeneration (Kelly et al., 1995). In another echinoderm species, the purple sea urchin *Strongylocentrotus purpuratus*, studies have also suggested that fasting reduces bacteremia in the coelomic fluid and increases spine regeneration (Scholnick and Winslow, 2020).

Organ regeneration in echinoderms- While regeneration of the digestive tract luminal layer has been studied in several model systems, regeneration of the complete intestinal organ has been the focus of work in an understudied group of animals: the Holothuroidea (Echinodermata) (**Figure 3B**). Several factors make holothurians or sea cucumbers the ideal model system to study the role of the microbiota on regenerative processes. The main one is their ability to eject their digestive tract in a process named evisceration, and to regenerate the entire organ in a period of about a month (Hyman, 1955; Byrne, 2001; Wilkie, 2001; Carnevali, 2006; García-Arrarás et al., 2018). This autotomy, with the subsequent regeneration, provides a unique "natural" model system where the process is part of the animal biology. Moreover, the cellular events that take place during the regeneration of the intestine in these animals have been well studied (García-Arrarás

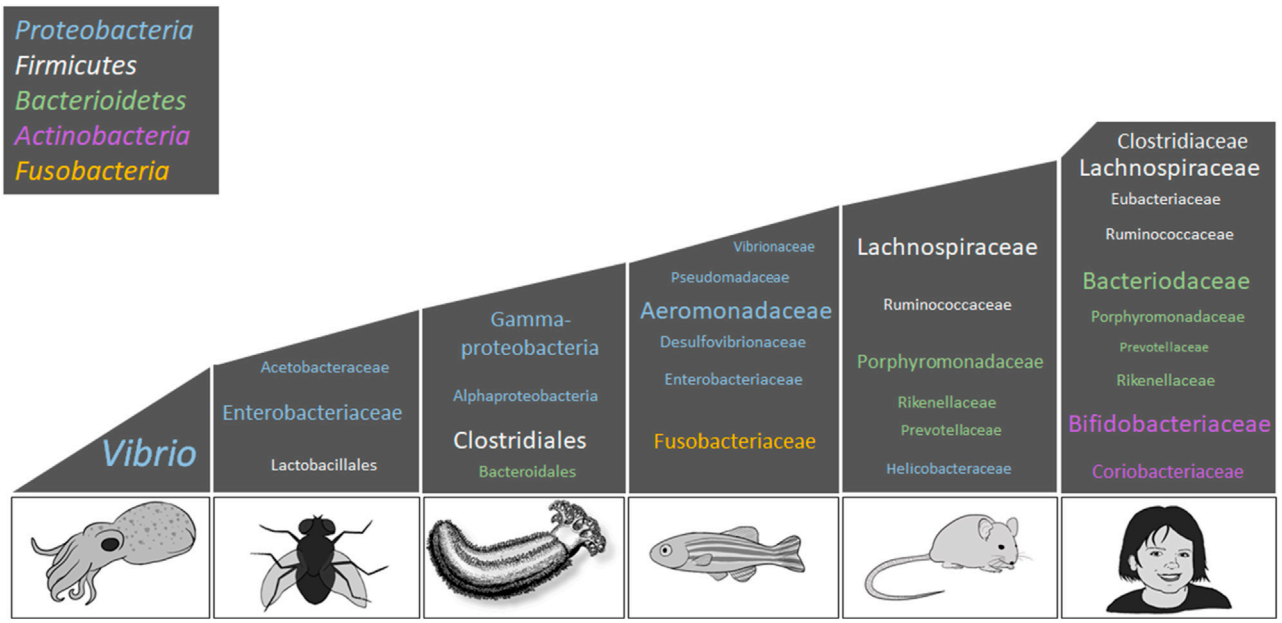


FIGURE 4 | *Bacterial composition associated with animal hosts.* This scheme presents the most representative taxa among the microbiota of *E. scolopes*, *D. melanogaster*, *H. glaberrima*, *D. rerio*, *M. musculus*, and *H. sapiens;* however, relative representation of these taxa may vary per individual. Top phyla among the animal kingdom includes Proteobacteria (blue), Firmicutes (white), Bacteroidetes (green), Actinobacteria (lilac), and Fusobacteria (yellow). The font size represents the relative abundance of the lower taxonomic levels. This figure is an adaptation of Kostic et al. (2013), and contains information from the following studies: Arumugam et al. (2011), Brinkman et al. (2011), Chandler et al. (2011), Roeselers et al. (2011), and Pagán-Jiménez et al. (2019). Images are original drawings by LD-D and AR-V.

et al., 1998; Quiñones et al., 2002; Mashanov et al., 2005; Candelaria et al., 2006; García-Arrarás et a., 2011; Mashanov and García-Arrarás, 2011; García-Arrarás et al., 2018) and the molecular basis for the regeneration is being actively investigated (Mashanov and García-Arrarás, 2011; García-Arrarás et al., 2018; Quispe-Parra et al., 2021). Echinoderms, being basal deuterostomes, occupy a key branch together with the chordate evolutionary tree, while at the same time are close to most other invertebrates. Moreover, the digestive tube is one of the best conserved organs, common to most metazoans. Thus, these animals can provide useful evolutionary insights into microbiome-host associations. Probably unknown to many, sea cucumbers also have a huge economic value, as part of an aquaculture industry centered in Asia. Thus, the microbiota-host relationships of these animals extend beyond the regenerative process and are studied in terms of health, growth, and other issues related to their nutritional value.

Comparison of Microbiota Structure Among Sea Cucumber Species

To study the role of the microbiota in intestinal regeneration, we need to first determine the components of the microbiota of our model organisms. Holothurians, as documented in all echinoderms studied to date, have a microbial diversity that is both relatively low and dominated by Proteobacteria. This has been shown in the sub-cuticle of the brittle stars *Ophiactis balli* and *Amphipholis squamate* (Burnett and McKenzie, 1997; Morrow et al., 2018), in the body wall, gonads, pyloric caeca, and coelomic fluid of multiple sea star species (Jackson et al., 2018), in the coelomic fluid, intestines, pharynx, and gut digesta of the sea urchin *Lytechinus variegatus* (Hakim et al., 2015; Hakim et al., 2016; Brothers et al., 2018) and in the intestine of the sea cucumbers *Apostichopus japonicus* and *Holothuria glaberrima* (Gao et al., 2014a; Gao et al., 2014b; Pagán-Jiménez et al., 2019).

The gut commensal microbes of sea cucumbers have been a focus of study during the last decade. The intestinal microbiota of three sea cucumber species: *A. japonicus*, *H. glaberrima,* and *Sclerodactyla briareus* have been described using 16S rRNA gene amplicon sequencing (Gao et al., 2014a; Gao et al., 2014b; Wang et al., 2018; Pagán-Jiménez et al., 2019; Weigel, 2020). Though the Proteobacteria and Bacteroidetes are among the most abundant phyla in all sea cucumber species (**Figure 4**), a difference in relative representation is seen among different species. Proteobacteria was the predominant phylum within the gut of the holothurian *A. japonicus*, while Gammaproteobacteria was the predominant bacterial class (Gao et al., 2014a; Gao et al., 2014b). A recent study in *S. briareus* supported these findings (Weigel, 2020). In the latter work, the taxonomic representation in the stomach and intestine from animals that were collected from different ponds or aquaria were evaluated and found that the mature intestine microbiota was composed primarily of Proteobacteria. In contrast, our group found that in the intestine of *H. glaberrima,* Firmicutes was the dominant phylum followed by Bacteroidetes, and then Proteobacteria (Pagán-Jiménez et al., 2019). The higher abundance of Firmicutes in *H. glaberrima*

TABLE 2 | Summary of current findings on sea cucumbers intestinal microbial communities.

Study	Sea cucumber model	Feeding behavior	Methods used for library preparation and analysis	Samples collected	Environmental samples	Control groups	Intestinal dominant bacteria	Regeneration stages	Temporal shifts associated to regeneration process
Gao et al. (2014a)	*A. japonicus* (posterior evisceration)	*deposit feeders*	*16S rRNA gene (V1-V3), 454 sequencing, grouped in OTUs*	*foregut and hindgut contents, and sediment*	*sediment*	*sediment*	*mostly Proteobacteria*	*not applicable*	*not applicable*
Wang et al. (2018)	*A. japonicus*	*deposit feeders*	*16S rRNA gene (V3-V4), Illumina HiSeq, grouped in OTUs*	*foregut, midgut, and hindgut with cloaca*	*none*	*1) non-eviscerated animals at the initial stage of experiment (plus 4days in "template culture") and 2) non-eviscerated animals at the final stage (55days)*	*Proteobacteria, Bacteroidetes, Euryarchaeota, and Firmicutes*	*1-, 5-, 15-, 25-, 35-, 45-, 55- dpe*	*Earlier regeneration stage (1–25- dpe): Proteobacteria in all samples, yet the sub-dominant phyla were different between samples. Later regeneration stage: (35–55- dpe) Proteobacteria and Bacteroidetes and the relative abundance of both reached above 95%*
Zhang et al. (2020)	*A. japonicus*	*deposit feeders*	*16S rRNA gene (V4–V5), Illumina HiSeq, grouped in OTUs*	*whole intestines of regenerating animals*	*not applicable*	*intestines of non-eviscerated sea cucumbers*	*Proteobacteria, Bacteroidetes, and Firmicutes*	*10-, 14-, 18-, and 21- dpe*	*Bacteroidetes' relative abundance increased on day 14 and day 18*
Yamazaki et al. (2016)	*A. japonicus*	*deposit feeders*	*16S rRNA gene (V1–V2) grouped in OTUs and ASVs*	*feces of eviscerated animals at different time points*	*not applicable*	*1) feces of non-eviscerated sea cucumbers at different time points and 2) feces from all animals at time point 0 (pre-evisceration)*	*families were explored: in most samples Rhodobacteraceae is dominant followed by Alteromonadaceae*	*samples collected at different time points, mainly 15-, 16-, 17-, 20-, 24-, and 28- dpe*	*The pre-evisceration fecal microbiota is significantly different from that of the feces post-evisceration One animal had a high abundance of the family* Colwelliaceae *in the feces collected pre-evisceration, yet the abundance drastically decreased after gut regeneration (around 17- dpe). Same thing happened with* Flavobacteriaceae *and* Rhodobacteraceae *with other two samples, respectively*
Pagán-Jiménez et al. (2019)	*H. glaberrima* (posterior evisceration)	*suspension feeders*	*16S rRNA gene (V4-V5), 454 sequencing, grouped in OTUs*	*anterior, medial, posterior, and seawater*	*seawater*	*1) seawater and 2) tissues from animals dissected in situ*	*Proteobacteria, Bacteroidetes, Fusobacteria, and Firmicutes*	*not applicable*	*not applicable*

(Continued on following page)

TABLE 2 | (*Continued*) Summary of current findings on sea cucumbers intestinal microbial communities.

Study	Sea cucumber model	Feeding behavior	Methods used for library preparation and analysis	Samples collected	Environmental samples	Control groups	Intestinal dominant bacteria	Regeneration stages	Temporal shifts associated to regeneration process
Weigel (2020)	*S. briareus* (anterior evisceration)	*deposit feeders*	*16S rRNA gene (V4), Illumina HiSeq, grouped in amplicon sequence variants (ASVs)*	*stomach and medial small intestine for mature and control individuals, stomach and whole intestine for regenerating individuals*	*seawater, sediment, algae Graciaria sp., and seagrass Zostera marina*	*1) initial condition control: samples of stomach and intestines after 2-days lab acclimation 2) tank control: stomach and intestine of animals eviscerated after 18 days in the tank without evisceration*	*mostly Proteobacteria in mature intestines. Mature intestines from tank control also had a high percentage of Epsilonbacteraeota*	*13-, 17-, 20- dpe*	*The regenerating intestine microbiomes were dominated by Proteobacteria, Bacteroidetes, and Epsilonbacteraeota similar to mature intestines (initial and tank controls) but have a higher diversity in lower taxonomic levels. Richness increased from evisceration to day 20dpe. regenerating intestines had higher evenness than mature intestines*

dpe, day post evisceration.

may be a key difference with other holothurians, however microbiota differences among holothurians are probably determined by the differences in habitat and/or feeding behaviors (**Table 2**).

Apart from differences among the gut microbiota of various species, discrepancies in the microbiota between areas of the gut have been observed. Some of these differences are seen in the relative abundances of the microbial community in various segments of the digestive tract. In *H. glaberrima* dissected *in situ* (as soon as collected from the intertidal space), both areas of the small intestine (comprised of anterior and the medial) showed a similar microbiota, composed mostly by members of the phylum Proteobacteria (Pagán-Jiménez et al., 2019). However, the large (posterior) intestine contained mostly Firmicutes. Beta analysis supported these results, revealing that the anterior, medial, and posterior intestine samples had significantly different microbial communities. In addition, differences between environmental microbiota and gut microbiota were documented. Both seawater microbial communities (collected *in situ* and the aquarium water) were more similar to the communities of the anterior and medial intestine, than that of the posterior intestine. These data suggested a distinctive microbiota in the large intestine. In species where the digestive tract includes a stomach, different bacterial communities were found between the stomach and the intestine (Weigel, 2020). Similarly, different microbiotas were found between the water and the internal organs. Thus, all published studies of sea cucumbers microbiota show significant differences between the seawater and the intestinal communities, and differences among the digestive tract structures themselves (Gao et al., 2014a; Gao et al., 2014b; León-Palmero et al., 2018; Pagan-Jimenez et al., 2019; Weigel, 2020). This contrast between marine animal organs and seawater microbial communities was found in other organisms including corals, sea urchins, sea stars and sea anemones (Ainsworth et al., 2015; Brothers et al., 2018; Jackson et al., 2018; León-Palmero et al., 2018).

It is imperative to mention that the experimental design for studies of the microbial communities in holothurians, including the dissections and tissue collections varied among the different studies. In some, the viscera were processed individually, while in others the intestine was not separated from the cloaca. Thus, in **Table 2** we summarized the similarities and contrast of holothurian microbiota studies.

Examining the Microbe-Echinoderm Associations

In *A. japonicus*, the link between microbial diversity and animal growth has been examined (Yamazaki et al., 2016). The metagenomes of feces of large and small sea cucumbers were sequenced, showing that larger and smaller animals had different microbiota, and that while the alpha diversity was similar, the relative abundance differed. The orders Rhodobacterales, Oceanospirillales, and Desulfobacterales were more abundant in larger animals. The long-term effects, in terms of growth and disease resistance, of disrupting the bacterial community of *A. japonicus* sea cucumbers by using antibiotics was also explored (Zhao et al., 2019). Interestingly, after administering different antibiotics (tetracycline, erythromycin, or norfloxacin), it was observed that some antibiotics increased the growth of the

animals yet weakened their immunological system. In a different study, Yamazaki and colleagues found that *Rhodobacterales* are the third most abundant order in the fecal microbiota of *A. japonicus,* and the relative abundances were significantly higher in larger animals than in smaller individuals (Yamazaki, et al., 2016). However, the subsequent article by Zhao and colleagues reported a decreased relative abundance of Rhodobacteraceae in *A. japonicus* juveniles when treated with either tetracycline or erythromycin, but an increase in sea cucumber survival and body weight (Zhao et al., 2019). The above summarized studies demonstrate how bacteria metabolic activity might play a key role in providing the energy source to hosts to facilitate or activate their cellular and molecular process.

Is the Echinoderm's Regenerative Capacity Influenced by the Microbiota?

Two types of studies explore if the microbiome influences the intestinal regeneration of holothurians. The first group of studies focuses on correlating changes in the microbiome with different stages of intestinal regeneration. The regenerating gut microbiome of *A. japonicus* was characterized, showing that Proteobacteria (Wang et al., 2018) or Actinobacteria (Zhang et al., 2020) were the dominant phyla from wound healing stage to lumen formation (early in their regeneration process), and at later stages of regeneration, Proteobacteria and Bacteroidetes became the dominant phyla (Wang et al., 2018; Zhang et al., 2020). This change suggested that during early stages of the regeneration process, the gut was populated mostly by the bacteria from the sediments and water, and then was gradually replaced by digestive-associated microbiota.

An expanded analysis of regenerating intestine microbiota in a different holothurian species, *S. briareus,* documented higher richness (on day 20 after evisceration) and evenness (on day 13–20 after evisceration), when compared to mature intestines (Weigel 2020). Moreover, an Alphaproteobacteria species abundant in mature intestine samples was not found in regenerating intestines. Regenerating stomachs were found to be more diverse in comparison to mature ones. Interestingly, beta-analysis plots showed that regenerating stomach and regenerating intestine were similar. Taxonomic representation and alpha diversity analysis revealed that the regeneration process was associated with a change in microbial community that recovered at the end of the regeneration process. In addition, tank residence, but not collection site, were suggested to affect gut microbial community, however changes in the regenerating microbes were not simply due to tank effects.

It is important to highlight these studies because they propose a correlation between the gut microbiota and the regeneration process. However, as mentioned before, these findings were shown mainly by using functional inferences from genomic data which do not strongly establish that the microbial community causes a particular effect ruling the intestinal regeneration associated events. For example, genomic data cannot distinguish if the organisms found in this community were even alive or if they were transient (ingested debris or indiscriminate colonization). Thus, the future of this field is beyond correlative analysis, and it requires experimentation that delves directly into the microbial community influence and if its modulation alters the effects on host's regeneration.

The second type of study, which precisely examined the role of the microbiome in holothurian gut regeneration, was recently published by our group (Díaz-Díaz, et al., 2021). Here, different antibiotic cocktails were used to cause dysbiosis and study the influence of the commensal community in the intestinal regeneration process. We observed that antibiotic treatments altered cellular processes associated with regeneration such as cellular dedifferentiation, extracellular matrix remodeling, and cell proliferation. To rule out that the antibiotics were exerting a direct effect on the holothurian tissues, we performed MTT assays on dissociated cells and explant cultures. *Ex vivo* experiments suggested that the antibiotics used did not directly alter the holothurian tissue metabolic activity, while being capable of inhibiting gut bacterial populations *in vitro.* Therefore, we proposed that the antibiotics are influencing *H. glaberrima* regeneration via the dysbiosis of the gut microbiota. Moreover, because *H. glaberrima* microbiota is mainly composed of Firmicutes (mostly Gram-positive bacteria) and Proteobacteria (mostly Gram-negative bacteria) (Pagán-Jiménez et al., 2019), and the cocktails targeting mostly the Gram-positive bacteria had the most detrimental effects over the intestinal regeneration, we suggest that Firmicutes may have a crucial role in the progression of the intestinal regeneration. Antibiotics have also been shown to have long-term effects on holothurian growth and disease resistance. In an experiment where antibiotics (tetracycline, erythromycin, or norfloxacin) were administered to disrupt the bacterial community, some antibiotics increased the growth of sea cucumbers, yet appear to inhibit the animal immune system (Zhao et al., 2019).

Furthermore, the role of the microbiota during regeneration could be addressed using other echinoderms. The crinoids, which are well known for their potential to regenerate their arms, can also lose and renew their entire digestive system (Dendy, 1886; Meyer, 1985; Meyer, 1988; Mozzi et al., 2006; Bobrovskaya and Dolmatov, 2014; Kalacheva et al., 2017). These studies describe the fast visceral regeneration potential in crinoids, such as *Antedon mediterranea*, *Antedon rosaceus*, *Himerometra robustipinna* and *Lamprometra palmata*, through histological and cytological analysis but were neglected for many years. We propose that echinoderms are promising models to elucidate if, and how, the regeneration events in the digestive system are influenced by the gut microbiota. Moreover, these organisms provide models whose findings on the whole organ regrowth are not limited solely to the study of the repair of the luminal epithelium layer of the intestine.

Nevertheless, we acknowledge that these models have some disadvantages. First, one deficiency for many of the echinoderms members is the lack of genomic and metagenomic data available. Second, as they are marine invertebrates, the structure and function of their microbiota might be very distinctive, in comparison to humans; colonized by species that are not observed in terrestrial vertebrates.

CONCLUSION

Microbiota effects on regenerating tissues are just beginning to be investigated. The initial findings strongly suggest that, indeed,

bacterial species composition is an important factor in the timing and effectiveness of the regenerative process. However, most of the available data is correlative and needs to be backed by functional studies. These correlative studies on microbial successions and the regeneration process do not demonstrate a causal effect on the intestinal regeneration exerted by the gut microbiota. Nonetheless they do provide some evidence that supports the hypothesis that the microbiota may be influencing regenerative events.

The challenge for future investigations is to identify the specific roles of the microbiota and the signaling pathways or physiological processes by which they might modulate regeneration. Central to this issue is the use of appropriate model systems in which to decipher the specifics of the microbe associations. We consider that *in vivo* examinations where the use of agents that modulate the microbiota, such as prebiotic, probiotic or antibiotics, will be crucial to understand the role of these microorganisms during gut repair mechanisms. Here, we have described various promising echinoderm models to decipher the role of the microbiota during intestinal regeneration, that encompasses the whole organ formation beyond the luminal epithelium repair and homeostasis. We propose that this need may be fulfilled in part by the sea cucumber intestinal regeneration model. The fact that the regenerating organ is a structure present in most metazoans and is the one organ where most microbiome studies have been made, makes this model particularly attractive to study host-microbiome interactions. Thus, we expect that studies with holothurians will provide groundbreaking knowledge on the field of microbiome-host associations and their impact on regenerative processes. However, this model also has some limitations. Among them, the need to improve the molecular tools available to study the specific functions of certain genes as well as the present limitations on identifying and characterizing many bacteria (and other components of the microbiota) that are difficult or impossible to grow in the laboratory. Nonetheless, we believe that comparative studies using the sea cucumber, as well as other models, will be transformative in defining the interactions of host-microbiome in regenerative processes.

AUTHOR CONTRIBUTIONS

LD-D and AR-V acquisition, analysis or interpretation of data for the work; LD-D and AR-V drafting the work, LD-D, AR-V, and JG-A revision and edition.

FUNDING

This research was funded by the National Science Foundation grant (NSFIOS-2100494) and the Puerto Rico Science, Technology and Research Trust (PRSTRT) grant 20270.001.000.XXXX.220.206350070017.00. LD-D and AR-V were funded by Research Training Initiative for Student Enhancement (RISE) program grant 5R25GM061151.

ACKNOWLEDGMENTS

We would like to acknowledge the people who kindly allow us to use their pictures in this review: **Figure 2A**: planaria's picture by Sr. Waldo Nell (retrieved form: https://www.flickr.com/photos/pwnell/33110930070/in/photolist-SrUaWE-7Tb3yC-dYwSwt-7e1Z8C-pydNx-DXtNWx-eR3dS4-2faoBGD-TS8NDw-ar6gCS-98Gf7j-2iUc5S-RBf6ic-2kErabH-cCvnr1-2ivr1Lb-2kErG19-eF21pi-2iPML2-2iUcio-T6b7c9-2kFfwPJ-2gieQcL-q4sX2j-rTbgJN-oY7DDk-LkhrbL-cwHeq9-7R8JV5-9MuHFM-9MuFUB-9Mxqe7-QYfTcJ-8QVidv-7R5sQV-q7qYC4-ppgA8R-qkXUUU-2kFbUJR-pp341C-bUQucV-uAw1fQ-rms2vX-5heWp1-nmZhDm-2f52EPc-5tH2Yj-azz8F1-eN3vNh-eiA7TD), **Figure 2B**: Adult zebrafish (*Danio rerio*) of AB strain. Top: female, bottom: male. Photography and postwork by Tohru Murakami, Gunma University Graduate School of Medicine, Japan (retrieved from: https://www.flickr.com/photos/8659392@N07/13896905021/in/dateposted-public/), **Figure 2C**: Axolotl photographed by David Shane, professor of Physics, Lansing Community College (retrieved from: https://www.flickr.com/photos/david_shane/6151631980/in/photolist-eSE6YY-2RxHYB-anAHSm-6owNsm-6owN3Q-5aUjGL-DUgzM-5xwbz9-RR7cMG-6k8wXU-cjsomA-8XHiw2-JSVwLA-b1cPhH-acM7Qx-22gq9BQ-jKo6Ag-5ejWZG-7W814u-b5BZ6a-Aw3CAA-dbFVVr-ga3Q52-PbKTj-o8fRou-9Gxggp-PbKSN-6L58D3-6qUJW3-9pG7Ro-8rLdzs-Przrow-NAGBT-JXMBsF-9g9tAJ-Ktf9Y7-4w5Kxe-4tdERU-521zsc-4eXZ48-4UNP9a-bfuKNB-5RY3qa-PbKT9-PbKSQ-aq317b-9KW5UH-23WC4PS-7dtcVs-7zCNbM). **Figure 2D** was assessed from NOAA Office of National Marine Sanctuaries (Public Domain, retrieved from https://www.flickr.com/photos/onms/27984972905/in/photolist-2jngRaw-a8nsFL-a8nsBd-JCWisR-2hJt2YU-fZaNi-at6gX5-GniC3z-2hjNEaC-29Py1gc). **Figure 2E** was taken by one of the authors of this review (LD-D).

REFERENCES

Abrams, G. D., Bauer, H., and Sprinz, H. (1963). Influence of the normal flora on Mucosal Morphology and Cellular Renewal in the Ileum. A Comparison of Germ-free and Conventional Mice. *Lab. Invest.* 12, 355–364.

Ainsworth, T. D., Krause, L., Bridge, T., Torda, G., Raina, J.-B., Zakrzewski, M., et al. (2015). The Coral Core Microbiome Identifies Rare Bacterial Taxa as Ubiquitous Endosymbionts. *Isme J.* 9, 2261–2274. doi:10.1038/ismej.2015.39

Akira, S., and Takeda, K. (2004). Toll-like Receptor Signalling. *Nat. Rev. Immunol.* 4, 499–511. doi:10.1038/nri1391

Alam, A., Leoni, G., Quiros, M., Wu, H., Desai, C., Nishio, H., et al. (2016). The Microenvironment of Injured Murine Gut Elicits a Local Pro-restitutive Microbiota. *Nat. Microbiol.* 1, 15021. doi:10.1038/nmicrobiol.2015.21

Alam, A., and Neish, A. (2018). Role of Gut Microbiota in Intestinal Wound Healing and Barrier Function. *Tissue Barriers* 6, e1539595. doi:10.1080/21688370.2018.1539595

Ammons, M. C. B., Morrissey, K., Tripet, B. P., Van Leuven, J. T., Han, A., Lazarus, G. S., et al. (2015). Biochemical Association of Metabolic Profile and Microbiome in Chronic Pressure Ulcer Wounds. *PLoS ONE* 10, e0126735. doi:10.1371/journal.pone.0126735

Arnold, C. P., Merryman, M. S., Harris-Arnold, A., McKinney, S. A., Seidel, C. W., Loethen, S., et al. (2016). Pathogenic Shifts in Endogenous Microbiota Impede Tissue Regeneration via Distinct Activation of TAK1/MKK/P38. *eLife* 5, e16793. doi:10.7554/eLife.16793

Arumugam, M., Raes, J., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., et al.MetaHIT Consortium (2011). Enterotypes of the Human Gut Microbiome. *Nature* 473, 174–180. doi:10.1038/nature09944

Arvizu, F., Aguilera, A., and Salgado, L. M. (2006). Activities of the Protein Kinases STK, PI3K, MEK, and ERK Are Required for the Development of the Head Organizer in Hydra Magnipapillata. *Differentiation* 74 (6), 305–312. doi:10.1111/j.1432-0436.2006.00078.x

Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., et al. (2008). ATP Drives Lamina Propria TH17 Cell Differentiation. *Nature* 455, 808–812. doi:10.1038/nature07240

Bäckhed, F., and Crawford, P. A. (2010). Coordinated Regulation of the Metabolome and Lipidome at the Host-Microbial Interface. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1801 (3), 240–245. doi:10.1016/j.bbalip.2009.09.009

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., et al. (2007). Identification of Stem Cells in Small Intestine and colon by Marker Gene Lgr5. *Nature* 449 (7165), 1003–1007. doi:10.1038/nature06196

Bartow-McKenney, C., Hannigan, G. D., Horwinski, J., Hesketh, P., Horan, A. D., Mehta, S., et al. (2018). The Microbiota of Traumatic, Open Fracture Wounds Is Associated with Mechanism of Injury. *Wound Rep. Reg.* 26 (2), 127–135. doi:10.1111/wrr.12642

Bates, J. M., Akerlund, J., Mittge, E., and Guillemin, K. (2007). Intestinal Alkaline Phosphatase Detoxifies Lipopolysaccharide and Prevents Inflammation in Zebrafish in Response to the Gut Microbiota. *Cell Host & Microbe* 2 (6), 371–382. doi:10.1016/j.chom.2007.10.010

Beumer, J., and Clevers, H. (2016). Regulation and Plasticity of Intestinal Stem Cells during Homeostasis and Regeneration. *Development* 143 (20), 3639–3649. doi:10.1242/dev.133132

Blutt, S. E., Klein, O. D., Donowitz, M., Shroyer, N., Guha, C., and Estes, M. K. (2019). Use of Organoids to Study Regenerative Responses to Intestinal Damage. *Am. J. Physiology-Gastrointestinal Liver Physiol.* 317 (6), G845–G852. doi:10.1152/ajpgi.00346.2018

Bobrovskaya, N. V., and Dolmatov, I. Y. (2014). Autotomy of the Visceral Mass in the Feather star Himerometra Robustipinna (Crinoidea, Comatulida). *Biol. Bull.* 226, 81–91. doi:10.1086/BBLv226n2p81

Boettcher, K. J., and Ruby, E. G. (1990). Depressed Light Emission by Symbiotic Vibrio Fischeri of the Sepiolid Squid *Euprymna scolopes*. *J. Bacteriol.* 172, 3701–3706. Accessed January 19, 2013, Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC213346/pdf/jbacter00121-0177.pdf. doi:10.1128/jb.172.7.3701-3706.1990

Bose, J. L., Rosenberg, C. S., and Stabb, E. V. (2008). Effects of luxCDABEG Induction in *Vibrio Fischeri*: Enhancement of Symbiotic Colonization and Conditional Attenuation of Growth in Culture. *Arch. Microbiol.* 190, 169–183. doi:10.1007/s00203-008-0387-1

Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I. G., et al. (2008). Lymphoid Tissue Genesis Induced by Commensals through NOD1 Regulates Intestinal Homeostasis. *Nature* 456 (7221), 507–510. doi:10.1038/nature07450

Brinkman, B. M., Hildebrand, F., Kubica, M., Goosens, D., Del Favero, J., Declercq, W., et al. (2011). Caspase Deficiency Alters the Murine Gut Microbiome. *Cell Death Dis* 2 (10), e220. doi:10.1038/cddis.2011.101

Brothers, C. J., van der Pol, W. J., Morrow, C. D., Hakim, J. A., Koo, H., and McClintock, J. B. (2018). Ocean Warming Alters Predicted Microbiome Functionality in a Common Sea Urchin. *Proc. R. Soc. B.* 285, 20180340. doi:10.1098/rspb.2018.0340

Bryant, D. M., Sousounis, K., Payzin-Dogru, D., Bryant, S., Sandoval, A. G. W., Martinez Fernandez, J., et al. (2017). Identification of Regenerative Roadblocks via Repeat Deployment of Limb Regeneration in Axolotls. *Npj Regen. Med.* 2 (1), 30. doi:10.1038/s41536-017-0034-z

Buchon, N., Broderick, N. A., Poidevin, M., Pradervand, S., and Lemaitre, B. (2009). Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation. *Cell Host & Microbe* 5, 200–211. doi:10.1016/j.chom.2009.01.003

Burnett, W. J., and McKenzie, J. D. (1997). Subcuticular Bacteria from the Brittle star Ophiactis Balli (Echinodermata: Ophiuroidea) Represent a New Lineage of Extracellular marine Symbionts in the Alpha Subdivision of the Class Proteobacteria. *Appl. Environ. Microbiol.* 63 (5), 1721–1724. doi:10.1128/aem.63.5.1721-1724.1997

Byndloss, M. X., Olsan, E. E., Rivera-Chávez, F., Tiffany, C. R., Cevallos, S. A., Lokken, K. L., et al. (2017). Microbiota-activated PPAR-γ Signaling Inhibits Dysbiotic Enterobacteriaceae Expansion. *Science* 357 (6351), 570–575. doi:10.1126/science.aam10.1126/science.aam9949

Byrne, M. (2001). The Morphology of Autotomy Structures in the Sea Cucumber Eupentacta Quinquesemita before and during Evisceration. *J. Exp. Biol.* 204, 849–863. doi:10.1242/jeb.204.5.849

Candelaria, A. G., Murray, G., File, S. K., and García-Arrarás, J. E. (2006). Contribution of Mesenterial Muscle Dedifferentiation to Intestine Regeneration in the Sea Cucumber Holothuria Glaberrima. *Cell Tissue Res* 325 (1), 55–65. doi:10.1007/s00441-006-0170-z

Carnevali, M. D. C. (2006). Regeneration in Echinoderms: Repair, Regrowth, Cloning. *ISJ* 3, 64–76. https://www.isj.unimore.it/index.php/ISJ/article/view/124.

Cebra, J. J., Periwal, S. B., Lee, G., Lee, F., and Shroff, K. E. (1998). Development and Maintenance of the Gut-Associated Lymphoid Tissue (GALT): the Roles of Enteric Bacteria and Viruses. *Develop. Immunol.* 6, 13–18. doi:10.1155/1998/68382

Chandler, J. A., Morgan Lang, J., Bhatnagar, S., Eisen, J. A., and Kopp, A. (2011). Bacterial Communities of Diverse Drosophila Species: Ecological Context of a Host-Microbe Model System. *Plos Genet.* 7, e1002272. doi:10.1371/journal.pgen.1002272

Charroux, B., and Royet, J. (2012). Gut-microbiota Interactions in Non-mammals: what Can We Learn from Drosophila? *Semin. Immunol.* 24 (1), 17–24. doi:10.1016/j.smim.2011.11.003

Cheesman, S. E., Neal, J. T., Mittge, E., Seredick, B. M., and Guillemin, K. (2011). Epithelial Cell Proliferation in the Developing Zebrafish Intestine Is Regulated by the Wnt Pathway and Microbial Signaling via Myd88. *Proc. Natl. Acad. Sci.* 108, 4570–4577. doi:10.1073/pnas.1000072107

Corby-Harris, V., Pontaroli, A. C., Shimkets, L. J., Bennetzen, J. L., Habel, K. E., and Promislow, D. E. L. (2007). Geographical Distribution and Diversity of Bacteria Associated with Natural Populations of *Drosophila melanogaster*. *Appl. Environ. Microbiol.* 73, 3470–3479. doi:10.1128/aem.02120-06

Cox, C. R., and Gilmore, M. S. (2007). Native Microbial Colonization of *Drosophila melanogaster* and its Use as a Model of *Enterococcus faecalis* Pathogenesis. *Infect. Immun.* 75, 1565–1576. doi:10.1128/iai.01496-06

Davidson, S. K., Koropatnick, T. A., Kossmehl, R., Sycuro, L., and McFall-Ngai, M. J. (2004). NO Means 'yes' in the Squid-vibrio Symbiosis: Nitric Oxide (NO) during the Initial Stages of a Beneficial Association. *Cell Microbiol* 6 (12), 1139–1151. doi:10.1111/j.1462-5822.2004.00429.x

De Schryver, P., Sinha, A. K., Kunwar, P. S., Baruah, K., Verstraete, W., Boon, N., et al. (2010). Poly-β-hydroxybutyrate (PHB) Increases Growth Performance and Intestinal Bacterial Range-Weighted Richness in Juvenile European Sea Bass, *Dicentrarchus labrax*. *Appl. Microbiol. Biotechnol.* 86, 1535–1541. doi:10.1007/s00253-009-2414-9

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., et al. (2014). Microbiota-generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits. *Cell* 156 (1-2), 84–96. doi:10.1016/j.cell.2013.12.016

Demircan, T., İlhan, A. E., Ovezmyradov, G., Öztürk, G., and Yıldırım, S. (2019). Longitudinal 16S rRNA Data Derived from Limb Regenerative Tissue Samples of Axolotl *Ambystoma mexicanum*. *Sci. Data* 6 (1), 70. doi:10.1038/s41597-019-0077-7

Dendy, A. (1886). On the Regeneration of the Visceral Mass in Antedon Rosaceus. *Stud. Biol. laboratories Owens Coll.* 1, 299–312.

Díaz-Díaz, L. M., Rosario-Meléndez, N., Rodríguez-Villafañe, A., Figueroa-Vega, Y. Y., Pérez-Villafañe, O. A., Colón-Cruz, A. M., et al. (2021). Antibiotics Modulate Intestinal Regeneration. *Biology* 10 (3), 236. doi:10.3390/biology10030236

Doino, J. A., and McFall-Ngai, M. J. (1995). A Transient Exposure to Symbiosis-Competent Bacteria Induces Light Organ Morphogenesis in the Host Squid. *Biol. Bull.* 189, 347–355. doi:10.2307/1542152

Douglas, A. E. (2009). The Microbial Dimension in Insect Nutritional Ecology. *Funct. Ecol.* 23, 38–47. doi:10.1111/j.1365-2435.2008.01442.x

Dowd, S. E., Sun, Y., Secor, P. R., Rhoads, D. D., Wolcott, B. M., James, G. A., et al. (2008). Survey of Bacterial Diversity in Chronic Wounds Using Pyrosequencing, DGGE, and Full Ribosome Shotgun Sequencing. *BMC Microbiol.* 8, 43. doi:10.1186/1471-2180-8-43

Falk, P. G., Hooper, L. V., Midtvedt, T., and Gordon, J. I. (1998). Creating and Maintaining the Gastrointestinal Ecosystem: what We Know and Need to Know from Gnotobiology. *Microbiol. Mol. Biol. Rev.* 62, 1157–1170. doi:10.1128/MMBR.62.4.1157-1170.1998

Fielman, K. T., Stancyk, S. E., Dobson, W. E., and Jerome Clements, L. A. (1991). Effects of Disc and Arm Loss on Regeneration byMicrophiopholis Gracillima (Echinodermata: Ophiuroidea) in Nutrient-free Seawater. *Mar. Biol.* 111, 121–127. doi:10.1007/bf01986353

Fraune, S., and Bosch, T. C. G. (2010). Why Bacteria Matter in Animal Development and Evolution. *Bioessays* 32 (7), 571–580. doi:10.1002/bies.200900192

Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., et al. (2014). The Short-Chain Fatty Acid Acetate Reduces Appetite via a central Homeostatic Mechanism. *Nat. Commun.* 5, 3611. doi:10.1038/ncomms4611

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., et al. (2013). Commensal Microbe-Derived Butyrate Induces the Differentiation of Colonic Regulatory T Cells. *Nature* 504, 446–450. doi:10.1038/nature12721

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., et al. (2009). The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses. *Immunity* 31 (4), 677–689. doi:10.1016/j.immuni.2009.08.020

Gao, F., Li, F., Tan, J., Yan, J., and Sun, H. (2014a). Bacterial Community Composition in the Gut Content and Ambient Sediment of Sea Cucumber Apostichopus Japonicus Revealed by 16S rRNA Gene Pyrosequencing. *PLoS ONE* 9, e100092. doi:10.1371/journal.pone.0100092

Gao, F., Tan, J., Sun, H., and Yan, J. (2014b). Bacterial Diversity of Gut Content in Sea Cucumber (*Apostichopus Japonicus*) and its Habitat Surface Sediment. *J. Ocean Univ. China* 13, 303–310. doi:10.1007/s11802-014-2078-7

Garcia-Arraras, J., and Dolmatov, I. (2010). Echinoderms: Potential Model Systems for Studies on Muscle Regeneration. *Cpd* 16 (8), 942–955. doi:10.2174/138161210790883426

García-Arrarás, J. E., Estrada-Rodgers, L., Santiago, R., Torres, I. I., Díaz-Miranda, L., and Torres-Avillán, I. (1998). Cellular Mechanisms of Intestine Regeneration in the Sea Cucumber, Holothuria Glaberrima Selenka (Holothuroidea:Echinodermata). *J. Exp. Zool* 281, 288–304. doi:10.1002/(sici)1097-010x(19980701)281:4<288::aid-jez5>3.0.co;2-k

García-Arrarás, J. E., Lázaro-Peña, M. I., and Díaz-Balzac, C. A. (2018). Holothurians as a Model System to Study Regeneration. *Results Probl. Cel Differ* 65, 255–283. doi:10.1007/978-3-319-92486-1_13

García-Arrarás, J. E., Valentín-Tirado, G., Flores, J. E., Rosa, R. J., Rivera-Cruz, A., San Miguel-Ruiz, J. E., et al. (2011). Cell Dedifferentiation and Epithelial to Mesenchymal Transitions during Intestinal Regeneration in H. Glaberrima. *BMC Develop. Biol.* 11, 61. doi:10.1186/1471-213X-11-61

Gardiner, M., Vicaretti, M., Sparks, J., Bansal, S., Bush, S., Liu, M., et al. (2017). A Longitudinal Study of the Diabetic Skin and Wound Microbiome. *PeerJ* 5, e3543. doi:10.7717/peerj.3543

Gardner, S. E., Hillis, S. L., Heilmann, K., Segre, J. A., and Grice, E. A. (2013). The Neuropathic Diabetic Foot Ulcer Microbiome Is Associated with Clinical Factors. *Diabetes* 62 (3), 923–930. doi:10.2337/db12-0771

Gijzen, H. J., and Barugahare, M. (1992). Contribution of Anaerobic Protozoa and Methanogens to Hindgut Metabolic Activities of the American Cockroach, *Periplaneta americana*. *Appl. Environ. Microbiol.* 58 (8), 2565–2570. doi:10.1128/aem.58.8.2565-2570.1992

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., et al. (2006). Metagenomic Analysis of the Human Distal Gut Microbiome. *Science* 312 (5778), 1355–1359. doi:10.1126/science.1124234

Guo, S., and Dipietro, L. A. (2010). Factors Affecting Wound Healing. *J. Dent Res.* 89 (3), 219–229. doi:10.1177/0022034509359125

Ha, E.-M., Oh, C.-T., Ryu, J.-H., Bae, Y.-S., Kang, S.-W., Jang, I.-h., et al. (2005). An Antioxidant System Required for Host protection against Gut Infection in Drosophila. *Develop. Cel* 8 (1), 125–132. doi:10.1016/j.devcel.2004.11.007

Hakim, J. A., Koo, H., Dennis, L. N., Kumar, R., Ptacek, T., Morrow, C. D., et al. (2015). An Abundance of Epsilonproteobacteria Revealed in the Gut Microbiome of the Laboratory Cultured Sea Urchin, *Lytechinus variegatus*. *Front. Microbiol.* 6, 343. doi:10.3389/fmicb.2015.01047

Hakim, J. A., Koo, H., Kumar, R., Lefkowitz, E. J., Morrow, C. D., Powell, M. L., et al. (2016). The Gut Microbiome of the Sea urchin,*Lytechinus variegatus*, from its Natural Habitat Demonstrates Selective Attributes of Microbial Taxa and Predictive Metabolic Profiles. *FEMS Microbiol. Ecol.* 92, fiw146. doi:10.1093/femsec/fiw146

Hannigan, G. D., Hodkinson, B. P., McGinnis, K., Tyldsley, A. S., Anari, J. B., Horan, A. D., et al. (2014). Culture-independent Pilot Study of Microbiota Colonizing Open Fractures and Association with Severity, Mechanism, Location, and Complication from Presentation to Early Outpatient Follow-Up. *J. Orthop. Res.* 32 (4), 597–605. doi:10.1002/jor.22578

Hansen, A. K., Hansen, C. H. F., Krych, L., and Nielsen, D. S. (2014). Impact of the Gut Microbiota on Rodent Models of Human Disease. *Wjg* 20 (47), 17727–17736. doi:10.3748/wjg.v20.i47.17727

Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., and Gordon, J. I. (2001). Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. *Science* 291, 881–884. doi:10.1126/science.291.5505.881

Hoskins, D., Stancyk, S., and Decho, A. (2003). Utilization of Algal and Bacterial Extracellular Polymeric Secretions (EPS) by the deposit-feeding Brittlestar Amphipholis Gracillima (Echinodermata). *Mar. Ecol. Prog. Ser.* 247, 93–101. doi:10.3354/meps247093

Hou, Q., Ye, L., Huang, L., and Yu, Q. (2017). The Research Progress on Intestinal Stem Cells and its Relationship with Intestinal Microbiota. *Front. Immunol.* 8, 599. doi:10.3389/fimmu.2017.00599

Hou, Q., Ye, L., Liu, H., Huang, L., Yang, Q., Turner, J., et al. (2018). Lactobacillus Accelerates ISCs Regeneration to Protect the Integrity of Intestinal Mucosa through Activation of STAT3 Signaling Pathway Induced by LPLs Secretion of IL-22. *Cell Death Differ* 25 (9), 1657–1670. doi:10.1038/s41418-018-0070-2

Huseini, H. F., Rahimzadeh, G., Fazeli, M. R., Mehrazma, M., and Salehi, M. (2012). Evaluation of Wound Healing Activities of Kefir Products. *Burns* 38 (5), 719–723. doi:10.1016/j.burns.2011.12.005

Hyman, L. (1955). *The Invertebrates. IV. Echinodermata*. New York: The Celomate Bilateria McGraw-Hill Book Co. Inc.

Iacob, S., Iacob, D. G., and Luminos, L. M. (2019). Intestinal Microbiota as a Host Defense Mechanism to Infectious Threats. *Front. Microbiol.* 9, 3328. doi:10.3389/fmicb.2018.03328

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., et al. (2009). Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* 139 (3), 485–498. doi:10.1016/j.cell.2009.09.033

Iwanaga, S., and Lee, B.-L. (2005). Recent Advances in the Innate Immunity of Invertebrate Animals. *BMB Rep.* 38, 128–150. doi:10.5483/bmbrep.2005.38.2.128

Jackson, E. W., Pepe-Ranney, C., Debenport, S. J., Buckley, D. H., and Hewson, I. (2018). The Microbial Landscape of Sea Stars and the Anatomical and Interspecies Variability of Their Microbiome. *Front. Microbiol.* 9, 1829. doi:10.3389/fmicb.2018.01829

James, G. A., Swogger, E., Wolcott, R., Pulcini, E. d., Secor, P., Sestrich, J., et al. (2008). Biofilms in Chronic Wounds. *Wound Repair Regen.* 16, 37–44. doi:10.1111/j.1524-475X.2007.00321.x

Janssens, S., and Beyaert, R. (2003). Role of Toll-like Receptors in Pathogen Recognition. *Clin. Microbiol. Rev.* 16 (4), 637–646. doi:10.1128/CMR.16.4.637-646.2003

Jendrossek, D., and Pfeiffer, D. (2014). New Insights in the Formation of Polyhydroxyalkanoate Granules (Carbonosomes) and Novel Functions of Poly(3-Hydroxybutyrate). *Environ. Microbiol.* 16, 2357–2373. doi:10.1111/1462-2920.12356

Jones, B. W., and Nishiguchi, M. K. (2004). Counterillumination in the Hawaiian Bobtail Squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar. Biol.* 144, 1151–1155. Accessed January 19, 2013. Available at: http://www.medmicro.wisc.edu/labs/mcfall_ruby_papers/pdf/2004/Jones_Nishiguchi_2004_Biol.pdf. doi:10.1007/s00227-003-1285-3

Kalacheva, N. V., Eliseikina, M. G., Frolova, L. T., and Dolmatov, I. Y. (2017). Regeneration of the Digestive System in the Crinoid Himerometra Robustipinna Occurs by Transdifferentiation of Neurosecretory-like Cells. *PLOS ONE* 12 (7), e0182001. doi:10.1371/journal.pone.01820010.1371/journal.pone.0182001

Kandori, H., Hirayama, K., Takeda, M., and Doi, K. (1996). Histochemical, Lectin-Histochemical and Morphometrical Characteristics of Intestinal Goblet Cells of Germfree and Conventional Mice. *Exp. Anim.* 45, 155–160. doi:10.1538/expanim.45.155

Kanno, A., Ozawa, T., and Umezawa, Y. (2011). Detection of Protein-Protein Interactions in Bacteria by GFP-Fragment Reconstitution. *Methods Mol. Biol.* 705, 251–258. doi:10.1007/978-1-61737-967-3_15

Kanther, M., Sun, X., Mühlbauer, M., Mackey, L. C., Flynn, E. J., 3rd, Bagnat, M., et al. (2011). Microbial Colonization Induces Dynamic Temporal and Spatial Patterns of NF-Kb Activation in the Zebrafish Digestive Tract. *Gastroenterology* 141, 197–207. doi:10.1053/j.gastro.2011.03.042

Karlsson, F. H., Fåk, F., Nookaew, I., Tremaroli, V., Fagerberg, B., Petranovic, D., et al. (2012). Symptomatic Atherosclerosis Is Associated with an Altered Gut Metagenome. *Nat. Commun.* 3, 1245. doi:10.1038/ncomms2266

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., and Gordon, J. I. (2011). Human Nutrition, the Gut Microbiome and the Immune System. *Nature* 474, 327–336. doi:10.1038/nature10213

Kaufman, M. G., and Klug, M. J. (1991). The Contribution of Hindgut Bacteria to Dietary Carbohydrate Utilization by Crickets (Orthoptera: Gryllidae). *Comp. Biochem. Physiol. A: Physiol.* 98 (1), 117–123. doi:10.1016/0300-9629(91)90588-4

Kawai, T., and Akira, S. (2010). The Role of Pattern-Recognition Receptors in Innate Immunity: Update on Toll-like Receptors. *Nat. Immunol.* 11, 373–384. doi:10.1038/ni.1863

Kellow, N. J., Coughlan, M. T., and Reid, C. M. (2013). Metabolic Benefits of Dietary Prebiotics in Human Subjects: A Systematic Review of Randomised Controlled Trials. *Br. J. Nutr.* 111, 1147–1161. doi:10.1017/S0007114513003607

Kelly, M. S., Barker, M. F., McKenzie, J. D., and Powell, J. (1995). The Incidence and Morphology of Subcuticular Bacteria in the Echinoderm Fauna of New Zealand. *Biol. Bull.* 189 (2), 91–105. doi:10.2307/1542459

Khalturin, K., Panzer, Z., Cooper, M., and Bosch, T. (2004). Recognition Strategies in the Innate Immune System of Ancestral Chordates. *Mol. Immunol.* 41, 1077–1087. doi:10.1016/j.molimm.2004.06.010

Knoll, A. H. (2003). *The First Three Billion Years of Evolution on Earth*. Princeton/Oxford: Princeton Univ. Press, 277.Life on a Young Planet

Knoll, A. H. (2011). The Multiple Origins of Complex Multicellularity. *Annu. Rev. Earth Planet. Sci.* 39, 217–239. doi:10.1146/annurev.earth.031208.100209

Koropatnick, T. A., Engle, J. T., Apicella, M. A., Stabb, E. V., Goldman, W. E., and McFall-Ngai, M. J. (2004). Microbial Factor-Mediated Development in a Host-Bacterial Mutualism. *Science* 306, 1186–1188. doi:10.1126/science.1102218

Koropatnick, T. A., Kimbell, J. R., and McFall-Ngai, M. J. (2007). Responses of Host Hemocytes during the Initiation of the Squid-Vibrio Symbiosis. *Biol. Bull.* 212 (1), 29–39. doi:10.2307/25066578

Kostic, A. D., Howitt, M. R., and Garrett, W. S. (2013). Exploring Host-Microbiota Interactions in Animal Models and Humans. *Genes Dev.* 27 (7), 701–718. doi:10.1101/gad.212522.112

Lam, E. K. Y., Yu, L., Wong, H. P. S., Wu, W. K. K., Shin, V. Y., Tai, E. K. K., et al. (2007). Probiotic Lactobacillus Rhamnosus GG Enhances Gastric Ulcer Healing in Rats. *Eur. J. Pharmacol.* 565, 171–179. doi:10.1016/j.ejphar.2007.02.050

Lamarcq, L. H., and McFall-Ngai, M. J. (1998). Induction of a Gradual, Reversible Morphogenesis of its Host's Epithelial brush Border by Vibrio Fischeri. *Infect. Immun.* 66 (2), 777–785. doi:10.1128/IAI.66.2.777-785.1998

Lancaster, M. A., and Knoblich, J. A. (2014). Organogenesis in a Dish: Modeling Development and Disease Using Organoid Technologies. *Science* 345 (6194), 1247125. doi:10.1126/science.1247125

Lee, Y.-S., Kim, T.-Y., Kim, Y., Lee, S.-H., Kim, S., Kang, S. W., et al. (2018). Microbiota-derived Lactate Accelerates Intestinal Stem-Cell-Mediated Epithelial Development. *Cell Host & Microbe* 24, 833–846. e6. doi:10.1016/j.chom.2018.11.002

León-Palmero, E., Joglar, V., Álvarez, P. A., Martín-Platero, A., Llamas, I., and Reche, I. (2018). Diversity and Antimicrobial Potential in Sea Anemone and Holothurian Microbiomes. *PLoS One* 13, e0196178. doi:10.1371/journal.pone.0196178

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Human Gut Microbes Associated with Obesity. *Nature* 444 (7122), 1022–1023. doi:10.1038/4441022a

Liu, S.-H., Huang, Y.-C., Chen, L. Y., Yu, S.-C., Yu, H.-Y., and Chuang, S.-S. (2018). The Skin Microbiome of Wound Scars and Unaffected Skin in Patients with Moderate to Severe burns in the Subacute Phase. *Wound Rep. Reg.* 26 (2), 182–191. doi:10.1111/wrr.12632

Loesche, M., Gardner, S. E., Kalan, L., Horwinski, J., Zheng, Q., Hodkinson, B. P., et al. (2017). Temporal Stability in Chronic Wound Microbiota Is Associated with Poor Healing. *J. Invest. Dermatol.* 137, 237–244. doi:10.1016/j.jid.2016.08.009

Long, S. L., Gahan, C. G. M., and Joyce, S. A. (2017). Interactions between Gut Bacteria and Bile in Health and Disease. *Mol. Aspects Med.* 56, 54–65. doi:10.1016/j.mam.2017.06.002

Macpherson, A. J., and Harris, N. L. (2004). Interactions between Commensal Intestinal Bacteria and the Immune System. *Nat. Rev. Immunol.* 4, 478–485. doi:10.1038/nri1373

Madison, L. L., and Huisman, G. W. (1999). Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *Microbiol. Mol. Biol. Rev.* 63, 21–53. doi:10.1128/MMBR.63.1.21-53.1999

Maheswary, T., Nurul, A. A., and Fauzi, M. B. (2021). The Insights of Microbes' Roles in Wound Healing: A Comprehensive Review. *Pharmaceutics* 13 (7), 981. doi:10.3390/pharmaceutics13070981

Mashanov, V. S., Dolmatov, I. Y., and Heinzeller, T. (2005). Transdifferentiation in Holothurian Gut Regeneration. *Biol. Bull.* 209 (3), 184–193. doi:10.2307/3593108

Mashanov, V. S., and García-Arrarás, J. E. (2011). Gut Regeneration in Holothurians: a Snapshot of Recent Developments. *Biol. Bull.* 221 (1), 93–109. doi:10.1086/BBLv221n1p93

Mashanov, V. S., Zueva, O., and García-Arrarás, J. E. (2014). Postembryonic Organogenesis of the Digestive Tube: Why Does It Occur in Worms and Sea Cucumbers but Fail in Humans?. *Develop. Biol.* 108, 185–216. doi:10.1016/B978-0-12-391498-9.00006-1

McFall-Ngai, M., Heath-Heckman, E. A. C., Gillette, A. A., Peyer, S. M., and Harvie, E. A. (2012). The Secret Languages of Coevolved Symbioses: Insights from the *Euprymna Scolopes-Vibrio Fischeri* Symbiosis. *Semin. Immunol.* 24, 3–8. doi:10.1016/j.smim.2011.11.006

McFall-Ngai, M. J., and Ruby, E. G. (1998). Sepiolids and Vibrios: When First They Meet. *BioScience* 48, 257–265. doi:10.2307/1313352

McFall-Ngai, M. J., and Ruby, E. G. (1991). Symbiont Recognition and Subsequent Morphogenesis as Early Events in an Animal-Bacterial Mutualism. *Science* 254 (5037), 1491–1494. doi:10.1126/science.1962208

Metcalfe, C., Kljavin, N. M., Ybarra, R., and de Sauvage, F. J. (2014). Lgr5+ Stem Cells Are Indispensable for Radiation-Induced Intestinal Regeneration. *Cell Stem Cell* 14 (2), 149–159. doi:10.1016/j.stem.2013.11.008

Meyer, D. L. (1988). "Crinoids as Renewable Resource: Rapid Regeneration of the Visceral Mass in a Tropical Reef-Dwelling Crinoid from Australia," in *Echinoderm Biology*. Editors RD Burke, PV Mladenov, P Lambert, and RL Parsley (Rotterdam: Balkema), 519–522.

Meyer, D. L. (1985). Evolutionary Implications of Predation on Recent Comatulid Crinoids from the Great Barrier Reef. *Paleobiology* 11, 154–164. doi:10.1017/s0094837300011477

Milligan-Myhre, K., Charette, J. R., Phennicie, R. T., Stephens, W. Z., Rawls, J. F., Guillemin, K., et al. (2011). Study of Host-Microbe Interactions in Zebrafish. *Methods Cel Biol* 105, 87–116. doi:10.1016/B978-0-12-381320-6.00004-7

Miquel, S., Martín, R., Rossi, O., Bermúdez-Humarán, L., Chatel, J., Sokol, H., et al. (2013). *Faecalibacterium Prausnitzii* and Human Intestinal Health. *Curr. Opin. Microbiol.* 16 (3), 255–261. PMID 23831042. doi:10.1016/j.mib.2013.06.003

Morales-Jiménez, J., Zúñiga, G., Villa-Tanaca, L., and Hernández-Rodríguez, C. (2009). Bacterial Community and Nitrogen Fixation in the Red Turpentine Beetle, *Dendroctonus Valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microb. Ecol.* 58, 879–891. doi:10.1007/s00248-009-9548-2

Morrow, K. M., Tedford, A. R., Pankey, M. S., and Lesser, M. P. (2018). A Member of the Roseobacter Clade, *Octadecabacter* sp., Is the Dominant Symbiont in the Brittle star *Amphipholis Squamata*. *FEMS Microbiol. Ecol.* 94, 1967. doi:10.1093/femsec/fiy030

Mozzi, D., Dolmatov, I., Bonasoro, F., and Carnevali, M. (2006). Visceral Regeneration in the Crinoid Antedon Mediterranea: Basic Mechanisms, Tissues and Cells Involved in Gut Regrowth. *Cent. Eur. J. Biol.* 1, 609–635. doi:10.2478/s11535-006-0042-2

Naito, T., Mulet, C., De Castro, C., Molinaro, A., Saffarian, A., Nigro, G., et al. (2017). Lipopolysaccharide from Crypt-specific Core Microbiota Modulates the Colonic Epithelial Proliferation-To-Differentiation Balance. *MBio* 8, e01680–17. doi:10.1128/mBio.01680-17

Najdegerami, E. H., Tran, T. N., Defoirdt, T., Marzorati, M., Sorgeloos, P., Boon, N., et al. (2012). Effects of Poly-β-Hydroxybutyrate (PHB) on Siberian sturgeon

(*Acipenser baerii*) Fingerlings Performance and its Gastrointestinal Tract Microbial Community. *FEMS Microbiol. Ecol.* 79, 25–33. doi:10.1111/j.1574-6941.2011.01194.x

Narayanan, K. B., and Park, H. H. (2015). Toll/interleukin-1 Receptor (TIR) Domain-Mediated Cellular Signaling Pathways. *Apoptosis* 20, 196–209. PMID: 25563856. doi:10.1007/s10495-014-1073-1

Narbonne, G. M. (2005). The Ediacara Biota: Neoproterozoic Origin of Animals and Their Ecosystems. *Annu. Rev. Earth Planet. Sci.* 33, 421–442. doi:10.1146/annurev.earth.33.092203.122519

Neal, J. T., Peterson, T. S., Kent, M. L., and Guillemin, K. (2013). *H. pylori* Virulence Factor CagA Increases Intestinal Cell Proliferation by Wnt Pathway Activation in a Transgenic Zebrafish Model. *Dis. Model. Mech.* 6 (3), 802–810. doi:10.1242/dmm.011163

Neish, A. S. (2009). Microbes in Gastrointestinal Health and Disease. *Gastroenterology* 136 (1), 65–80. doi:10.1053/j.gastro.2008.10.080

Nhan, D. T., Wille, M., De Schryver, P., Defoirdt, T., Bossier, P., and Sorgeloos, P. (2010). The Effect of Poly β-hydroxybutyrate on Larviculture of the Giant Freshwater Prawn Macrobrachium Rosenbergii. *Aquaculture* 302, 76–81. doi:10.1016/j.aquaculture.2010.02.011

Nie, L., Cai, S.-Y., Shao, J.-Z., and Chen, J. (2018). Toll-Like Receptors, Associated Biological Roles, and Signaling Networks in Non-mammals. *Front. Immunol.* 9, 1523. doi:10.3389/fimmu.2018.01523

Nigro, G., Rossi, R., Commere, P.-H., Jay, P., and Sansonetti, P. J. (2014). The Cytosolic Bacterial Peptidoglycan Sensor Nod2 Affords Stem Cell protection and Links Microbes to Gut Epithelial Regeneration. *Cell Host & Microbe* 15, 792–798. doi:10.1016/j.chom.2014.05.003

Nyholm, S. V., Deplancke, B., Gaskins, H. R., Apicella, M. A., and McFall-Ngai, M. J. (2002). Roles of Vibrio Fischeri and Nonsymbiotic Bacteria in the Dynamics of Mucus Secretion during Symbiont Colonization of the *Euprymna scolopes* Light Organ. *Appl. Environ. Microbiol.* 68, 5113–5122. doi:10.1128/aem.68.10.5113-5122.2002

Nyholm, S. V., and McFall-Ngai, M. (2004). The Winnowing: Establishing the Squid-*Vibrio* Symbiosis. *Nat. Rev. Microbiol.* 2, 632–642. doi:10.1038/nrmicro957

Nyholm, S. V., Stabb, E. V., Ruby, E. G., and McFall-Ngai, M. J. (2000). Establishment of an Animal-Bacterial Association: Recruiting Symbiotic Vibrios from the Environment. *Proc. Natl. Acad. Sci.* 97, 10231–10235. doi:10.1073/pnas.97.18.10231

O'Hara, A. M., and Shanahan, F. (2006). The Gut flora as a Forgotten Organ. *EMBO Rep.* 7 (7), 688–693. doi:10.1038/sj.embor.7400731

Pagán-Jiménez, M., Ruiz-Calderón, J. F., Domínguez-Bello, M. G., and García-Arrarás, J. E. (2019). Characterization of the Intestinal Microbiota of the Sea Cucumber *Holothuria Glaberrima*. *PLoS ONE* 14 (1), e0208011. doi:10.1371/journal.pone.0208011

Peck, B. C. E., Shanahan, M. T., Singh, A. P., and Sethupathy, P. (20172017). Gut Microbial Influences on the Mammalian Intestinal Stem Cell Niche. *Stem Cell Int.* 2017, 1–17. doi:10.1155/2017/5604727

Pellegatta, T., Saler, M., Bonfanti, V., Nicoletti, G., and Faga, A. (2016). Novel Perspectives on the Role of the Human Microbiota in Regenerative Medicine and Surgery. *Biomed. Rep.* 5, 519–524. doi:10.3892/br.2016.778

Peral, M. C., Martinez, M. A. H., and Valdez, J. C. (2009). Bacteriotherapy withLactobacillus Plantarumin burns. *Int. Wound J.* 6 (1), 73–81. doi:10.1111/j.1742-481X.2008.00577.x

Petersen, C. P., and Reddien, P. W. (2009). A Wound-Induced Wnt Expression Program Controls Planarian Regeneration Polarity. *Proc. Natl. Acad. Sci.* 106 (40), 17061–17066. doi:10.1073/pnas.0906823106

Pull, S. L., Doherty, J. M., Mills, J. C., Gordon, J. I., and Stappenbeck, T. S. (2005). Activated Macrophages Are an Adaptive Element of the Colonic Epithelial Progenitor Niche Necessary for Regenerative Responses to Injury. *Proc. Natl. Acad. Sci.* 102, 99–104. doi:10.1073/pnas.0405979102

Qin, J., Li, R., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., et al.MetaHIT Consortium (2010). A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing. *Nature* 464, 59–65. doi:10.1038/nature08821

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A Metagenome-wide Association Study of Gut Microbiota in Type 2 Diabetes. *Nature* 490, 55–60. doi:10.1038/nature11450

Quiñones, J. L., Rosa, R., Ruiz, D. L., and García-Arrarás, J. E. (2002). Extracellular Matrix Remodeling and Metalloproteinase Involvement During Intestine Regeneration in the Sea Cucumber *Holothuria glaberrima*. *Develop. Biol.* 250 (1), 181–197. doi:10.1006/dbio.2002.0778

Quispe-Parra, D. J., Medina-Feliciano, J. G., Cruz-González, S., Ortiz-Zuazaga, H., and García-Arrarás, J. E. (2021). Transcriptomic Analysis of Early Stages of Intestinal Regeneration in Holothuria Glaberrima. *Sci. Rep.* 11 (1), 346. doi:10.1038/s41598-020-79436-2

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R. (2004). Recognition of Commensal Microflora by Toll-like Receptors Is Required for Intestinal Homeostasis. *Cell* 118, 229–241. doi:10.1016/j.cell.2004.07.002

Rawls, J. F., Samuel, B. S., and Gordon, J. I. (2004). From the Cover: Gnotobiotic Zebrafish Reveal Evolutionarily Conserved Responses to the Gut Microbiota. *Proc. Natl. Acad. Sci.* 101, 4596–4601. doi:10.1073/pnas.0400706101

Reddy, P. C., Gungi, A., and Unni, M. (2019). Cellular and Molecular Mechanisms of Hydra Regeneration. *Results Probl. Cel. Differ.* 68, 259–290. doi:10.1007/978-3-030-23459-1_12

Reinhardt, C., Reigstad, C. S., and Bäckhed, F. (2009). Intestinal Microbiota during Infancy and its Implications for Obesity. *J. Pediatr. Gastroenterol. Nutr.* 48 (3), 249–256. doi:10.1097/mpg.0b013e318183187c

Ren, C., Webster, P., Finkel, S. E., and Tower, J. (2007). Increased Internal and External Bacterial Load during Drosophila Aging without Life-Span Trade-Off. *Cel Metab.* 6, 144–152. doi:10.1016/j.cmet.2007.06.006

Roach, J. C., Glusman, G., Rowen, L., Kaur, A., Purcell, M. K., Smith, K. D., et al. (2005). The Evolution of Vertebrate Toll-like Receptors. *Proc. Natl. Acad. Sci.* 102, 9577. doi:10.1073/pnas.0502272102

Rodrigues, J. C. V., Locali, E. C., Freitas-Astua, J., and Kitajima, E. W. (2005). Transmissibility of Citrus Leprosis Virus by Brevipalpus Phoenicis to Solanum Violaefolium. *Plant Dis.* 89 (8), 911. doi:10.1094/PD-89-0911B

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., et al. (2011). Evidence for a Core Gut Microbiota in the Zebrafish. *ISME J.* 5, 1595–1608. doi:10.1038/ismej.2011.38

Roh, S. W., Sung, Y., Nam, Y.-D., Chang, H.-W., Kim, K.-H., Yoon, J.-H., et al. (2008). Arthrobacter Soli Sp. nov., a Novel Bacterium Isolated from Wastewater Reservoir Sediment. *J. Microbiol.* 46, 40–44. doi:10.1007/s12275-007-0239-8

Round, J. L., and Mazmanian, S. K. (2009). The Gut Microbiota Shapes Intestinal Immune Responses during Health and Disease. *Nat. Rev. Immunol.* 9 (5), 313–323. doi:10.1038/nri2515

Ruby, E. G., and Lee, K.-H. (1998). The Vibrio Fischeri-Euprymna scolopes Light Organ Association: Current Ecological Paradigms. *Appl. Environ. Microbiol.* 64 (3), 805–812. doi:10.1128/AEM.64.3.805-812.1998

Ryu, J.-H., Kim, S.-H., Lee, H.-Y., Bai, J. Y., Nam, Y.-D., Bae, J.-W., et al. (2008). Innate Immune Homeostasis by the Homeobox Gene Caudal and Commensal-Gut Mutualism in Drosophila. *Science* 319 (5864), 777–782. doi:10.1126/science.1149357

Sailaja, B. S., He, X. C., and Li, L. (2016). The Regulatory Niche of Intestinal Stem Cells. *J. Physiol.* 594 (17), 4827–4836. doi:10.1113/jp271931

Santos, A. J. M., Lo, Y.-H., Mah, A. T., and Kuo, C. J. (2018). The Intestinal Stem Cell Niche: Homeostasis and Adaptations. *Trends Cell Biology* 28 (12), 1062–1078. doi:10.1016/j.tcb.2018.08.001

Satake, H., and Sekiguchi, T. (2012). Toll-like Receptors of Deuterostome Invertebrates. *Front. Immun.* 3, 34. doi:10.3389/fimmu.2012.00034

Sato, T., Stange, D. E., Ferrante, M., Vries, R. G. J., Van Es, J. H., Van den Brink, S., et al. (2011). Long-term Expansion of Epithelial Organoids from Human Colon, Adenoma, Adenocarcinoma, and Barrett's Epithelium. *Gastroenterology* 141, 1762–1772. doi:10.1053/j.gastro.2011.07.050

Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., et al. (2009). Single Lgr5 Stem Cells Build Crypt-Villus Structures *In Vitro* without a Mesenchymal Niche. *Nature* 459, 262–265. doi:10.1038/nature07935

Savage, D. C., Siegel, J. E., Snellen, J. E., and Whitt, D. D. (1981). Transit Time of Epithelial Cells in the Small Intestines of Germfree Mice and Ex-Germfree Mice Associated with Indigenous Microorganisms. *Appl. Environ. Microbiol.* 42, 996–1001. doi:10.1128/aem.42.6.996-1001.1981

Scholnick, D. A., and Winslow, A. E. (2020). The Role of Fasting on Spine Regeneration and Bacteremia in the Purple Sea Urchin *Strongylocentrotus purpuratus*. *PLOS ONE* 15 (2), e0228711. doi:10.1371/journal.pone.0228711

Seite, S., Flores, G. E., Henley, J. B., Martin, R., Zelenkova, H., Aguilar, L., et al. (2014). Microbiome of Affected and Unaffected Skin of Patients with Atopic Dermatitis before and after Emollient Treatment. *J. Drugs Dermatol.* 13, 1365–1372.

Shin, S. C., Kim, S.-H., You, H., Kim, B., Kim, A. C., Lee, K.-A., et al. (2011). Drosophila Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling. *Science* 334 (6056), 670–674. doi:10.1126/science.1212782

Snel, J., Heinen, P. P., Blok, H. J., Carman, R. J., Duncan, A. J., Allen, P. C., et al. (1995). Comparison of 16S rRNA Sequences of Segmented Filamentous Bacteria Isolated from Mice, Rats, and Chickens and Proposal of "Candidatus Arthromitus". *Int. J. Syst. Bacteriol.* 45 (4), 780–782. doi:10.1099/00207713-45-4-780

Sommer, F., and Bäckhed, F. (2013). The Gut Microbiota - Masters of Host Development and Physiology. *Nat. Rev. Microbiol.* 11, 227–238. doi:10.1038/nrmicro2974

Stappenbeck, T. S., Hooper, L. V., and Gordon, J. I. (2002). Nonlinear Partial Differential Equations and Applications: Developmental Regulation of Intestinal Angiogenesis by Indigenous Microbes via Paneth Cells. *Proc. Natl. Acad. Sci.* 99 (24), 15451–15455. doi:10.1073/pnas.202604299

Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T., et al. (2004). Aberrant Expansion of Segmented Filamentous Bacteria in IgA-Deficient Gut. *Pnas* 101 (7), 1981–1986. doi:10.1073/pnas.0307317101

Swanson, P. A., 2nd, Kumar, A., Samarin, S., Vijay-Kumar, M., Kundu, K., Murthy, N., et al. (2011). Enteric Commensal Bacteria Potentiate Epithelial Restitution via Reactive Oxygen Species-Mediated Inactivation of Focal Adhesion Kinase Phosphatases. *Proc. Natl. Acad. Sci.* 108 (21), 8803–8808. doi:10.1073/pnas.1010042108

Szathmáry, E., and Smith, J. M. (1995). The Major Evolutionary Transitions. *Nature* 374, 227.

Thomas, H. (2016). Microbiota Promote Gut Healing. *Nat. Rev. Gastroenterol. Hepatol.* 13, 189. doi:10.1038/nrgastro.2016.31

Tischer, S., Reineck, M., Söding, J., Münder, S., and Böttger, A. (2013). Eph Receptors and Ephrin Class B Ligands Are Expressed at Tissue Boundaries in *Hydra vulgaris*. *Int. J. Dev. Biol.* 57 (9-10), 759–765. doi:10.1387/ijdb.130158ab

Troll, J. V., Bent, E. H., Pacquette, N., Wier, A. M., Goldman, W. E., Silverman, N., et al. (2010). Taming the Symbiont for Coexistence: A Host PGRP Neutralizes a Bacterial Symbiont Toxin. *Environ. Microbiol.* 12 (8), 2190–2203. doi:10.1111/j.1462-2920.2009.02121.x

Tu, S., and Johnson, S. L. (2011). Fate Restriction in the Growing and Regenerating Zebrafish Fin. *Develop. Cel.* 20 (5), 725–732. doi:10.1016/j.devcel.2011.04.013

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., Gordon, J. I., et al. (2007). The Human Microbiome Project. *Nature* 449 (7164), 804–810. doi:10.1038/nature06244

Turnbaugh, P. J., Bäckhed, F., Fulton, L., and Gordon, J. I. (2008). Diet-induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host & Microbe* 3, 213–223. doi:10.1016/j.chom.2008.02.015

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A Core Gut Microbiome in Obese and Lean Twins. *Nature* 457 (7228), 480–484. doi:10.1038/nature07540

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An Obesity-Associated Gut Microbiome with Increased Capacity for Energy Harvest. *Nature* 444 (7122), 1027–1031. doi:10.1155/2015/80624810.1038/nature05414

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.-M., Lemaitre, B., et al. (2000). Tissue-specific Inducible Expression of Antimicrobial Peptide Genes in Drosophila Surface Epithelia. *Immunity* 13 (5), 737–748. doi:10.1016/s1074-7613(00)00072-8

Uribe, A., Alam, M., Johansson, O., Midtvedt, T., and Theodorsson, E. (1994). Microflora Modulates Endocrine Cells in the Gastrointestinal Mucosa of the Rat. *Gastroenterology* 107, 1259–1269. doi:10.1016/0016-5085(94)90526-6

Valdéz, J. C., Peral, M. C., Rachid, M., Santana, M., and Perdigón, G. (2005). Interference of Lactobacillus Plantarum with *Pseudomonas aeruginosa In Vitro* and in Infected burns: the Potential Use of Probiotics in Wound Treatment. *Clin. Microbiol. Infect.* 11 (6), 472–479. doi:10.1111/j.1469-0691.2005.01142.x

Vieira, W. A., Wells, K. M., and McCusker, C. D. (2020). Advancements to the Axolotl Model for Regeneration and Aging. *Gerontology* 66, 212–222. doi:10.1159/000504294

Vijay-Kumar, M., Aitken, J. D., Carvalho, F. A., Cullender, T. C., Mwangi, S., Srinivasan, S., et al. (2010). Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-like Receptor 5. *Science* 328 (5975), 228–231. doi:10.1126/science.1179721

Visick, K. L., Foster, J., Doino, J., McFall-Ngai, M., and Ruby, E. G. (2000). *Vibrio Fischeri* Lux Genes Play an Important Role in Colonization and Development of the Host Light Organ. *J. Bacteriol.* 182, 4578–4586. doi:10.1128/JB.182.16.4578-4586.2000

Wagner, C. L., Taylor, S. N., and Johnson, D. (2008). Host Factors in Amniotic Fluid and Breast Milk that Contribute to Gut Maturation. *Clinic Rev. Allerg Immunol.* 34, 191–204. doi:10.1007/s12016-007-8032-3

Wang, B., Yao, M., Lv, L., Ling, Z., and Li, L. (2017). The Human Microbiota in Health and Disease. *Engineering* 3 (1), 71–82. doi:10.1016/J.ENG.2017.01.008

Wang, L., Zhao, X., Xu, H., Bao, X., Liu, X., Chang, Y., et al. (2018). Characterization of the Bacterial Community in Different Parts of the Gut of Sea Cucumber (Apostichopus Japonicus) and its Variation during Gut Regeneration. *Aquac. Res.* 49, 1987–1996. doi:10.1111/are.13654

Wang, Y., Zhang, S., Li, H., Wang, H., Zhang, T., Hutchinson, M. R., et al. (2020). Small-Molecule Modulators of Toll-like Receptors. *Acc. Chem. Res.* 20 (5), 1046–1055. doi:10.1021/acs.accounts.9b00631

Wang, Y., and Zhang, Y. (2015). Investigation of Gut-Associated Bacteria inTenebrio molitor(Coleoptera: Tenebrionidae) Larvae Using Culture-dependent and DGGE Methods. *Ann. Entomol. Soc. Am.* 108 (5), 941–949. doi:10.1093/aesa/sav079

Weigel, B. L. (2020). Sea Cucumber Intestinal Regeneration Reveals Deterministic Assembly of the Gut Microbiome. *Appl. Environ. Microbiol.* 86, e00489–20. doi:10.1128/AEM.00489-20

Weinstock, G. M. (2012). Genomic Approaches to Studying the Human Microbiota. *Nature* 489 (7415), 250–256. doi:10.1038/nature11553

Wilkie, I. C. (2001). Autotomy as a Prelude to Regeneration in Echinoderms. *Microsc. Res. Tech.* 55, 369–396. doi:10.1002/jemt.1185

Wolcott, R. D., Hanson, J. D., Rees, E. J., Koenig, L. D., Phillips, C. D., Wolcott, R. A., et al. (2016). Analysis of the Chronic Wound Microbiota of 2,963 Patients by 16S rDNA Pyrosequencing. *Wound Rep. Reg.* 24, 163–174. doi:10.1111/wrr.12370

Wong, C. N. A., Ng, P., and Douglas, A. E. (2011). Low-diversity Bacterial Community in the Gut of the Fruitfly *Drosophila melanogaster*. *Environ. Microbiol.* 13 (7), 1889–1900. doi:10.1111/j.1462-2920.2011.02511.x

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A., and Jenkins, D. J. A. (2006). Colonic Health: Fermentation and Short Chain Fatty Acids. *J. Clin. Gastroenterol.* 40, 235–243. doi:10.1097/00004836-200603000-00015

Yamazaki, Y., Meirelles, P. M., Mino, S., Suda, W., Oshima, K., Hattori, M., et al. (2016). Individual *Apostichopus Japonicus* Fecal Microbiome Reveals a Link with Polyhydroxybutyrate Producers in Host Growth Gaps. *Sci. Rep.* 6, 21631. doi:10.1038/srep21631

Yin, Y., Wang, Y., Zhu, L., Liu, W., Liao, N., Jiang, M., et al. (2013). Comparative Analysis of the Distribution of Segmented Filamentous Bacteria in Humans, Mice and Chickens. *ISME J.* 7 (3), 615–621. doi:10.1038/ismej.2012.128

Yokoyama, H. (2008). Initiation of Limb Regeneration: the Critical Steps for Regenerative Capacity. *Dev. Growth Differ.* 50 (1), 13–22. doi:10.1111/j.1440-169X.2007.00973.x

Zaborin, A., Krezalek, M., Hyoju, S., Defazio, J. R., Setia, N., Belogortseva, N., et al. (2017). Critical Role of Microbiota within Cecal Crypts on the Regenerative Capacity of the Intestinal Epithelium Following Surgical Stress. *Am. J. Physiol. Gastrointest. Liver Physiol.* 312, G112–G122. doi:10.1152/ajpgi.00294.2016

Zhang, H., Wang, Q., Zhao, J., Liu, S., Zhang, L., Zhao, Y., et al. (2020). Quantitative Microbiome Profiling Links Microbial Community Variation to the Intestine Regeneration Rate of the Sea Cucumber Apostichopus Japonicus. *Genomics* 112, 5012–5020. doi:10.1016/j.ygeno.2020.09.017

Zhao, Y., Wang, Q., Liu, H., Li, B., and Zhang, H. (2019). High-Throughput Sequencing of 16S rRNA Amplicons Characterizes Gut Microbiota Shift of Juvenile Sea Cucumber Apostichopus Japonicus Feeding with Three Antibiotics. *J. Ocean. Limnol.* 37, 1714–1725. doi:10.1007/s00343-019-8308-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

REVIEW
published: 01 February 2022
doi: 10.3389/fevo.2022.783818

Comparative Study in Zebrafish and Medaka Unravels the Mechanisms of Tissue Regeneration

Kaushik Chowdhury[1,2], *Shi Lin*[1,3] *and Shih-Lei Lai*[1,2]*

[1] *Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan,* [2] *Taiwan International Graduate Program in Molecular Medicine, National Yang Ming Chiao Tung University and Academia Sinica, Taipei, Taiwan,* [3] *Department of Integrative Biology and Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, CA, United States*

Tissue regeneration has been in the spotlight of research for its fascinating nature and potential applications in human diseases. The trait of regenerative capacity occurs diversely across species and tissue contexts, while it seems to decline over evolution. Organisms with variable regenerative capacity are usually distinct in phylogeny, anatomy, and physiology. This phenomenon hinders the feasibility of studying tissue regeneration by directly comparing regenerative with non-regenerative animals, such as zebrafish (*Danio rerio*) and mice (*Mus musculus*). Medaka (*Oryzias latipes*) is a fish model with a complete reference genome and shares a common ancestor with zebrafish approximately 110–200 million years ago (compared to 650 million years with mice). Medaka shares similar features with zebrafish, including size, diet, organ system, gross anatomy, and living environment. However, while zebrafish regenerate almost every organ upon experimental injury, medaka shows uneven regenerative capacity. Their common and distinct biological features make them a unique platform for reciprocal analyses to understand the mechanisms of tissue regeneration. Here we summarize current knowledge about tissue regeneration in these fish models in terms of injured tissues, repairing mechanisms, available materials, and established technologies. We further highlight the concept of inter-species and inter-organ comparisons, which may reveal mechanistic insights and hint at therapeutic strategies for human diseases.

Keywords: zebrafish, medaka (*Oryzias latipes*), tissue regeneration, heart, retina, fin, evolution, comparative genomics

OPEN ACCESS

Edited by:
Mathilda Mommersteeg, University of Oxford, United Kingdom

Reviewed by:
Yuki Shimizu, National Institute of Advanced Industrial Science and Technology, Japan
Lazaro Centanin, Heidelberg University, Germany

***Correspondence:**
Shih-Lei Lai
ben.s.lai@ibms.sinica.edu.tw

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

Received: *27 September 2021*
Accepted: *10 January 2022*
Published: *01 February 2022*

Citation:
Chowdhury K, Lin S and Lai S-L (2022) Comparative Study in Zebrafish and Medaka Unravels the Mechanisms of Tissue Regeneration. Front. Ecol. Evol. 10:783818. doi: 10.3389/fevo.2022.783818

INTRODUCTION: TISSUE REGENERATION IN MODEL ORGANISMS

Reparative regeneration refers to replacing damaged or lost body parts with new tissue, an injury response that restores the tissue homeostasis and function in the optimal scenario (Iismaa et al., 2018). By studying regeneration, scientists can devise biological concepts for tissue repairing and apply them to traumatic injury and degenerative diseases in humans/patients. The studies encompass the strategy to stimulate the repair mechanism to replace the damaged tissues and organs, involve cross-discipline practices, and serves as a bridge between developmental biology and clinical study. A common way to study regeneration is to introduce experimental injury to the model animals and observe how they repair the tissue and recover from the injury.

In the Kingdom Animalia, the loss of regenerative ability coincides with the evolution of new and complex cell and tissue types (Brockes et al., 2001; Maginnis, 2006; Bely and Nyberg, 2010; Elchaninov et al., 2021). Anatomically simple organisms, such as hydra and planarians, can regenerate their entire body when cut into multiple pieces (Reddien and Alvarado, 2004; Reddy et al., 2019). Considerable members of early branching vertebrate lineages (like bony fish and amphibians) can also regrow various organs upon experimental injuries (Yun, 2015; Khyeam et al., 2021). In striking contrast, mammals (like mice and humans), retain a limited regenerative capacity only in some tissues and organs (Iismaa et al., 2018; **Figure 1**). The differences in regeneration capacity solely rely on the cellular source for replenishing lost or damaged tissue, which may come from one of three mechanisms, including the *proliferation* of progenitor/stem cells, *dedifferentiation* of mature cells into progenitors, and *transdifferentiation* from one cell type to another (Jopling et al., 2011). Regenerative species usually possess the pool of progenitor cells or the potential of dedifferentiation and transdifferentiation upon activating the regenerative program within the injured tissue. Taking the heart for example, most cardiomyocytes stop proliferating soon after birth, and there is no stem-like or progenitor cell population identified in the adult hearts (Bely and Nyberg, 2010; Steinhauser and Lee, 2011; Mollova et al., 2013; Bergmann et al., 2015). Instead, mature cardiomyocytes were stimulated to dedifferentiate, proliferate, and re-differentiate to replenish the lost tissue upon injury (Eschenhagen et al., 2017). Over the years, investigations across the animal kingdom have led us to compile a list of masters in tissue regeneration who also have comparable organ systems to humans, including zebrafish (Marques et al., 2019), newts (Laube et al., 2006), and Axolotl (Cano-Martinez et al., 2010; Simon and Tanaka, 2013; **Figure 1**).

Since regenerative capacity exists unevenly among species and their respective organs, an exciting way to uncover the mechanisms of tissue regeneration is by comparing the repair processes in animals with differential regenerative capacities. Such comparisons have been carried out in two ways: "Inter-species" comparing the repair of the same tissue/organ that is regenerative in one species (could also be age or living condition) but non-regenerative in another; Or "inter-organ" comparing two regenerative tissues within the same species to identify a central regenerative program (Potts et al., 2021). However, the regenerative species are usually quite distant in phylogeny, anatomy, and physiology from those non-regenerative ones, such as zebrafish and mice. It is thus essential to find more comparable species to overcome these shortcomings. In search of such comparative systems that can justify the disadvantages of comparing fish with mammals, zebrafish and medaka represent a more simplistic and feasible platform for comparing tissue repair and regeneration.

Zebrafish and medaka are two commonly used vertebrate models in biomedical research, given the homology with mammals and the availability of a wide range of research tools (Furutani-Seiki and Wittbrodt, 2004). Despite diverged 115–200 Mya, zebrafish and medaka are similar in anatomy, physiology, and genetics, with many conserved gene regulatory elements. In addition, zebrafish shares more than 70% of homologous genes with humans, and conserved signaling pathways and metabolic networks, making it a valuable model for biomedical research (Howe et al., 2013). Interestingly, medaka possesses regenerative capacity in fin (Katogi et al., 2004), kidney (Watanabe et al., 2009), liver (Van Wettere et al., 2013), pancreas (Otsuka and Takeda, 2017), lateral line neuromasts (Seleit et al., 2017b), and gills (Stolper et al., 2019) but is impaired to regenerate the heart (Ito et al., 2014; Lai et al., 2017), retina (Lust and Wittbrodt, 2018), brain (Shimizu and Kawasaki, 2021), and posterior lateral line (pLL) nerve cells (Seleit et al., 2022). This uneven regenerative capacity across organs is in sharp contrast with zebrafish, which can regenerate almost all organs, including the heart (Poss et al., 2002), retina (Vihtelic and Hyde, 2000; Sherpa et al., 2008), brain (Kroehne et al., 2011; Marz et al., 2011; Kishimoto et al., 2012), spinal cord (Becker et al., 1997; Ghosh and Hui, 2018), notochord (Garcia et al., 2017; Lopez-Baez et al., 2018), fin (Poss et al., 2003), kidney (Diep et al., 2011), liver (Sadler et al., 2007), pancreas (Moss et al., 2009), gills (Mierzwa et al., 2020), and lateral line (Hair cells) (Lush and Piotrowski, 2014; Cruz et al., 2015). These features make them great models for studying tissue regeneration by inter-species comparisons. Even for the inter-organ comparisons, common vs. tissue-specific regenerative programs could be revealed in zebrafish, while tissue-specific injury responses relevant to regeneration may be explored in medaka, which will be further elaborated in this review.

Here, we highlight the potential of this comparative platform by summarizing the current knowledge from published work, available tools and techniques, and elaborate on current limitations and future outlooks. This platform may provide a new opportunity for investigating the intrinsic mechanisms of tissue regeneration at the organism level and in an unbiased manner. The constraints and triggers of tissue regeneration may further translate toward novel therapeutics for related human diseases.

EVOLUTION OF THE REGENERATIVE CAPACITY

Deciphering the underlying mechanisms of tissue regeneration across phylogeny requires the integrative knowledge of evolutionary biology since the trait (regeneration) changes over the course of evolution (Zattara et al., 2019). This phenomenon is due to the fact that maintaining regenerative capacities requires selective pressures, in terms of the frequency and severity of major damages in an extreme living environment (Morgan, 1901; Lin et al., 2017; Elchaninov et al., 2021). These damages may compromise the fitness of the organisms, but they are not always detrimental for survival and propagation as a selective pressure (Fox and McCoy, 2000; Bernardo and Agosta, 2005). As a result, a critical phylogenetic trend identified across the animal phyla reveals declined regenerative capacity instead of preservation (Bely, 2010; Bely and Nyberg, 2010).

Various theories have been proposed to explain the declined regenerative capacity, including low damage intensity over the evolution mentioned above and the changes in adaptive value

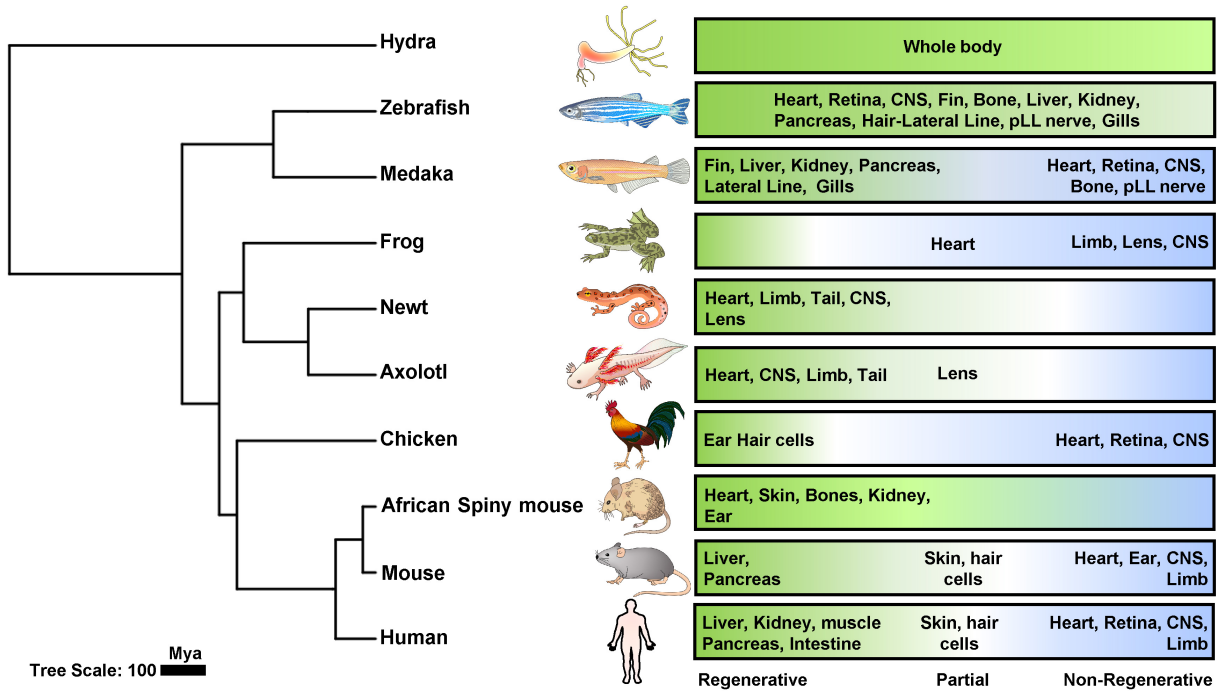


FIGURE 1 | Phylogenetic analysis of tissue regeneration. Phylogenetic tree of animal models tested for regenerative capacities including cnidaria, teleost's, amphibians, aves and mammals were generated using TimeTree (TimeTree::The Timescale of Life) (Kumar et al., 2017) and later organized by iTOL (Letunic and Bork, 2021). Regenerative (green), partial (white), and non-regenerative (blue) organs were depicted in the panels for each category.

of organs (Elchaninov et al., 2021). In addition, the loss of capacity in myocardial regeneration in adult mammals could be an evolutionary trade-off related to energy metabolism (Elhelaly et al., 2016). In a different context, the regenerative capacity of limbs in amphibians but lost in other tetrapods may result from the semiautonomous module of limb development, so their limb may regenerate as a separate organ in adults without the interactions with other transient structures during development (Galis et al., 2003). Another interesting observation is that the declined regenerative capacity seems to inversely correlate with complex immune systems during development and evolution (Mescher and Neff, 2005). For example, the development and maturation of the immune system strongly correlate with the decline of regenerative capacity during frog metamorphosis (Robert and Ohta, 2009; Godwin and Rosenthal, 2014) and mammalian cardiac maturation (Porrello et al., 2011; Vivien et al., 2016). Thus, the selection pressure of the immune system may have underlying influences on the regenerative capacity that reflect at the tissue or organismic levels. However, the immune system does not always obstruct regeneration. It even acts as a critical tissue regeneration component as a coordinated innate immune response is indispensable for regenerating the axolotl limb and neonatal mouse heart (Godwin et al., 2013; Aurora et al., 2014). Moreover, the current knowledge indicates the capacity to regenerate is not only confined to organ-specific or tissue-specific levels but a coordinated involvement of systemic responses (Aurora et al., 2014; Lai et al., 2017; Sanz-Morejon et al., 2019; Bevan et al., 2020).

It is known that the loss/gain of tissue regenerative ability has evolved independently several times over the course of evolutionary history (Zattara et al., 2019). Given this, it is of paramount importance to examine and compare regeneration in a lineage-specific context (Dwaraka and Voss, 2021). Despite the availability of systematic reviews on the evolutionary origin of regeneration, only a handful of studies have addressed the regenerative potential in a lineage-specific context and have reconstructed routes of the ancestral states with the organ of interests (Zattara et al., 2019; Dwaraka and Voss, 2021). Fortunately, growing research groups are proposing comparative analyses of tissue regeneration across animal phylogeny. Comparative phylogenetic studies investigating the regenerative capacity of diverse animal taxa bring invaluable insights into the origin and preservation of regeneration throughout evolution. Novel inferences may be drawn only by comparing a wide range of organisms covering major branches/lineages of interest.

Such a study was recently reported by Hirose et al. (2019) who used cardiomyocyte ploidy as an indicator of heart regeneration and assessed the ploidy of cardiomyocytes in 41 vertebrate species. They found that the diploid cardiomyocyte frequency inversely correlated with the energy metabolism process modulated by the thyroid signaling, an evolutionary trade-off for acquiring endothermy in mammals compared to fish (Hirose et al., 2019). Evolutionary trade-offs are the manifestation of loss or gain of a particular functional trait caused by opposing selections resulting from different environments at an apparent cost (Agrawal et al., 2010). More

examples of the trade-off between regenerative capacity and metabolism can be observed in Mexican cavefish (Stockdale et al., 2018). Stockdale et al. (2018) reported that the surface- and cavefish possessed similar levels of cardiomyocyte proliferative capacity, but the cave-fish showed differential upregulation of immune and scarring responses with downregulated metabolic genes compared to their surface-dwelling counterparts. These switch in metabolic regulation might play an essential role in the regenerative capacity of the cavefish when it fails to regenerate its heart and instead forms a fibrotic scar overtime. Furthermore, this intra-species comparative study nicely depicts that successful heart regeneration relies on the interplay of cardiomyocytes (CM) proliferation and scarring, which is absent in the cave-dwelling species (Stockdale et al., 2018). Moreover, the current evidence for heart regeneration further suggests that the loss of mammalian regenerative capacity is a one-trait evolutionary trade-off for higher energy metabolism in cardiac output and failure in cardiomyocyte proliferation as a capacity for heart regeneration (Elhelaly et al., 2016). The switch in metabolic reprogramming can be further correlated with cardiomyocyte proliferation observed during zebrafish heart regeneration switching from oxidative phosphorylation to glycolysis (Honkoop et al., 2019). These events, in turn, if activated in mouse hearts by ErbB2 signaling, can induce cardiomyocyte proliferation and improve functional recovery post-ischemic injury (Honkoop et al., 2019). Interestingly, medaka possesses the potential for testing these theories and makes people wonder the mechanisms underlying their uneven regenerative capacity amongst different organs, especially when compared to zebrafish.

ZEBRAFISH AND MEDAKA: POWERFUL MODELS FOR COMPARATIVE STUDY

Among various model systems used to study regeneration, fish species are extensively investigated. Fish is a phylogenetically "inclusive" term that encompasses four major vertebrate lineages: Sarcopterygii (lobe-finned fish), Actinopterygii (ray-finned fish), Chondrichthyes (cartilaginous fish), and Agnatha (jawless fish). Interestingly, these primitive vertebrates exhibit uneven regenerative capacity among different organs, living conditions, and between phylogenetically close species, making them perfect models to reveal how regeneration works and how to preserve or rehabilitate it in other vertebrates that have lost the capacity. Among actinopterygian fish, zebrafish are natives of the river basins in India and a well-established animal model used extensively for scientific research since the 1980s (Streisinger et al., 1981, 1986). Over the decades, zebrafish has stood out as a powerful tool for studying developmental biology, evolution, human genetics, and diseases. The advantages of the zebrafish model include a small size for manipulation, short reproductive cycle, large clutches of embryos, rapid development, cheap maintenance, comparable organs to mammals, and fully sequenced genome with well-annotated genes (Gemberling et al., 2013; Howe et al., 2013; Beffagna, 2019). Most importantly, zebrafish regenerate almost all organs upon experimental injury (Marques et al., 2019). On the other hand, medaka species are small egg-laying freshwater teleost fish home to Asia with native diversity from Japan, Korea, Taiwan, and China (Hilgers and Schwarzer, 2019). They live in rice paddy fields, rivers, and creeks in Japan, thus also named "Japanese Rice fish." As a resident of the temperate zone, medaka can tolerate a temperature range from 4 to 40°C for both embryos and adults in the wild. In particular, Japanese medaka (*Oryzias latipes*) is highly tolerant to inbreeding, ideal for laboratory conditions with 14 h light and 10 h dark circles for mating conditions, with simple dietary and habitat requirements (Kirchmaier et al., 2015). They were established as a genetic model as early as 1975 (Yamamoto, 1975) and were one of the first model organisms for genetic manipulations (Ozato et al., 1986). Additionally, medaka is an ideal model organism owning to short development (7–9 days) and reproduction cycle (2–3 months), fully sequenced genome (three strains), and a transparent body throughout the juvenile stage (Ishikawa, 2000; Wittbrodt et al., 2002; Kirchmaier et al., 2015). Although zebrafish and medaka are distant relatives that got separated around 110-200 million years ago during evolution (Wittbrodt et al., 2002), they are similar in size, anatomy, and physiology, allowing them to be raised in the same laboratory conditions in terms of feeding, light-dark cycle, water temperature/quality, and propagation (Furutani-Seiki and Wittbrodt, 2004). In addition to orthologous gene-sets for genome-wide profiling and reciprocal analyses, many materials and methods can be applied equally to both zebrafish and medaka, making them ideal for comparative studies than more distantly related species (**Figure 2**).

Inter-Species Comparisons: Cardiac Regeneration

As one of the most vital organs, mammalian hearts have a minimal capacity for regeneration upon disease or injury in the post-natal period, especially for replenishing cardiac muscle cells (cardiomyocytes, CMs). Instead, the infarcted hearts undergo fibrotic repair, which in turn deteriorates tissue contractility and function, eventually leading to heart failure and organismal death (Kong et al., 2014). In contrast to the limited regenerative capacity of adult mammals, certain fishes and amphibians, and even neonatal mice can regenerate their hearts after injury (Vivien et al., 2016). This is an excellent example of how the regenerative capacity of hearts exists unevenly across species and developmental stages, as mammals possess the regenerative capacity only for a short time window after birth (Porrello et al., 2011; Haubner et al., 2012, 2016). These mammalian and non-mammalian models provide unique opportunities to study the intrinsic capacity and mechanisms of heart regeneration. Amongst, extensive knowledge was gained from the zebrafish studies. Since the ground-breaking discovery of zebrafish heart regeneration by Poss et al. (2002), researchers have made in-depth investigations to understand the mechanisms of heart regeneration in zebrafish (Marques et al., 2019; Jaźwińska and Blanchoud, 2020; Potts et al., 2021). Briefly, zebrafish hearts mount a robust immune response in the recruitment of macrophages and neutrophils immediately after injury (Lai et al., 2017) and fast revascularization that expands

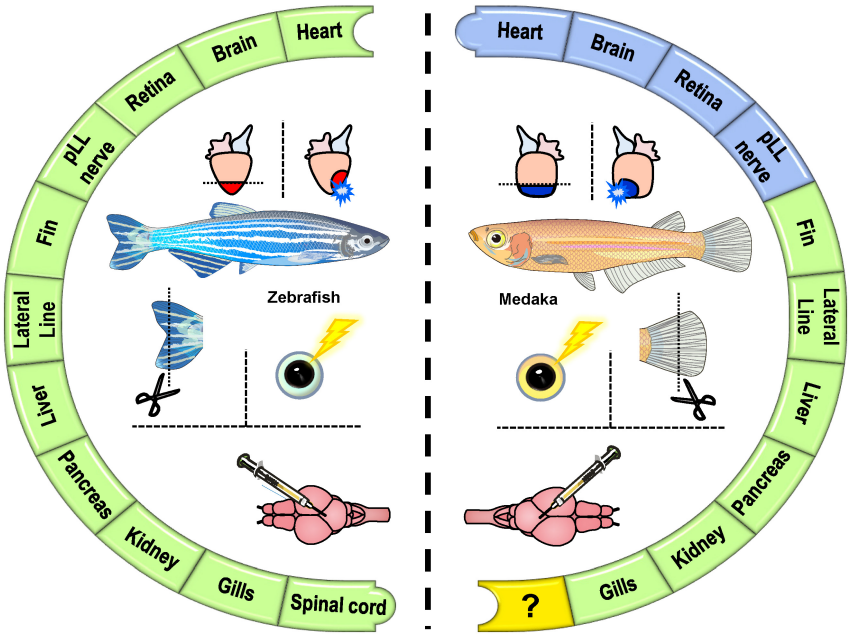


FIGURE 2 | The comparative analyses of tissue regeneration in zebrafish and medaka. A summary of the regenerative (green) vs. non-regenerative (blue) organs and the comparisons (mostly inter-species) of tissue regeneration in zebrafish and medaka.

superficially and intraventricularly and serve as the scaffold for CM repopulation (Marín-Juez et al., 2016, 2019). Almost concurrently, epicardium activates and expands by proliferation to cover the injured area and serve as a signaling hub to stimulate CM de-differentiation and proliferation in the border zone of the injured area (Kikuchi et al., 2010, 2011a,b; Jopling et al., 2011; González-Rosa et al., 2012; Cao and Poss, 2018). Newly formed CMs gradually replace scar tissue coincident with ECM remodeling (Sanchez-Iranzo et al., 2018) and scar resolution (Bevan et al., 2020; Simoes et al., 2020), eventually restoring the morphology and function of the heart. Conserved processes have been shown in neonatal mouse heart regeneration, particularly the source of regenerated CMs (Porrello et al., 2011; Vivien et al., 2016). Furthermore, hints gained from zebrafish studies have been applied to mice models to accelerate cardiac repair (Chen et al., 2016; Honkoop et al., 2019). A comparative study in zebrafish and mouse injured hearts even revealed microRNA dynamics that may regulate CM proliferation and cardiac repair (Crippa et al., 2016). However, considering the taxonomy distance between zebrafish and mice, a comparative approach may be more feasible for more closely related species with similar physiology and structure.

Unlike zebrafish, medaka showed impaired heart regeneration, indicated by a lack of revascularization, low CM proliferation, and a permanent fibrotic scar in the injured area after resection (Ito et al., 2014). To understand the differences in cardiac repair in zebrafish and medaka, Lai and colleagues performed a global transcriptomic analysis and revealed a robust immune response and angiogenic revascularization exist preferentially in zebrafish (Lai et al., 2017). Coincidently, they observed a reduced macrophage infiltration and prolonged neutrophil recruitment/retention in medaka hearts compared to zebrafish. The blunted immune response in medaka encouraged the investigation of the acute immune response and timely macrophage recruitment in heart regeneration. Indeed, in a loss-of-function setting, delayed macrophage recruitment by clodronate liposome pre-depletion abolished the regenerative capacity in zebrafish (Lai et al., 2017), which correspond nicely with findings in neonatal mice (Aurora et al., 2014). These results support an essential role of macrophage function in heart regeneration across species and encore the requirement of the macrophage function in the regeneration of other organs, including fin, retina, optic tectum, brain, and spinal cord reviewed elsewhere (Var and Byrd-Jacobs, 2020). They further identified the immunostimulant poly I:C as one of the upstream candidates that may trigger the differential transcriptomic response found between zebrafish and medaka. Indeed, they further showed that stimulating immune response by poly I:C administration promotes heart regeneration in medaka in a gain-of-function setting (Lai et al., 2017). These results support the strength of comparative analysis using fish models to gain knowledge conserved across species and identify pro-regenerative factors. However, it remains unclear how poly I:C (or other immunostimulants) promote heart regeneration in medaka and whether the same principle may apply to mammals, awaiting further investigation.

Inter-Species Comparisons: Retina Regeneration

The capacity to regenerate retinal neurons after injury also varies drastically among vertebrate species. While mammalian Müller

glia (MG) do not spontaneously regenerate lost retinal neurons, zebrafish MG cells possess a robust capacity to regenerate all retinal cell types and recover their visual ability (Vihtelic and Hyde, 2000; Sherpa et al., 2008; Goldman, 2014; Gorsuch and Hyde, 2014; Lenkowski and Raymond, 2014). Several pluripotent factors, including *ascl1a, lin-28,* and *sox2,* regulate the dedifferentiation, reprogramming, and proliferation of MG cells into various retinal cell types during retina regeneration in zebrafish (Ramachandran et al., 2010a; Gorsuch et al., 2017). Sox2 is also one of the four Yamanaka factors that induced pluripotent stem cell status (Takahashi et al., 2007). In addition to retina, retinal pigment epithelium (RPE) regeneration was also recently described in zebrafish (Leach et al., 2021). Similar to heart regeneration, the immune response, particularly the macrophages and microglia cells, responds to injury and plays a critical role in retina and RPE regeneration, potentially associated with phagocytotic debris clearance and cytokine secretion (Mitchell et al., 2019; Leach et al., 2021).

During retina development, medaka neural stem cells behave similarly to those in zebrafish (Martinez-Morales et al., 2009; Centanin et al., 2011, 2014). However, Lust and Wittbrodt discovered that medaka showed limited regenerative capacity in the retina. The MG cells proliferate but fail to self-renew and reprogram, eventually giving rise to only photoreceptor cells (Lust and Wittbrodt, 2018). Moreover, by comparing medaka with zebrafish, they identified that medaka MG cells fail to maintain *sox2* expression after injury and demonstrated that sustained *sox2* expression in medaka MGs confers regenerative response (Lust and Wittbrodt, 2018). Similar to the above-mentioned reciprocal analyses in heart regeneration, Lust and Wittbrodt were able to identify the critical factor and demonstrated the functional relevance of *sox2* expression in promoting retina regeneration.

More recently, Hoang et al. (2020) identified the evolutionarily conserved and species-specific gene regulatory networks that control the quiescent, reactive, and proliferative MG transition after retinal injury in another cross-species comparison between mice, chick, and zebrafish. They further demonstrated that deleting the factors maintaining the quiescent state may promote MG reprogramming into regeneration-competent cells in adult mice (Hoang et al., 2020).

Inter-Species Comparisons: Central Nervous System Regeneration

Unlike mammals, zebrafish respond to injury or degeneration by inducing specific neurogenic programs and constitutive neurogenesis for tissue regeneration (Diotel et al., 2020). Learning the regenerative mechanisms occurring in zebrafish will be invaluable for developing therapeutics for brain injury and degenerative diseases. In contrast to target-oriented studies, new knowledge may come from side-by-side and unbiased comparisons of animal models with divergent regenerative capacities. Unfortunately, mice brains and zebrafish brains show distinct features other than regenerative capacity, including the overall anatomy and neurogenic niches, thus preventing direct comparisons (Diotel et al., 2020; Labusch et al., 2020). Therefore, it is relevant to explore the mechanisms of brain regeneration in more closely related models.

The regenerative capacity of zebrafish central nervous system (CNS) has been investigated in the optic tectum (Ito et al., 2010; Shimizu et al., 2018; Lindsey et al., 2019) and telencephalon (Kroehne et al., 2011; Marz et al., 2011; Kishimoto et al., 2012). In the adult zebrafish CNS, both MG and the radial glia (RG) cells are activated to proliferate and differentiate into new neuronal cells following injury (Raymond et al., 2006; Ito et al., 2010; Shimizu et al., 2018). These regenerative responses seem to be induced and facilitated by immune responses (Kyritsis et al., 2012; Caldwell et al., 2019), while a specific inflammatory signaling cascade is stimulated by microglia during zebrafish brain repair (Kanagaraj et al., 2020).

A comparative study of brain regeneration in medaka and zebrafish has also been reported very recently (Shimizu and Kawasaki, 2021). Medaka shares a similar brain structure with zebrafish and neural stem cells (NSCs) niche for brain development and growth (Adolf et al., 2006; Grandel et al., 2006; Alunni et al., 2010; Kuroyanagi et al., 2010). Main NSCs exist in the optic tectum of both zebrafish and medaka, including the proliferative neuroepithelial-like stem (NE) cells and the quiescent RG cells (Alunni et al., 2010; Ito et al., 2010; Takeuchi and Okubo, 2013; Dambroise et al., 2017). However, medaka could not regenerate their optic tectum after stab injury and thus leaving a permanent scar (Shimizu and Kawasaki, 2021). In medaka, RG cells were similarly activated for proliferation upon tectum injury, but they failed to differentiate into neuron cells. Unlike the scenario in the retina, *sox2* is substantially expressed in both zebrafish and medaka optic tectum and does not associate with the differential regenerative ability. Instead, the expression of pro-regenerative transcriptional factors *ascl1a* and *oct4* were missing in the medaka. As a result, glial scar-like structures composed of GFAP+ radial fibers filled the injured area of the medaka optical tectum. Follow-up studies might be required to test the functional relevance of the ectopic expression of *ascl1a* and *oct4* in promoting RG differentiation and optic tectum regeneration in medaka.

Inter-Species Comparisons: Fin Regeneration

Among various tissues and organs, appendage regeneration draws major attention early on in the field as teleost fish, urodeles, and amphibians all can regenerate their fins, arms, and legs following amputation (Daponte et al., 2021). Compared to limbs of urodeles and amphibians, fish fin structure is simpler and consists of bony fin rays covered by thin epidermal cells (Grandel and Schulte-Merker, 1998). Nevertheless, limb and fin are homologous tissue across vertebrate species (Yano and Tamura, 2013). A forward-genetic screen done in zebrafish identified a novel and conserved regulator of appendage patterning. When mutated, zebrafish formed limb-like bones in fins, suggesting the conservation in skeleton development and the potential of fin-to-limb transition (Hawkins et al., 2021). The regenerative capacity of the fish fin was examined as early as the 1700s to understand appendage regeneration with

the first reports by French naturalist Broussonet (Broussonet, 1786; Broussonet, 1789). Like the limb regeneration in urodeles and amphibians, zebrafish repair their caudal fin by blastema-mediated epimorphic regeneration (Poss et al., 2003). In this context, blastema originates from dedifferentiated mesenchymal cells and is the primary source for growing new tissues, including bone, nerve, and vessel (Poss et al., 2003; Straube and Tanaka, 2006; Pfefferli and Jazwinska, 2015). Conserved pathways, including the Wnt/β-catenin pathway, were shown to regulate appendage regeneration across different vertebrate species, including zebrafish, Xenopus, and axolotl (Kawakami et al., 2006; Yokoyama et al., 2007).

Like zebrafish, medaka also regenerates their fin after amputation via blastema-mediated epimorphic regeneration (Katogi et al., 2004; Nakatani et al., 2007). It will be interesting to learn if medaka fin regeneration shares a conserved regenerative program with zebrafish in a cross-species study. In addition, inflammation and macrophages play a central role in both heart and fin regeneration in zebrafish but seem deficient/blunt in medaka hearts upon injury (Petrie et al., 2014; Lai et al., 2017). One may wonder whether systemic inflammation and immune response contribute differently upon fin and heart injury in medaka, which will be further discussed and await future investigation.

Overall, all these studies highlight the strength of inter-species comparisons between zebrafish (regenerative) and medaka (non-regenerative) organs to identify (and in some cases also to validate) the potential triggers of tissue regeneration. It is worth mentioning that other comparisons of organisms/conditions exhibiting diverse regenerative capacity have also been reported. For example, Stockdale and colleagues identified genes fundamental to heart regeneration by comparing the injury response of regenerative Astyanax mexicanus surface fish with their non-regenerative counterparts Pachón cave-dwelling fish (Stockdale et al., 2018). Following such regenerative traits among other species, researchers have also explored the evolutionary concept of regeneration amongst other teleost species (**Table 1**). In addition, comparing the transcriptomes and open chromatin landscapes of the cardiac cells isolated from the regenerative neonatal vs. non-regenerative adult mice hearts, Wang Z. et al. (2020) revealed the gene regulatory networks in diverse cardiac cell types and extracellular mediators for cardiomyocyte proliferation, angiogenesis, and fibroblast activation. Furthermore, mammals like the African spiny mouse (*Acomys*) can regenerate their ear, skin, heart, and bones in contrast to the house mouse *(Mus musculus),* providing more opportunities for cross-species analyses (Seifert et al., 2012; Matias Santos et al., 2016; Simkin et al., 2017; Qi et al., 2021).

Comparing organisms with similar regenerative properties may also identify the conserved regenerative programs. For example, from an inter-species comparison of zebrafish and African killifish *(Nothobranchius furzeri)* following fin amputation, Wang and colleagues identified the evolutionary conserved regenerative response elements (RRE) (Wang W. et al., 2020). Activation of *inhba*, a gene downstream of the RRE, is essential for both fin and heart regeneration and requires the binding motifs of activator protein 1 (AP-1) complex. Such enhancer is also present in mammals, shares Ap-1 binding motifs, and responds to injury, although it cannot promote regeneration. These results suggest that RREs might have been repurposed in regeneration-incompetent animals during evolution and only promote tissue repair but not regeneration (Yang and Kang, 2019; Wang W. et al., 2020). Of note, killifish possess the regenerative capacity in multiple organs, including the heart (Wang W. et al., 2020), fin (Wendler et al., 2015), and brain (Van Houcke et al., 2021) while being phylogenetically

TABLE 1 | Fish models and organs for regeneration research.

Species	Organs
Zebrafish (*Danio rerio)*	Heart (Poss et al., 2002) Retina (Vihtelic and Hyde, 2000; Sherpa et al., 2008) Brain (Kroehne et al., 2011; Marz et al., 2011; Kishimoto et al., 2012) Fin (Poss et al., 2003) Kidney (Diep et al., 2011) Liver (Sadler et al., 2007) Pancreas (Moss et al., 2009) Notochord (Garcia et al., 2017; Lopez-Baez et al., 2018) Lateral line (Hair cells) (Lush and Piotrowski, 2014; Cruz et al., 2015) Gills (Mierzwa et al., 2020) Intestine (Schall et al., 2015) Spinal cord (Becker et al., 1997; Ghosh and Hui, 2018)
Giant Danio (*Devario aequipinnatus*)	Heart (Lafontant et al., 2012) Lateral Line (Mekdara et al., 2018)
Goldfish (*Carassius auratus*)	Heart (Grivas et al., 2014) Retina (Raymond et al., 1988) Fin (Jh, 1947; Darnet et al., 2019) Spinal cord (Bernstein, 1964)
Grass carp (*Ctenopharyngodon idella*)	Heart (Long et al., 2019) Gonads (Underwood et al., 1986)
Medaka (*Oryzias latipes*)	Heart (Ito et al., 2014; Lai et al., 2017) Retina (Lust and Wittbrodt, 2018) Brain (optic tectum) (Shimizu and Kawasaki, 2021) Caudal Fin (Katogi et al., 2004) Kidney (Watanabe et al., 2009) Liver (Van Wettere et al., 2013) Pancreas (Otsuka and Takeda, 2017) Notochord (Seleit et al., 2020) Lateral Line (Seleit et al., 2017b) Posterior lateral line (pLL) nerve (Seleit et al., 2022) Gill (Stolper et al., 2019)
African Killifish (*Nothobranchius furzeri)*	Fin (Wendler et al., 2015) Heart and fin (Wang W. et al., 2020) Brain (Van Houcke et al., 2021)
Platyfish (*Xiphophorus maculatus*)	Fin (Offen et al., 2008)
Atlantic Salmon (*Salmo salar L.*)	Heart (Ferguson et al., 2005) Skin (Sveen et al., 2019)
Mexican cave/surface fish (*Astyanax mexicanus*)	Heart and Fin (Stockdale et al., 2018)
Senegal bichir *(Polypterus senegalus)*	Heart (Kikuchi et al., 2011b) Pectoral Fin (Cuervo et al., 2012)

TABLE 2 | Injury models developed in zebrafish and medaka.

Tissue type	Injury Type	Zebrafish references	Medaka references
Heart	Resection	Poss et al., 2002	Ito et al., 2014
	Cryoinjury	Chablais et al., 2011; Gonzalez-Rosa et al., 2011; Schnabel et al., 2011	Lai et al., 2017
	Genetic ablation	Cardiomyocytes (Wang J. et al., 2011)	Not available
Fin	Resection	Géraudie et al., 1994; Poleo et al., 2001	Katogi et al., 2004
	Cryoinjury	Chassot et al., 2016	Not available
Retina	Light, Laser	Vihtelic and Hyde, 2000; DiCicco et al., 2014	Lust and Wittbrodt, 2018
	Stabbing	Senut et al., 2004	
	Chemical	Fimbel et al., 2007; Powell et al., 2016	Not available
Brain	Stabbing	Kroehne et al., 2011; Marz et al., 2011	Shimizu and Kawasaki, 2021
	Traumatic Brain Injury	Maheras et al., 2018	Not available
	Genetic ablation	Hypocretin Neurons (Elbaz et al., 2012); Radial glial specific (Shimizu et al., 2015); dopaminergic neuron-specific (Godoy et al., 2015)	Not available
Spinal Cord	Transection	Becker et al., 1997	Not available
Bone	Genetic ablation	Osteoblasts (Singh et al., 2012)	Osteoblasts (Willems et al., 2012)
Notochord	Laser	Goldstein and Fishman, 1998	Seleit et al., 2020
	Stabbing	Lopez-Baez et al., 2018	Not available
	Genetic ablation	Vacuolated cells (Garcia et al., 2017)	Not available
Lateral Line	Chemical	Harris et al., 2003	Not available
	Laser Ablation	Schuck and Smith, 2009; Cruz et al., 2015	Seleit et al., 2017b, 2022
Kidney	Chemical	Reimschuessel and Williams, 1995	Watanabe et al., 2009
Liver	Chemical	Cox et al., 2014	Van Wettere et al., 2013
	Resection	Sadler et al., 2007	Not available
	Genetic ablation	Hepatocytes (Curado et al., 2007)	Not available
Gills	Resection	Mierzwa et al., 2020	Stolper et al., 2019
	Cryoinjury	Ramel et al., 2021	Not available
Pancreas	Genetic ablation	Beta cells (Pisharath et al., 2007)	Beta cells (Otsuka and Takeda, 2017)
Intestine	Resection	Schall et al., 2015	Not available

closer to medaka (Terzibasi et al., 2007), represent an alternative model for inter-species comparison.

Inter-Organ Comparisons in Medaka and Zebrafish (Regenerative Programs, Regulatory Elements, and Systemic Immune Responses)

In addition to the availability of progenitor/stem cell populations in each tissue/organ, we wonder how systemic responses, including immune response, neural innervation, hormonal regulation, metabolic shift, contribute differently to the respective injured tissues/organs of the same organism and lead to uneven regenerative capacity. The problem is especially apparent when one teleost zebrafish can regenerate organs such as the heart, retina, and brain, while another teleost medaka cannot. Taking the heart for example, the blunt immune response seems to be the major obstacle for medaka to initiate regenerative programs, but how does the same systemic (immune) response sustain the regeneration of other organs, for example, the fin? Are there tissue-specific contributions/responses, for example, residential immune cells, tissue-specific injury response elements, or even changes in the epigenomic landscape? These questions may be best addressed in medaka where the organ-specific regenerative capacities are uneven and well studied, including the regenerative fin (Katogi et al., 2004), kidney (Watanabe et al., 2009), liver (Van Wettere et al., 2013), and pancreas (Otsuka and Takeda, 2017), and non-regenerative heart (Ito et al., 2014; Lai et al., 2017), retina (Lust and Wittbrodt, 2018), and brain (Shimizu and Kawasaki, 2021). The potential findings can be cross-species compared and further validated in zebrafish loss-of-function and medaka gain-of-function experiments. A similar concept could also apply to other species. Differential regenerative capacity can be observed in lower vertebrates where some lizards can replace their tail but not their limb (Alibardi and Toni, 2005). Even in mice, the uneven regenerative capacity exists ranging from active (intestine and skin), partial (liver, pancreas, muscle), to none (CNS, heart, and most other internal organs) (Iismaa et al., 2018; **Figure 1**).

Even between two regenerative organs, researchers have identified common and tissue-specific regeneration responsive elements/enhancers by inter-organ comparisons. For example, Kang et al. (2016) have performed such a comparative study identifying a tissue regeneration enhancer element (TREE) from zebrafish heart and fin, which locates upstream of *lepb* gene and activates following injury. This element could also be activated in neonatal mouse tissues upon injury and may be engineered to modulate the regenerative potential of vertebrate organs. Another study by Pfefferli and Jaźwińska (2017) identified a 3.18 kb regulatory element upstream of *ctgfa* gene, named as *careg* element that drives the regenerative response in both zebrafish

TABLE 3 | Visualization tools developed in zebrafish and medaka.

Tissue type	Cell-type	Zebrafish transgenic lines (References)	Medaka transgenic lines (References)
Blood vessels	Pan-endothelial cells	*Tg(tie2:EGFP)* (Motoike et al., 2000) *Tg(fli1a:EGFP)*^{y1} (Lawson and Weinstein, 2002)	*Tg(tie2:GFP)* (Nakatani et al., 2008) *Tg(fli1:GFP)* (Moriyama et al., 2010)
	Arterial- endothelial cells	*Tg(kdrl:mCherry)*^{is5} (Wang et al., 2010)	*Tg(kdrl:DsRed2)* (TG1252, NBRP)
Lymphatic vessels	Pan-lymphatic cells	*Tg(lyve1:DsRed2)*^{nz101} (Okuda et al., 2012) *TgBAC(flt4:Citrine)*^{hu7135} (Gordon et al., 2013) *Tg(mrc1a:egfp)*^{y251} (Jung et al., 2017)	*Tg(flt4-EGFP)* (Deguchi et al., 2012)
Heart cells	Pan- cardiomyocytes	*Tg(cmlc2:DsRed2-Nuc)* (Rottbauer et al., 2002) *Tg(myl7:EGFP)*^{twu26} (Huang et al., 2003)	*Cab-Tg(zfmlc2-5.1k:DsRed2-nuc)* (Taneda et al., 2010); Anti-MyHC (MF20) (Ito et al., 2014)
	Dedifferentiating-cardiomyocytes	*Tg(gata4:EGFP)*^{ae1} (Kikuchi et al., 2010) *TgBAC(nppa:mCitrine)* (Honkoop et al., 2019)	Not available
	Epicardium	*Tg(wt1b:GFP)* (Perner et al., 2007) *Tg(tcf21:nucEGFP)*^{pd41} (Wang J. et al., 2011)	Not available
	Endocardium	*Tg(flt1:YFP)*^{hu4624} (Hogan et al., 2009) Anti-Raldh2 (Kikuchi et al., 2011b)	*Tg(raldh2-GFP)* (Ito et al., 2014)
Muscles and whole body	Skeletal muscles	*Tg(mylz2:gfp)* (Ju et al., 2003)	*Tg(mylz2:gfp)* (Zeng et al., 2005)
	Ubiquitous	*Tg(actc1b:GFP)* (Higashijima et al., 1997) *Tg(bactin2:switch)* (Bertrand et al., 2010) *Tg(-3.5ubi:EGFP)* (Mosimann et al., 2011)	*Tg(pOBA-GFP)* (Hamada et al., 1998) *Tg(EF-1α-A-GFP)* (Kinoshita et al., 2000) *Tg(CMV-EGFP-ITR)* (Chou et al., 2001) *Tg(β-actin-EGFP-ITR)* (Chou et al., 2001) *Wimbledon* (Centanin et al., 2011) Gaudi Toolkit (Centanin et al., 2014)
Skin	Epithelial cells	*Tg(krt4:nlsEGFP)*^{cy34} (Chen et al., 2011)	*Tg(krt8:rfp)* (Zeng et al., 2005) *Tg(K15:H2B-EGFP)* (Seleit et al., 2017a,b) *Tg(K15:H2B-EGFP)* and *Tg(K15:LifeAct-tRFP)* *(Seleit et al., 2022)*
Fibroblasts	Activated fibroblasts	*Tg(postnb:citrine)*^{cn6} (Sanchez-Iranzo et al., 2018)	Not available
	Collagen producing fibroblasts	*Tg(col1a2:loxP-mCherry-NTR)*^{cn11} (Sanchez-Iranzo et al., 2018)	Not available
	Fibroblasts	*Tg1(-6.8wt1a:EGFP)*^{li7Tg} (Bollig et al., 2009)	Not available
Immune cells	Macrophages	*Tg(mpeg1:EGFP)*^{gl22} (Ellett et al., 2011) *Tg(mpeg1.4:mCherry-F)*^{ump2} (Bernut et al., 2014) *Tg(mfap4:tdTomato-CAAX)*^{xt6} (Walton et al., 2015)	*Tg(mpeg1:mCherry)* (Phan et al., 2020) Isolectin B4 (Lai et al., 2017)
	Mononuclear phagocyte system	*Tg(ptprc:DsRed)*^{sd3} (Bertrand et al., 2008) *Tg(mhc2dab:GFP)*^{sd6} (Wittamer et al., 2011)	*Tg(Cxcr3a:GFP)* (Aghaallaei et al., 2010)
	Pro-inflammatory cells	*Tg(tnfa:EGFP-F)*^{ump5Tg} (Nguyen-Chi et al., 2015) *Tg(irg1:EGFP)* (Sanderson et al., 2015)	Not available
	Neutrophils	*TgBAC(mpx:GFP)*^{i114} (Renshaw et al., 2006)	*Tg(FmpoP::EB3-EGFP/FmpoP::RFP-Lifeact)* (Crespo et al., 2014) *Tg(FmpoP::mCherry)* (TG1044, NBRP)
	T-cells	*Tg(lck:lck-EGFP)*^{cz2} (Langenau et al., 2004) *Tg(ikzf1:GFP)*^{fr24} (Bajoghli et al., 2009)	*Tg(lck:gfp)* (Bajoghli et al., 2015)
	T-regulatory cells	*TgBAC(foxp3a:EGFP)* (Hui et al., 2017)	Not available
	Progenitors and thymocytes	*Tg(rag1:GFP)* (Jessen et al., 1999) *Tg(rag2:GFP)* (Jessen et al., 2001)	*Tg(rag1-egfp)* (Li et al., 2007) *Tg(ccr9a:gfp)* and *Tg(rag2:gfp-pest)* (Bajoghli et al., 2015)
	B-cells	*Tg(Cau.Ighv-ighm:EGFP)*^{sd19} (Page et al., 2013) *Tg(cd79a:GFP)* and *Tg(cd79b:GFP)* (Liu et al., 2017)	Not available
Blood	Erythrocyte	*Tg(gata1:DsRed)*^{sd2} (Traver et al., 2003) *Tg(runx1P1:EGFP) and Tg(runx1P2:EGFP)* (Yi Ni Lam et al., 2009)	*Tg(fli::GFP;gata1::GFP)* (Schaafhausen et al., 2013)
	Thrombocytes	*Tg(CD41:GFP)* (Lin et al., 2005)	Not available
Eyes	Rod cells	*Tg(XlRho:EGFP)*^{fl1} (Fadool, 2003)	Not available
	Müller glia cells	*Tg(gfap:EGFP)*^{nt11} (Thummel et al., 2008)	*Tg(rx2:H2B-RFP)* (Inoue and Wittbrodt, 2011) *Tg(rx2:lifeact-EGFP)* and *Tg(rx2:H2B-EGFP)* (Lust and Wittbrodt, 2018)
	Photoreceptor	*Tg(-5.5opn1sw1:EGFP)*^{kj9} (Takechi et al., 2003)	
	Retinal pigment epithelium (RPE)	*Tg(rpe65a:EGFP)* (Collery et al., 2016)	

(Continued)

TABLE 3 | (Continued)

Tissue type	Cell-type	Zebrafish transgenic lines (References)	Medaka transgenic lines (References)
CNS	Pan-neurons	*Tg(huC:GFP)* (Park et al., 2000)	*Tg(kif5a:gfp)* (Kawasaki et al., 2012)
	Radial glial cells	*Tg(gfap:GFP)*$^{mi200-1}$ (Raymond et al., 2006) *Tg(cyp19a1b:cyp19a1b-GFP)* (Tong et al., 2009)	Anti-Gfap immunostaining, *Tg(cyp19a1b-GFP)* (Takeuchi and Okubo, 2013)
	Neural stem cells	*Tg(-1.7Cau.Tuba1:GFP)* (Goldman et al., 2001)	*Tg(rx2::H2B-RFP)* (Inoue and Wittbrodt, 2011; Reinhardt et al., 2015) *Tg(wdr12:GFP)* (Dambroise et al., 2017) *Tg(cndp::eGFP-caax)* (Becker et al., 2021)
	Oligodendrocyte	*Tg(olig2:DsRed2)* and *Tg(sox10:mRFP)* (Kucenas et al., 2008)	Not available
Notochord	Sheath cells	*Tg(col9a2:GFPCaaX)*^{pd1151} (Garcia et al., 2017)	*Tg(desmogon:EGFP)* (Seleit et al., 2020)
	Vacuolated cells	*Tg(col8a1a:GFPCaaX)* (Garcia et al., 2017)	
	Intervertebral disk	*Tg(twhh:gfp)* (Du and Dienhart, 2001)	Not available
Lateral Line	Neuromast	*Tg(brn3c:GAP43-GFP)*^{s356t} (Xiao et al., 2005)	*Tg(eya1:EGFP), Tg(eya1:mECFP), Tg(K15:H2B-EGFP),* and *Tg(K15:H2B-RFP)* (Seleit et al., 2017a,b) *Tg(K15:LifeAct-tRFP)* (Seleit et al., 2022)
Fin and Bone	Osteoblasts and precursors	*Tg(sp7:EGFP)*^{b1212} (DeLaurier et al., 2010)	*Tg(osx-mCherry)* (Renn and Winkler, 2009) *Tg(col10a1:nlGFP)* (Renn et al., 2013)
	Osteoclasts	*TgBAC(ctsk:Citrine)* (Bussmann and Schulte-Merker, 2011)	*Tg(ctsk:mEGFP)* (To et al., 2012) *Tg(TRAP:GFP)* (Chatani et al., 2011)
Pancreas	Pancreatic endocrine cells	*Tg(-6.5pdx1:GFP)* (Huang et al., 2001) *Tg(-8.5nkx2.2a:GFP)* (Zecchin et al., 2007) *Tg(-4.0ins:GFP)* (Huang et al., 2001)	*Tg(pdx1-EGFP)* (Otsuka et al., 2015) *Tg(insulin-EGFP-NTR)* (Otsuka et al., 2015)
	Pancreatic exocrine cells	*Tg(elaA:gfp)* (Wan et al., 2006)	*Tg(ptf1a-mCherry)* (Otsuka et al., 2015)
Liver	Hepatocytes	*Tg(-2.8fabp10a:EGFP)* (Her et al., 2003) *Tg(-1.7apoa2:GFP)* (Wang R. et al., 2011)	*Tg(chg-L1.5 kb/GFP-emgb/RFP)* (Ueno et al., 2004)

fin and heart via TGFβ/Activin-β signaling pathway. This type of study paved the way for identifying evolutionarily conserved RREs, which can also be analyzed in medaka to decipher how these RREs exist and regulate the regenerative programs in a tissue-specific manner.

MATERIALS AND METHODS AVAILABLE FOR COMPARATIVE STUDY IN ZEBRAFISH AND MEDAKA

Here, we highlight the materials and methods selected from published studies to accelerate comparative studies in tissue regeneration using zebrafish and medaka, including the injury methods, visualization of gene expression and specific cell types, and functional assays by drug delivery and genetic modifications. Online resources and new experimental models will also be summarized in this section.

Injury Models

Various injury models have been established in fish models to introduce tissue injury and investigate the reparative process. In addition to the feasibility and reproducibility, these methods were often established based on the similarity to the human diseases or trauma conditions to gain translational value (**Table 2**). The resection or amputation model is one of the most commonly used injury models, involving surgical removal of a part of the tissue for observing the restoration of size, morphology/structure, and function of the injured tissue. Resection is straightforward, cheap, and reproducible and has been widely adopted to fin (Poss et al., 2003; Nakatani et al., 2007) and heart (Poss et al., 2002; Ito et al., 2014) regeneration studies in both zebrafish and medaka. However, resection is often accompanied by excessive bleeding and open wounds, which leads to infection and high mortality. Also, resection is sometimes infeasible for internal organs or tissues those unexposed or too small. Similar models include stabbing and transection, which works by surgically disrupting the integrity without removing any tissue and are well established in the retina, brain, and spinal cord regeneration in both zebrafish (Becker et al., 1997; Marz et al., 2011; Shimizu et al., 2018) and medaka (Shimizu and Kawasaki, 2021).

Slightly different from resection, researchers adapted the cauterization method in fish models to mimic the fibrotic repair in mammalian organs, which is highly associated with the inflammatory response (Strungs et al., 2013; Polizzotti et al., 2016). The cauterization technique involves burning or freezing the target tissue with electric or metal probes and introducing necrotic and apoptotic cell death. Cryoinjury is popular in heart regeneration studies in both zebrafish (Gonzalez-Rosa et al., 2011; Schnabel et al., 2011; Dyck et al., 2020) and medaka (Lai et al., 2017) as it mimics the myocardial infarction in mammals better than resection model (Chablais et al., 2011; Darehzereshki et al., 2015). This technique can also be applied to external organs, such as the fin (Chassot et al., 2016) and gills (Ramel et al., 2021). However, cauterization is technically challenging and less reproducible compared to resection. Also, the wound usually takes a longer time to recover as the cell debris needs to be cleared before regeneration occurs (Schnabel et al., 2011). To increase the

accuracy and reproducibility, cauterization can also be performed by using high-powered lasers in fish tissues, including the retina (Conedera et al., 2017; Lust and Wittbrodt, 2018) and skin (Richardson et al., 2013). The target tissue has to be exposed or transparent for laser penetration in this case. Overall, most physical injuries are invasive to cause high mortality and take practice to be consistent.

Apart from physical injury models, genetic cell ablation models in fish were established by expressing enzymes that catabolize cytotoxic products added to the system or induce cell death directly (**Table 2**). The former method was developed by expressing the bacterial enzyme Nitroreductase (NTR), which alone is not toxic but can catabolize the prodrug metronidazole (Mtz) to induce cytotoxicity (Lindmark and Müller, 1976). This system can achieve spatial (tissue-specific expression of NTR) and temporal control (the timing of adding Mtz), and labeling the target cells (co-expression with reporter system) at the same time. NTR/Mtz system has been used to tease out the functions of specific cell types in a complex process of organ regeneration in zebrafish, including the heart (Curado et al., 2007; Wang et al., 2013; Zhang et al., 2013), fin (Petrie et al., 2014), pancreatic β-cells (Pisharath et al., 2007), bone (Willems et al., 2012) and RPE regeneration (Hanovice et al., 2019). This system is also applicable to medaka, demonstrated by accessing regeneration capacity using NTR/Mtz mediated genetic ablation of the pancreatic β-cell population (Otsuka and Takeda, 2017), osteoblasts (Willems et al., 2012), and bone progenitor cells (Dasyani et al., 2019) in fin regeneration.

The latter genetic ablation tool to study zebrafish development and regeneration involves diphtheria toxin A (DTA) expression under a tissue-specific promoter, exampled by *crystallin* promoter-driven DTA expression in lens (Kurita et al., 2003), *elastase A* promoter-driven DTA expression in exocrine pancreas (Wan et al., 2006), and *myl7* promoter-driven DTA expression in cardiomyocytes (Wang J. et al., 2011). Though the DTA approach lacks temporal control for activation, it is highly toxic for killing the target cells efficiently. In a modified method, the temporal control can be achieved by expressing the human diphtheria toxin receptor (DTR) and further activate cytotoxicity by diphtheria toxin injection (Jimenez et al., 2021). The genetic ablation models are technically simpler with faster recovery. These models can be used combined with physical injury to tease the role of a specific cell type during complex organ regeneration. For example, Sanchez-Iranzo et al. (2018) depleted fibroblast cells after cardiac cryoinjury and demonstrated how fibroblasts contribute to heart regeneration. Overall, most of these injury models developed in zebrafish may also apply to medaka in comparative studies of tissue regeneration.

Visualization Tools

One of the best attributes of using zebrafish and medaka as research models is the tools and techniques available for visualizing specific cell types and biological processes *in vivo*. Here, we summarize the visualization tools that have been established in fish models, especially in zebrafish and correspondingly in medaka, with a focus on the transgenic reporter lines (**Table 3**) and alternative approches.

Since fluorescence reporter driven by tissue-specific gene promoter is applicable and efficient in zebrafish and medaka, many transgenic lines have been generated to study specific tissues/organs in development and diseases. Here we summarize tissue-specific reporters described in major tissue regeneration studies previously mentioned in **Table 3**. In addition to tissue-specific reporter lines, researchers can also use antibodies against cell-specific transcription factors or cytosolic proteins to assess cellular dynamics in growth, development, and regeneration. For example, Mef-2 (sc-313), nkx2.5 (GTX128357) or MF20 (Fischman, D.A., DSHB) antibody can be used in combination with proliferation/cell cycle markers PCNA (GTX124496), Anti-phospho-Histone H3 (Ser10) (06-670, Merck), and BrdU/EdU (C10086, ThermoFisher, Eugene, OR, United States) to label the proliferating CMs (Chablais et al., 2011; Chablais and Jaźwińska, 2012a). Similarly, transgenic medaka lines have also been generated to facilitate cardiac research using zebrafish *cmlc2* regulatory elements for myocardial expression (Taneda et al., 2010). In addition to labeling cardiac tissues, researchers have successfully developed medaka reporters utilizing zebrafish skeletal specific *mylz2* promoter to label skeletal muscles (Zeng et al., 2005). Vice versa, medaka *mylz2* promoter can also recapitulate GFP expression in zebrafish (Zeng et al., 2005). On the same note, medaka β-*actin* promoter can drive ubiquitous gene expression in both medaka and zebrafish (Yoshinari et al., 2012), while zebrafish *krt8* promoter can label both skin and intestinal epithelium in medaka, as almost identical to zebrafish (Zeng et al., 2005). These examples showed that the transcriptional regulation of many genes is highly conserved in both species, with many tissue-specific transgenes developed to facilitate research, as summarized in **Table 3**.

Restoring vasculature and circulation is one of the first steps during tissue regeneration (Jung and Kleinheinz, 2013). Both these blood vessels and lymphatic vessels that regulate tissue homeostasis and immune cell trafficking can be visualized by reporters and have been used extensively for regenerative studies in zebrafish (**Table 3**). To name a few of the most commonly used reporter lines, *tie2* reporter for pan-endothelial cells (Motoike et al., 2000), *fli1a* reporter for endothelial and endocardial cells (Lawson and Weinstein, 2002), *kdrl* reporter for arterial vessels (Wang et al., 2010), and *lyve1* (Okuda et al., 2012) reporter for lymphatic vessels in zebrafish. Correspondingly, a wide range of transgenic reporter strains has been generated in medaka based on orthologous gene promoters (**Table 3**). Moreover, one can also use staining methods to label the vasculature when transgenic animals are inaccessible. One of the widely adopted vasculature staining methods involves alkaline phosphatase (AP) staining, which relies on the endogenous AP activity to convert NBT/BCIP into purple precipitates in endothelial cells for rapid visualization in larvae (Childs et al., 2002) and heart (Lai et al., 2017). In addition, the Fli1 antibody (ab133485) can be used to mark endothelial cell nuclei in zebrafish by immunostaining (Bensimon-Brito et al., 2020). Aside from post-fixation staining, angiography can be achieved by fluorescent dextran injection into the circulation of larval (Hoeppner et al., 2015; Takanezawa et al., 2021) and adult fish (Pugach et al., 2009).

Immune response, especially inflammatory cell infiltration and resolution, is a critical component of tissue regeneration to prevent infection, clear damaged tissue, maintain tissue integrity, and sometimes even is associated with the fibrotic response and cell proliferation (Julier et al., 2017). On top of the tremendous capacity in regeneration, zebrafish possess both innate and adaptive immunity comparable to mammals (Trede et al., 2004), making it a powerful model to study the role of immune response in tissue repair and regeneration (Var and Byrd-Jacobs, 2020). Inflammatory cells, including neutrophils and macrophages, are among the first responders recruited to the injured tissue by chemokines and damage-associated molecular patterns (DAMPs) (McDonald et al., 2010; Soehnlein and Lindbom, 2010). Some of the zebrafish reporter lines have been used to visualize these innate immune cells, including fluorescent genes expression driven by neutrophil-specific *mpx* promoter (Renshaw et al., 2006) and macrophages specific *mpeg1.1/mpeg1.4* (Ellett et al., 2011) and *mfap4* (Walton et al., 2015) promoters. Taking advantage of the transparent tissue at the larval stage, zebrafish have been extensively used for studying the dynamic and function of these inflammatory cells in tissue repair/regeneration (Li et al., 2012). Apart from the innate immune system, some adaptive immune cell reporters were also established in zebrafish (**Table 3**). Materials for zebrafish immune research have also been previously reviewed (Martins et al., 2019). The immune system in medaka is less studied compared to zebrafish. Still, some immune cell reporters have been generated in medaka based on zebrafish orthologous genes (**Table 3**), including neutrophil-specific *mpo* reporter (alias to *mpx*) (Grabher et al., 2007; Crespo et al., 2014), macrophages specific *mpeg1.1* reporter (Phan et al., 2020), and pan mononuclear phagocytes *cxcr3.2* reporter (Aghaallaei et al., 2010). For the adaptive immune cells, medaka gained interest for studying T-cell development where Tg lines were developed respectively (Bajoghli et al., 2019). Given the importance of immune response in tissue repair/regeneration and the amount of knowledge gained in zebrafish, it is pretty evident that corresponding transgenic reporter lines in medaka await future development for comparative studies. Specifically, it would be interesting to learn more about the critical roles of immune cells that plays similarly or differently in these two model systems.

Due to the limited resource of antibodies against fish proteins and reporters for labeling immune cells, other approaches can be applied to label and even isolate immune cells. For example, isolectin B4 (IB4) and liposome-uptake may label macrophage and other phagocytes in both zebrafish and medaka. In a comparative study, IB4 labels mainly macrophages in zebrafish and medaka and show colocalized signals with zebrafish mpeg1 reporter signals (Lai et al., 2017). On the other hand, DiI liposomes can label the phagocytes efficiently in both zebrafish and medaka based on their properties of macrophage ablation when loaded with clodronate (Lai et al., 2017). Despite in limited numbers, some antibodies work in both fish models in labeling the immune cells, including Lcp1 (GTX124420) (Redd et al., 2006), Lyz (GTX132379) for leukocytes, Spi/Pu.1 for myeloid cells (GTX128266), Mpx (GTX128379) for neutrophils (Lai et al., 2017), Mpeg1 (GTX54246) for macrophages (Simoes et al., 2020), and anti-4C4 for microglia (Becker and Becker, 2001).

Here, we have tabulated the most widely used fish reporter and transgenic lines in **Table 3**. More transgenic lines that label different tissues can be easily looked up in the Zebrafish Information Network (ZFIN[1]) and accessed from the Zebrafish International Resource Center (ZIRC[2]) and the European Zebrafish Resource Center (EZRC[3]), or the NBRP Medaka website[4]. Overall, common regulatory elements between zebrafish and medaka support that the activation of zebrafish-specific factors can be well recapitulated in the medaka and vice-versa. This evidence further highlights the conserved gene-regulatory networks between zebrafish and medaka, making them excellent models to perform comparative studies in tissue regeneration.

Genetic Manipulations in Zebrafish and Medaka

Taking advantage of being model organisms and a broad research community, zebrafish and medaka are well-equipped with tools for genetic manipulations for generating animal models for specific cell ablations, visualization, and functional manipulations introduced previously. Here, we summarize these toolsets established in zebrafish and medaka (**Table 4**) to investigate the cellular and molecular mechanisms of tissue regeneration.

Genetic manipulation in forward genetic screening has been established in fish models early on (Driever et al., 1996; Wienholds et al., 2003). As a standard method, ENU (ethylnitrosourea) treatment introduced point mutations via base alkylation that give rise to single base mutations (often called ENU mutants) in zebrafish (Driever et al., 1996) and medaka (Loosli et al., 2000; Furutani-Seiki et al., 2004). Lacking efficient methods for targeted gene mutagenesis used to be a weak spot of zebrafish reverse genetics, but was recently overcome by the invention of zinc-finger nucleases (ZFN) (Doyon et al., 2008), transcription activator-like effector nucleases (TALENs) (Huang et al., 2011; Bedell et al., 2012), and the Clustered, Regularly Interspaced, Short Palindromic Repeat (CRISPR)/CRISPR-associated 9 (Cas9) technology (Hruscha et al., 2013; Hwang et al., 2013). CRISPR became the favorable genetic manipulation strategy given its ease to generate and assemble, and application in almost all eukaryotic cells. In addition to gene knockout/mutagenesis, CRISPR technology was further modified for generating knock-in/transgenic zebrafish carrying reporter/functional genes under endogenous gene regulation (Kimura et al., 2014). Like zebrafish, genome editing using TALEN (Ansai et al., 2013, 2014) and CRISPR/Cas9 (Ansai and Kinoshita, 2014) have been established in medaka. CRISPR/Cas9 mediated gene knock-in via NHEJ was used to generate transgenic and mutant medaka with a high germline transmission rate (Watakabe et al., 2018). In addition, the knock-in method using CRISPR was also applied to generate conditional knockout zebrafish by targeted insertion of loxP sites

[1]https://zfin.org/

[2]https://zebrafish.org/home/guide.php

[3]https://www.ezrc.kit.edu/

[4]https://shigen.nig.ac.jp/medaka/

TABLE 4 | Genetic manipulation tools in zebrafish and medaka.

Tools	Zebrafish (References)	Medaka (References)
ENU (ethylnitrosourea) mutagenesis	Driever et al., 1996	Loosli et al., 2000; Furutani-Seiki et al., 2004
ENU tiling	Moens et al., 2008	Taniguchi et al., 2006
ENU screens	Kettleborough et al., 2013	Furutani-Seiki et al., 2004
ZFN (zinc-finger nucleases)	Meng et al., 2008	Ansai et al., 2012
TALENs (transcription activator-like effector nucleases)	Huang et al., 2011; Sander et al., 2011	Ansai et al., 2013, 2014
CRISPR/Cas9: NHEJ	Hruscha et al., 2013; Hwang et al., 2013	Ansai and Kinoshita, 2014
CRISPR/Cas9: HDR	Kimura et al., 2014	Murakami et al., 2017
Tol2 transposon system	Kawakami and Shima, 1999	Koga et al., 2002; Kawakami, 2007
TgBAC cloning	Suster et al., 2011	Nakamura et al., 2008
I-SceI meganuclease	Grabher et al., 2004	Thermes et al., 2002; Grabher and Wittbrodt, 2007
Frog Prince	Miskey et al., 2003	Sano et al., 2009
Ac/Ds system	Ng and Gong, 2011; Froschauer et al., 2012	Emelyanov et al., 2006
Sleeping Beauty	Davidson et al., 2003	Grabher et al., 2003
PhiC31	Mosimann et al., 2013	Kirchmaier et al., 2013
Morpholinos	Nasevicius and Ekker, 2000	Carl et al., 2002
Toolkits for transactivation	GENEWELD toolbox (Wierson et al., 2020)	Gaudi toolbox (Centanin et al., 2014)
Cre/loxP system	Thummel et al., 2005; Le et al., 2007	Okuyama et al., 2013
Gal4/UAS	Asakawa and Kawakami, 2008	Grabher and Wittbrodt, 2004
Tet system	Knopf et al., 2010	Hosoya et al., 2021
Mtz/NTR ablation	Curado et al., 2007	Willems et al., 2012
DTA ablation	Kurita et al., 2003	Not available
siRNA mediated transient knockdown	Xiao et al., 2018	Not available
Viral mediated transduction	Gulías et al., 2019	Suehiro et al., 2010

(Burg et al., 2018). Instead of NHEJ mediated knock-in which is error-prone, Wierson et al. (2020) have further developed knock-in method based on homology mediated end joining (HMEJ) repair for a more efficient and precise genome editing in zebrafish known as the GENEWELD method. Likewise, homology-directed repair (HDR) mediated knock-in strategies were also feasible in medaka (Murakami et al., 2017).

This concurrent development of technologies in zebrafish and medaka highlights the reciprocal nature of exchanging tools and methods between these model systems, facilitating the advancement of scientific research. Besides mutagenesis, genetic manipulation via stable transgenesis was first demonstrated in medaka (Ozato et al., 1986). Later, Kawakami and Shima (1999) identified the *Tol2* transposon system in Medaka and adapted this system in zebrafish for transgenesis which revolutionized the field. To better recapitulate the endogenous gene expression patterns, insertion of BAC constructs by *Tol2* transposase has been widely used in generating zebrafish reporter lines (Suster et al., 2011). Despite the fact that *Tol2* was originally identified in medaka, it is more efficient in zebrafish (Kawakami, 2007). Therefore, another method for insertional transgenesis was developed in fish models using I-SceI meganuclease and greatly facilitated transgenesis in medaka (Thermes et al., 2002; Grabher and Wittbrodt, 2008).

As applications, spatial (e.g., specific tissue) and temporal regulation of ectopic gene expression can be achieved by combining transgenic lines generated by the above-mentioned methods (**Tables 4**, **5**). For example, the Cre/lox system is widely used to perform reverse genetics, ectopic gene expression, and lineage tracing experiments in multicellular organisms, including zebrafish (Felker and Mosimann, 2016). To achieve spatial-temporal control, the Cre recombinase gene is fused with a human estrogen receptor (ER) domain and expressed under tissue-specific promoter, resulting in recombination of lox sequences in specific tissue upon estrogen stimulation (Metzger et al., 1995; Feil et al., 1996). Since then, there have been growing numbers of tissue-specific Cre lines generated in zebrafish (Jungke et al., 2013, 2015). Within the scope of tissue repair and regeneration, we have summarized a list of Cre driver and switch lines applied in previously described studies (**Table 5**). Additionally, robust co-expression of multiple genes following switch cassette can be accomplished by placing polycistronic ORFs separated by short viral 2A peptides (Provost et al., 2007). In contrast to zebrafish, there is way fewer medaka Cre/lox transgenic lines that we found and summarized with respect to the comprehensive list in zebrafish with available databases like CreZoo (Jungke et al., 2013, 2015). Fortunately, the advances of CRISPR technology allow genetic manipulation in both fish models and generate powerful tools for fate mapping and functional experiments (Liu et al., 2019). Overall, our review encompasses a portion of tools and strategies commonly used in zebrafish and medaka, which were extensively reviewed elsewhere for medaka (Kirchmaier et al., 2015) and zebrafish (Sassen and Köster, 2015), respectively.

Delivery of Pharmaceutical Reagents

Since zebrafish and medaka are routinely used for drug screening and validations, developing various routes of pharmaceutic administration is essential (**Table 6**). As these fish are tiny compared to mice, the development of administration methods requires further optimization to mimic the delivery route in mice.

Intraperitoneal (IP) injection was one of the first delivery methods introduced to deliver reagents in adult zebrafish and is adapted from veterinary practice in bigger fishes (Kinkel et al., 2010). The injection is performed using a 31G needle with a small injection volume (usually < 10–15 μl) into the abdominal cavity posterior to the pelvic girdle and midline to the pelvic fins in zebrafish (Kinkel et al., 2010). The reagents administrated by IP injection will distribute majorly to the spleen and liver within 72 h and later into the circulation, making it a favorable method for studying biochemical modulation in

TABLE 5 | The Cre drivers and switch lines for studying tissue regeneration.

Tissue type	Zebrafish transgenic lines (References)	Medaka transgenic lines (References)
Cardiomyocytes	*Tg(myl7:creERT2)* (Kikuchi et al., 2010) *Tg(gata4:creERT2)* (Kikuchi et al., 2011a)	Not available
Skeletal muscles	*Tg(cry:mCherry;-1.9mylz2:CreERT2)* (Mukherjee and Liao, 2018)	*Tg(myl2::nlsCreCherry)* (TG938, NBRP)
Endothelial cells	*Tg(fli1a:CreERT2)^{cn9}* (Sanchez-Iranzo et al., 2018)	Not available
Epicardial cells	*Tg(tcf21:CreERT2)^{pd42Tg}* (Kikuchi et al., 2011a)	Not available
Neuronal cells	*Tg(Cau.tuba1a:CreERT2, Cau.tuba1a:CFP)$^{mi19/+}$* (Ramachandran et al., 2010b)	*Tg(rx2::CreERT2)* (Reinhardt et al., 2015) Tg(*K15:Ert2-Cre)* (Seleit et al., 2017b) *Tg(cndp::CreERT2)* (Becker et al., 2021)
Macrophages	*Tg(mpeg:Cre)^{fh506}* (Roh-Johnson et al., 2017) *Tg(mfap4:iCre:p2A-tdTomato)^{xt8}* (Walton et al., 2015)	Not available
Fibroblast and collagen producing cells	*Tg(periostin:CreERT2)^{cn7} and Tg(wt1a:CreERT2)^{cn10}* (Sanchez-Iranzo et al., 2018)	Not available
Bone	*Tg(Ola.Sp7:CreERT2-P2A-mCherry)^{tud8}* (Knopf et al., 2011)	Not available
Heat-shock (temporal)	*Tg(hsp70l:mCherry, Cre-ERT2)^{tud104}* (Hans et al., 2011)	Gaudi$^{HspCRE.A}$ (Centanin et al., 2014)
Pan-cells switch type	*(ubi:Switch) and Tg(-3.5ubb:CreErT2, myl7:EGFP)^{cz1702}* (Mosimann et al., 2011) *Tg(bactin2:loxp-DsRed-STOP-loxp-EGFP)* (Kikuchi et al., 2010)	*GaudiRSG toolkit* (Centanin et al., 2014) *Tg(Olactb:loxP-dsR2-loxP-EGFP)* (Yoshinari et al., 2012)
Switch ablation line	*Tg(bactin2:loxP-mCherry-STOP-loxP-DTA176)^{pd36}* (Wang J. et al., 2011)	Not available

zebrafish over multiple injections or an extended period (Ruyra et al., 2014). IP is widely used for systemic administration of drugs, small-molecule inhibitors, nanoparticles, reagents in regeneration studies, including clodronate liposomes for ablating macrophages (de Preux Charles et al., 2016), poly I:C for immune-stimulation in medaka (Lai et al., 2017), mTOR-inhibitor rapamycin for autophagy inhibition (Chavez et al., 2020), IWR-1-endo for Wnt inhibition (Chen et al., 2009; Zhao et al., 2019), and tamoxifen for Cre-mediated recombination (Hans et al., 2009).

On the other hand, intravenous (IV) injection is effective for drug delivery in mice but has been technically challenging for zebrafish, due to the small vessel diameter and poor vasculature visibility. To improve vessel visibility, transparent *casper* fishes can be injected intravenously through their cardinal vein described in cancer research in zebrafish (Benjamin and Hynes, 2017). As alternative methods for direct access to the circulation, the intra-cardiac injection has been tested but showed high mortality (White et al., 2008), while the retro-orbital (RO) injection is suitable for delivering both reagents and cells into the blood circulation (Pugach et al., 2009; Simoes et al., 2020).

For more tissue/organ-localized delivery methods, intra-tissue injections may be applied. For example, intrathoracic (IT) injections were developed in zebrafish to test the effects of exogenous factors on adult heart regeneration, including nanoparticles encapsulated siRNAs (Xiao et al., 2018; Bise and Jazwinska, 2019). Cerebroventricular microinjection (CVMI) was also developed to deliver reagents to the adult zebrafish brain (Kizil and Brand, 2011). Intravitreal injections for the targeted delivery to the vitreous space of the retina (Fimbel et al., 2007) and intraspinal injection to the spinal cord were also developed in zebrafish (Wehner et al., 2018).

In contrast to invasive methods, which sometimes lead to tissue damage and mortality, a straightforward and convenient way for pharmaceutic delivery is immersion/incubation. Although immersion/incubation can be applied to both larvae and adults, it is more costly for incubating adults due to a large amount of reagent needed to reach the same dose. The biodistribution from immersion varies among reagents and different stages. In adults, the reagent is mainly intake through gills and ingestion, then absorbed/digested in the intestine and liver, which resembles oral uptake in the mice model (Ruyra et al., 2014). Examples of incubation experiments include tamoxifen treatment for Cre activation and Erbb2 inhibitor AG1478 in heart regeneration of both zebrafish larvae and adults (de Koning et al., 2015; Gemberling et al., 2015). Incubation of Alk5/4 inhibitor SB431542 has also been used in adult fin (Jaźwińska et al., 2007) and heart (Chablais and Jaźwińska, 2012b) to understand the dynamics of fibrosis in tissue regeneration.

Overall, based on the size and anatomic similarities between zebrafish and medaka, most of these delivery methods (**Table 6**) can be adapted in medaka and are regularly used in our laboratory as well as in other studies (Maekawa et al., 2016; Lai et al., 2017; Marín-Juez et al., 2019).

Genomics Resources for Zebrafish and Medaka

Due to the interest in exploring cellular and molecular mechanisms throughout the last few decades, both zebrafish (∼1,412 Mb) (Howe et al., 2013) and medaka genome (∼800 Mb) (Kasahara et al., 2007; Kobayashi and Takeda, 2008) have been sequenced and are publicly available in the databases, including Ensembl for zebrafish[5] (see footnote 1) and medaka[6] (**Table 7**). Like zebrafish, medaka has emerged as one of the most popular and influential animal models to investigate development and disease. At the genomic level, the regenerative capacity of zebrafish might rely on gene regulatory networks, which might be repressed in other non-regenerative

[5]http://asia.ensembl.org/Danio_rerio/Info/Index

[6]https://asia.ensembl.org/Oryzias_latipes/Info/Annotation

TABLE 6 | Tutorials/protocols for injury and drug delivery for zebrafish and medaka.

Description	Website	References
Dissection of different organs from the Adult Zebrafish	Dissection of Organs from the Adult Zebrafish | Protocol (jove.com)	Gupta and Mullins, 2010
Dissection of the Adult Zebrafish Kidney	Dissection of the Adult Zebrafish Kidney | Protocol (jove.com)	Gerlach et al., 2011
Induction of myocardial infarction in adult zebrafish using cryoinjury	Induction of Myocardial Infarction in Adult Zebrafish Using Cryoinjury | Protocol (jove.com)	Chablais and Jaźwińska, 2012a
Brain injury model by stabbing in Adult Zebrafish	Stab Wound Injury of the Zebrafish Adult Telencephalon: A Method to Investigate Vertebrate Brain Neurogenesis and Regeneration | Protocol (jove.com)	Schmidt et al., 2014
Spinal cord injury by transection in larval zebrafish	Spinal Cord Transection in the Larval Zebrafish | Protocol (jove.com) Zebrafish In Situ Spinal Cord Preparation for Electrophysiological Recordings from Spinal Sensory and Motor Neurons | Protocol (jove.com)	Briona and Dorsky, 2014; Moreno et al., 2017
Laser-induced retinal injury model in zebrafish	Müller Glia Cell Activation in a Laser-induced Retinal Degeneration and Regeneration Model in Zebrafish | Protocol (jove.com)	Conedera et al., 2017
Examining muscle regeneration in zebrafish models of muscle disease	Examining Muscle Regeneration in Zebrafish Models of Muscle Disease | Protocol (jove.com)	Au - Montandon et al., 2021
Hepatocyte-specific ablation in zebrafish to study biliary-driven liver regeneration	Hepatocyte-specific Ablation in Zebrafish to Study Biliary-driven Liver Regeneration | Protocol (jove.com)	Choi et al., 2015
Intraperitoneal injection in zebrafish	Intraperitoneal Injection: A Method of Solution Delivery into the Abdominal Cavity of an Adult Zebrafish | Protocol (jove.com)	Kinkel et al., 2010
Intrathoracic injection for the study of adult zebrafish heart	Intrathoracic Injection for the Study of Adult Zebrafish Heart | Protocol (jove.com)	Bise and Jazwinska, 2019
Retro-orbital injection in adult zebrafish	Retro-orbital Injection in Adult Zebrafish | Protocol (jove.com)	Pugach et al., 2009
Nanoparticle-mediated siRNA gene-silencing in adult zebrafish heart	Nanoparticle-mediated siRNA Gene-silencing in Adult Zebrafish Heart | Protocol (jove.com)	Xiao et al., 2018
CRISPR/Cas9-generated gene knockouts in zebrafish	Efficient Production and Identification of CRISPR/Cas9-generated Gene Knockouts in the Model System Danio rerio | Protocol (jove.com)	Sorlien et al., 2018
Imaging blood vessels and lymphatic vessels in the zebrafish	Methods in Cell Biology | The Zebrafish - Cellular and Developmental Biology, Part A Cellular Biology | ScienceDirect.com by Elsevier	Jung et al., 2016
Microinjection of medaka embryos for use as a model genetic organism	Microinjection of Medaka Embryos for use as a Model Genetic Organism | Protocol (jove.com)	Porazinski et al., 2010b
Dechorionation of medaka embryos and cell transplantation for the generation of chimeras	Dechorionation of Medaka Embryos and Cell Transplantation for the Generation of Chimeras | Protocol (jove.com)	Porazinski et al., 2010a
Medaka: Biology, Management, and Experimental protocols	Volume 1 and 2 (https://onlinelibrary.wiley.com/doi/book/10.1002/9781119575399#)	Kinoshita et al., 2009; Murata et al., 2019

animal models (Yang and Kang, 2019). Early findings in zebrafish suggest epigenetic modifications ranging from histone modifications to initiation of enhancer-induced activation of regenerative programs (Kang et al., 2016; Pfefferli and Jaźwińska, 2017). Growing evidence support the compatibility of zebrafish and medaka for comparative transcriptomic analyses (Tena et al., 2014; Lai et al., 2017). Moreover, with the advances of epigenetic profiling, medaka has also been explored for epigenetic changes associated with embryogenesis, development, and evolution (Nakamura et al., 2014; Tena et al., 2014; Ichikawa et al., 2017; Marletaz et al., 2018; Uesaka et al., 2019; Li et al., 2020). These properties may bring medaka as a new teleost model in epigenetics for comparative studies in tissue regeneration.

Hybrid/Chimera Fish and Cell Transplantations

Among various teleost fishes that come in different shapes and sizes, zebrafish and medaka have a similar developmental process overall. However, medaka shows a slower pace and hatch at 9 days compared to 3 days in zebrafish. Considering the similarities and differences between zebrafish and medaka, inter-species blastula transplantation was explored to study the genetic developmental timing during organogenesis (Hong et al., 2012; Fuhrmann et al., 2020). Generating chimeric organisms named "Zebraka" or "Medrafish" involves cell transplantation at the blastula stage and ectopic formation of chimeric organs like retina from zebrafish donor cells in the medaka host. This approach helps researchers determine the transcriptional dynamics of retinal organogenesis and further state the existence of organ-intrinsic mechanisms independent of the development pace of the host (Fuhrmann et al., 2020). It will be intriguing to consider the differential regenerative capacity of each organ in these hybrid animals. For example, if the heart was contributed by zebrafish donor cells and grows in medaka, would it still be regenerative (and why)? Hybrid animals may help researchers dissect the intrinsic and extrinsic properties of tissue regeneration, and zebrafish and medaka chimeras may provide a unique opportunity for such study.

TABLE 7 | Resources for zebrafish and medaka research.

Fish	Resource	Description	Website and references
Zebrafish	The Zebrafish Information Network (ZFIN)	Central database of zebrafish resources, studies and protocols	http://zfin.org/ (Ruzicka et al., 2019)
	Ensembl: *Danio rerio*	Genome assembly, GRCz11	http://asia.ensembl.org/Danio_rerio/Info/Index
	zfRegeneration	Dataset for zebrafish associated regeneration studies	http://www.zfregeneration.org/ (Nieto-Arellano and Sanchez-Iranzo, 2019)
	CreZoo	Database of Zebrafish Cre driver lines	Zebrafish CreZoo (Jungke et al., 2013)
	Zebrafish International Resource Center	Zebrafish stock center, United States	https://zebrafish.org/home/guide.php
	European Zebrafish Resource Center (EZRC)	Zebrafish stock center, KIT-Europe	https://www.ezrc.kit.edu/index.php
	The Taiwan Zebrafish Core Facility (TZCF)	Core facility for Zebrafish stock	http://www.tzcf-tzenh.org/ (You et al., 2016)
	NBRP-Zebrafish	Japan stock center for zebrafish resource	https://shigen.nig.ac.jp/zebra/index_en.html
	China Zebrafish Resource Center (CZRC)	Zebrafish resources, developing new lines and technology	http://en.zfish.cn/
Medaka	NBRP medaka	Central repository and achieve for medaka resources	https://shigen.nig.ac.jp/medaka/\penalty-\@M (Sasado et al., 2010)
	NBRP strains	Repository for strains and transgenics	https://shigen.nig.ac.jp/medaka/strain/strainTop.jsp
	Ensembl: Japanese medaka HdrR	Gene assembly and gene annotation (ASM223467v1)	http://asia.ensembl.org/Oryzias_latipes/Info/Index
	NBRP genome tools	Genome Mapping	http://viewer.shigen.info/medakavw/mapview/
	MEPD	Gene expression data by *in situ* hybridization	http://mepd.cos.uni-heidelberg.de/mepd/ (Alonso-Barba et al., 2016)
	mODP	OMICs data and epigenetic modification database	http://tulab.genetics.ac.cn/modp/#/Browser?species=medaka (Li et al., 2020)
Both Models	CCTop	CRISPR/Cas9 design tool	https://cctop.cos.uni-heidelberg.de:8043/index.html (Stemmer et al., 2015)
	CRISPRscan	CRISPR/Cas9 design tool	https://www.crisprscan.org/ (Moreno-Mateos et al., 2015)

Similar ideas have been exploited in the intra-species blastula transplantation in embryonic and adoptive transfer in adult zebrafish to determine the cellular contribution of specific biological processes (cell-autonomous vs. non-cell-autonomous actions). The concept was recently adopted in a heart regeneration study where macrophages were isolated from zebrafish larvae donors and adoptively transferred into adult hosts. Larval macrophages were found to infiltrate the injured hearts and contribute to scar formation by directly secreting collagens in adults (Simoes et al., 2020). Similar strategies may pave the way for future research, in which zebrafish donor cells might be transferred in medaka host or vice-versa and determine the cellular contributions in tissue regeneration.

LIMITATIONS, FUTURE DIRECTIONS, AND CONCLUDING REMARKS

In this review, we illustrated the concept of comparative study in tissue regeneration and highlighted the examples in zebrafish and medaka models. Zebrafish and medaka are phylogenetically close model organisms compared to animals from different phyla across the animal kingdom yet possess a distinct tissue regeneration capacity. Various tools and techniques commonly used in zebrafish and medaka were summarized here, supporting the unique strength of conducting comparative tissue regeneration research. Nevertheless, we cannot ignore some technical challenges and conceptual oversight that must be addressed or overcome.

On a technical aspect, orthologous gene annotation and identification are critical for inter-species comparisons. The Zebrafish genome has been well annotated and referenced due to a broad research interest with orthologous genes identified and translatable to the mammalian system (Howe et al., 2013). However, the medaka genome is less annotated than zebrafish. The limitation may sometimes be overcome by mapping the medaka genome against zebrafish to identify the orthologous genes. Though this might not be the optimal approach for genome annotation, it is currently the most feasible method to translate genetic information to the zebrafish and the mammalian context. Using such a method, Lai et al. (2017) have identified more than 15,000 orthologous genes across zebrafish and medaka in a comparative transcriptomic analysis.

On a conceptual aspect, it is still unclear how the trait of regeneration evolved along with all the other physiological and anatomical differences among species under the pressure of natural selection. Following the same logic, the similarities and differences between zebrafish and medaka might not necessarily associate with their regenerative capacity, which

confounds the causal relationship between candidate factors and tissue regeneration. In addition, the concept of zebrafish and medaka comparison is based on the existence of conserved mechanisms underly tissue regeneration between these organisms, which might not be accurate, and the same blindside also exists for other inter-species comparisons. In light of this, potential factors identified from the comparative analyses must be further examined in both gain-of-function and loss-of-function experiments to determine their exact role in tissue regeneration. Comparative studies in the heart and retina provide nice examples of such practice (Lai et al., 2017; Lust and Wittbrodt, 2018). To gain translational opportunities, candidate factors (biological processes/signaling pathways) should be further tested to regulate regenerative capacity in mammals. Overall, the comparative approach in different models may gain basic knowledge in tissue regeneration and hint at new therapeutic strategies.

Since the pioneer studies in zebrafish tissue regeneration, broad interests have been invested in the regenerative program and other mechanistic insights underlying various tissue regeneration, hoping that the knowledge will hint or translate into therapeutics in regenerative medicine. Although the medaka has only begun to enter the stage of regenerative biology, their similar characteristics to zebrafish and abundant resources as a model animal draw more and more attention for inter-species and inter-organ comparisons in tissue regeneration and await further exploration.

AUTHOR CONTRIBUTIONS

KC, SL, and S-LL contributed to the conception, writing and revision of the manuscript. KC drafted the manuscript and prepared the tables and figures. S-LL reviewed, edited, and oversaw the manuscript preparation. All the authors read and approved the submitted version.

FUNDING

The research in the S-LL group was supported by the Ministry of Science and Technology (MOST 108-2320-B-001-032-MY2), the IBMS/Academia Sinica (IBMS-CRC108-P03), and the Grand Challenge Project, Academia Sinica (AS-GC-110-L06) in Taiwan. KC is the recipient of the Academia Sinica-Taiwan International Graduate Program (AS-TIGP) Research Progress Fellowship.

ACKNOWLEDGMENTS

We thank Tsung-Hung Hung from the Medical Art Room, IBMS for the artwork, and I-Ting Lin, Khai-Lone Lim, Wei-Han Lang, and Ke-Hsuan Wei for their critical comments and suggestions during the manuscript preparation. We apologize to our colleagues whose articles we could not cite due to space limitations. We also thank the reviewers' time and efforts during the manuscript revision.

REFERENCES

Adolf, B., Chapouton, P., Lam, C. S., Topp, S., Tannhauser, B., Strahle, U., et al. (2006). Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev. Biol.* 295, 278–293. doi: 10.1016/j.ydbio.2006.03.023

Aghaallaei, N., Bajoghli, B., Schwarz, H., Schorpp, M., and Boehm, T. (2010). Characterization of mononuclear phagocytic cells in medaka fish transgenic for a cxcr3a:gfp reporter. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18079–18084. doi: 10.1073/pnas.1000467107

Agrawal, A. A., Conner, J. K., and Rasmann, S. (2010). "Tradeoffs and negative correlations in evolutionary ecology," in *Evolution Since Darwin: The First*, eds M. A. Bell, W. F. Eanes, D. J. Futuyma, and J. S. Levinton (Sunderland, MA: Sinauer Associates), 243–268.

Alibardi, L., and Toni, M. (2005). Wound keratins in the regenerating epidermis of lizard suggest that the wound reaction is similar in the tail and limb. *J. Exp. Zool. A. Comp. Exp. Biol.* 303, 845–860. doi: 10.1002/jez.a.213

Alonso-Barba, J. I., Rahman, R. U., Wittbrodt, J., and Mateo, J. L. (2016). MEPD: medaka expression pattern database, genes and more. *Nucleic Acids Res.* 44, D819–D821. doi: 10.1093/nar/gkv1029

Alunni, A., Hermel, J. M., Heuze, A., Bourrat, F., Jamen, F., and Joly, J. S. (2010). Evidence for neural stem cells in the medaka optic tectum proliferation zones. *Dev. Neurobiol.* 70, 693–713. doi: 10.1002/dneu.20799

Ansai, S., Inohaya, K., Yoshiura, Y., Schartl, M., Uemura, N., Takahashi, R., et al. (2014). Design, evaluation, and screening methods for efficient targeted mutagenesis with transcription activator-like effector nucleases in medaka. *Dev. Growth Differ.* 56, 98–107. doi: 10.1111/dgd.12104

Ansai, S., and Kinoshita, M. (2014). Targeted mutagenesis using CRISPR/Cas system in medaka. *Biol. Open* 3, 362–371. doi: 10.1242/bio.20148177

Ansai, S., Ochiai, H., Kanie, Y., Kamei, Y., Gou, Y., Kitano, T., et al. (2012). Targeted disruption of exogenous EGFP gene in medaka using zinc-finger nucleases. *Dev. Growth Differ.* 54, 546–556. doi: 10.1111/j.1440-169X.2012.01357.x

Ansai, S., Sakuma, T., Yamamoto, T., Ariga, H., Uemura, N., Takahashi, R., et al. (2013). Efficient targeted mutagenesis in medaka using custom-designed transcription activator-like effector nucleases. *Genetics* 193, 739–749. doi: 10.1534/genetics.112.147645

Asakawa, K., and Kawakami, K. (2008). Targeted gene expression by the Gal4-UAS system in zebrafish. *Dev. Growth Differ.* 50, 391–399. doi: 10.1111/j.1440-169X.2008.01044.x

Au - Montandon, M., Au - Currie, P. D., and Au - Ruparelia, A. A. (2021). Examining muscle regeneration in Zebrafish Models of Muscle Disease. *J. Vis. Exp.* 167:e62071. doi: 10.3791/62071

Aurora, A. B., Porrello, E. R., Tan, W., Mahmoud, A. I., Hill, J. A., Bassel-Duby, R., et al. (2014). Macrophages are required for neonatal heart regeneration. *J. Clin. Invest.* 124, 1382–1392. doi: 10.1172/JCI72181

Bajoghli, B., Aghaallaei, N., Hess, I., Rode, I., Netuschil, N., Tay, B. H., et al. (2009). Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. *Cell* 138, 186–197. doi: 10.1016/j.cell.2009.04.017

Bajoghli, B., Dick, A. M., Claasen, A., Doll, L., and Aghaallaei, N. (2019). Zebrafish and Medaka: two Teleost Models of T-Cell and Thymic Development. *Int. J. Mol. Sci.* 20:4179. doi: 10.3390/ijms20174179

Bajoghli, B., Kuri, P., Inoue, D., Aghaallaei, N., Hanelt, M., Thumberger, T., et al. (2015). Noninvasive in toto imaging of the thymus reveals heterogeneous migratory behavior of developing T cells. *J. Immunol.* 195, 2177–2186. doi: 10.4049/jimmunol.1500361

Becker, C., Lust, K., and Wittbrodt, J. (2021). Igf signaling couples retina growth with body growth by modulating progenitor cell division. *Development* 148:dev199133. doi: 10.1242/dev.199133

Becker, T., and Becker, C. G. (2001). Regenerating descending axons preferentially reroute to the gray matter in the presence of a general macrophage/microglial reaction caudal to a spinal transection in adult zebrafish. *J. Comp. Neurol.* 433, 131–147. doi: 10.1002/cne.1131

Becker, T., Wullimann, M. F., Becker, C. G., Bernhardt, R. R., and Schachner, M. (1997). Axonal regrowth after spinal cord transection in adult zebrafish. *J. Comp. Neurol.* 377, 577–595. doi: 10.1002/(sici)1096-9861(19970127)377:4<577::aid-cne8>3.0.co;2-#

Bedell, V. M., Wang, Y., Campbell, J. M., Poshusta, T. L., Starker, C. G., Krug, R. G. II, et al. (2012). *In vivo* genome editing using a high-efficiency TALEN system. *Nature* 491, 114–118.

Beffagna, G. (2019). Zebrafish as a smart model to understand regeneration after heart injury: how fish could help humans. *Front. Cardiovasc. Med.* 6:107. doi: 10.3389/fcvm.2019.00107

Bely, A. E. (2010). Evolutionary loss of animal regeneration: pattern and process. *Integr. Comp. Biol.* 50, 515–527. doi: 10.1093/icb/icq118

Bely, A. E., and Nyberg, K. G. (2010). Evolution of animal regeneration: re-emergence of a field. *Trends Ecol. Evol.* 25, 161–170. doi: 10.1016/j.tree.2009.08.005

Benjamin, D. C., and Hynes, R. O. (2017). Intravital imaging of metastasis in adult Zebrafish. *BMC Cancer* 17:660. doi: 10.1186/s12885-017-3647-0

Bensimon-Brito, A., Ramkumar, S., Boezio, G. L., Guenther, S., Kuenne, C., Helker, C. S., et al. (2020). TGF-β signaling promotes tissue formation during cardiac valve regeneration in adult zebrafish. *Dev. Cell* 52, 9–20.e7. doi: 10.1016/j.devcel.2019.10.027

Bergmann, O., Zdunek, S., Felker, A., Salehpour, M., Alkass, K., Bernard, S., et al. (2015). Dynamics of cell generation and turnover in the human heart. *Cell* 161, 1566–1575. doi: 10.1016/j.cell.2015.05.026

Bernardo, J., and Agosta, S. J. (2005). Evolutionary implications of hierarchical impacts of nonlethal injury on reproduction, including maternal effects. *Biol. J. Linn. Soc.* 86, 309–331.

Bernstein, J. J. (1964). Relation of spinal cord regeneration to age in adult goldfish. *Exp. Neurol.* 9, 161–174. doi: 10.1016/0014-4886(64)90014-7

Bernut, A., Herrmann, J. L., Kissa, K., Dubremetz, J. F., Gaillard, J. L., Lutfalla, G., et al. (2014). Mycobacterium abscessus cording prevents phagocytosis and promotes abscess formation. *Proc. Natl. Acad. Sci. U.S.A.* 111, E943–E952. doi: 10.1073/pnas.1321390111

Bertrand, J. Y., Chi, N. C., Santoso, B., Teng, S., Stainier, D. Y., and Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* 464, 108–111. doi: 10.1038/nature08738

Bertrand, J. Y., Kim, A. D., Teng, S., and Traver, D. (2008). CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. *Development* 135, 1853–1862. doi: 10.1242/dev.015297

Bevan, L., Lim, Z. W., Venkatesh, B., Riley, P. R., Martin, P., and Richardson, R. J. (2020). Specific macrophage populations promote both cardiac scar deposition and subsequent resolution in adult zebrafish. *Cardiovasc. Res.* 116, 1357–1371. doi: 10.1093/cvr/cvz221

Bise, T., and Jazwinska, A. (2019). Intrathoracic Injection for the Study of Adult Zebrafish Heart. *J. Vis. Exp.* 147:e59724. doi: 10.3791/59724

Bollig, F., Perner, B., Besenbeck, B., Kothe, S., Ebert, C., Taudien, S., et al. (2009). A highly conserved retinoic acid responsive element controls wt1a expression in the zebrafish pronephros. *Development* 136, 2883–2892. doi: 10.1242/dev.031773

Briona, L. K., and Dorsky, R. I. (2014). Spinal cord transection in the larval zebrafish. *J. Vis. Exp.* 87:51479. doi: 10.3791/51479

Brockes, J. P., Kumar, A., and Velloso, C. P. (2001). Regeneration as an evolutionary variable. *J. Anat.* 199(Pt 1–2), 3–11. doi: 10.1046/j.1469-7580.2001.19910003.x

Broussonet, M. (1786). Observations sur la régénérations de quelques parties du corps des poissons. *Hist. Acad. R. Des. Sci.* 105, 625–641.

Broussonet, M. (1789). *Memoir on the Regeneration of Certain Parts of the Bodies of Fishes*. (London: Printed for the Proprietors and Sold by C. Forster), 111–113.

Burg, L., Palmer, N., Kikhi, K., Miroshnik, E. S., Rueckert, H., Gaddy, E., et al. (2018). Conditional mutagenesis by oligonucleotide-mediated integration of loxP sites in zebrafish. *PLoS Genet.* 14:e1007754. doi: 10.1371/journal.pgen.1007754

Bussmann, J., and Schulte-Merker, S. (2011). Rapid BAC selection for tol2-mediated transgenesis in zebrafish. *Development* 138, 4327–4332. doi: 10.1242/dev.068080

Caldwell, L. J., Davies, N. O., Cavone, L., Mysiak, K. S., Semenova, S. A., Panula, P., et al. (2019). Regeneration of Dopaminergic Neurons in Adult Zebrafish depends on immune system activation and differs for distinct populations. *J. Neurosci.* 39, 4694–4713. doi: 10.1523/JNEUROSCI.2706-18.2019

Cano-Martinez, A., Vargas-Gonzalez, A., Guarner-Lans, V., Prado-Zayago, E., Leon-Oleda, M., and Nieto-Lima, B. (2010). Functional and structural regeneration in the axolotl heart (Ambystoma mexicanum) after partial ventricular amputation. *Arch. Cardiol. Mex.* 80, 79–86.

Cao, J., and Poss, K. D. (2018). The epicardium as a hub for heart regeneration. *Nat. Rev. Cardiol.* 15, 631–647. doi: 10.1038/s41569-018-0046-4

Carl, M., Loosli, F., and Wittbrodt, J. (2002). Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. *Development* 129, 4057–4063.

Centanin, L., Ander, J. J., Hoeckendorf, B., Lust, K., Kellner, T., Kraemer, I., et al. (2014). Exclusive multipotency and preferential asymmetric divisions in post-embryonic neural stem cells of the fish retina. *Development* 141, 3472–3482. doi: 10.1242/dev.109892

Centanin, L., Hoeckendorf, B., and Wittbrodt, J. (2011). Fate restriction and multipotency in retinal stem cells. *Cell Stem Cell* 9, 553–562. doi: 10.1016/j.stem.2011.11.004

Chablais, F., and Jaźwińska, A. (2012a). Induction of myocardial infarction in adult zebrafish using cryoinjury. *J. Vis. Exp.* 62:3666. doi: 10.3791/3666

Chablais, F., and Jaźwińska, A. (2012b). The regenerative capacity of the zebrafish heart is dependent on TGFβ signaling. *Development* 139, 1921–1930. doi: 10.1242/dev.078543

Chablais, F., Veit, J., Rainer, G., and Jaźwińska, A. (2011). The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. *BMC Dev. Biol.* 11:21. doi: 10.1186/1471-213X-11-21

Chassot, B., Pury, D., and Jazwinska, A. (2016). Zebrafish fin regeneration after cryoinjury-induced tissue damage. *Biol. Open* 5, 819–828. doi: 10.1242/bio.016865

Chatani, M., Takano, Y., and Kudo, A. (2011). Osteoclasts in bone modeling, as revealed by *in vivo* imaging, are essential for organogenesis in fish. *Dev. Biol.* 360, 96–109. doi: 10.1016/j.ydbio.2011.09.013

Chavez, M. N., Morales, R. A., Lopez-Crisosto, C., Roa, J. C., Allende, M. L., and Lavandero, S. (2020). Autophagy activation in Zebrafish heart regeneration. *Sci. Rep.* 10:2191. doi: 10.1038/s41598-020-59106-z

Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat. Chem. Biol.* 5, 100–107. doi: 10.1038/nchembio.137

Chen, C. F., Chu, C. Y., Chen, T. H., Lee, S. J., Shen, C. N., and Hsiao, C. D. (2011). Establishment of a transgenic zebrafish line for superficial skin ablation and functional validation of apoptosis modulators *in vivo*. *PLoS One* 6:e20654. doi: 10.1371/journal.pone.0020654

Chen, W. C., Wang, Z., Missinato, M. A., Park, D. W., Long, D. W., Liu, H. J., et al. (2016). Decellularized zebrafish cardiac extracellular matrix induces mammalian heart regeneration. *Sci. Adv.* 2:e1600844. doi: 10.1126/sciadv.1600844

Childs, S., Chen, J. N., Garrity, D. M., and Fishman, M. C. (2002). Patterning of angiogenesis in the zebrafish embryo. *Development* 129, 973–982.

Choi, T.-Y., Khaliq, M., Ko, S., So, J., and Shin, D. (2015). Hepatocyte-specific ablation in zebrafish to study biliary-driven liver regeneration. *J. Vis. Exp.* 99:e52785. doi: 10.3791/52785

Chou, C.-Y., Horng, L.-S., and Tsai, H.-J. (2001). Uniform GFP-expression in transgenic medaka (Oryzias latipes) at the F0 generation. *Transgenic Res.* 10, 303–315. doi: 10.1023/a:1016671513425

Collery, R. F., Volberding, P. J., Bostrom, J. R., Link, B. A., and Besharse, J. C. (2016). Loss of Zebrafish Mfrp causes nanophthalmia, hyperopia, and accumulation of subretinal macrophages. *Invest. Ophthalmol. Vis. Sci.* 57, 6805–6814. doi: 10.1167/iovs.16-19593

Conedera, F. M., Arendt, P., Trepp, C., Tschopp, M., and Enzmann, V. (2017). Muller Glia cell activation in a laser-induced retinal degeneration and regeneration model in Zebrafish. *J. Vis. Exp.* 128:e56249. doi: 10.3791/56249

Cox, A. G., Saunders, D. C., Kelsey, P. B. Jr., Conway, A. A., Tesmenitsky, Y., Marchini, J. F., et al. (2014). S-nitrosothiol signaling regulates liver development and improves outcome following toxic liver injury. *Cell Rep.* 6, 56–69. doi: 10.1016/j.celrep.2013.12.007

Crespo, C. L., Vernieri, C., Keller, P. J., Garre, M., Bender, J. R., Wittbrodt, J., et al. (2014). The PAR complex controls the spatiotemporal dynamics of F-actin

and the MTOC in directionally migrating leukocytes. *J. Cell Sci.* 127(Pt 20), 4381–4395. doi: 10.1242/jcs.146217

Crippa, S., Nemir, M., Ounzain, S., Ibberson, M., Berthonneche, C., Sarre, A., et al. (2016). Comparative transcriptome profiling of the injured zebrafish and mouse hearts identifies miRNA-dependent repair pathways. *Cardiovasc. Res.* 110, 73–84. doi: 10.1093/cvr/cvw031

Cruz, I. A., Kappedal, R., Mackenzie, S. M., Hailey, D. W., Hoffman, T. L., Schilling, T. F., et al. (2015). Robust regeneration of adult zebrafish lateral line hair cells reflects continued precursor pool maintenance. *Dev. Biol.* 402, 229–238. doi: 10.1016/j.ydbio.2015.03.019

Cuervo, R., Hernandez-Martinez, R., Chimal-Monroy, J., Merchant-Larios, H., and Covarrubias, L. (2012). Full regeneration of the tribasal Polypterus fin. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3838–3843. doi: 10.1073/pnas.1006619109

Curado, S., Anderson, R. M., Jungblut, B., Mumm, J., Schroeter, E., and Stainier, D. Y. (2007). Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev. Dyn.* 236, 1025–1035. doi: 10.1002/dvdy.21100

Dambroise, E., Simion, M., Bourquard, T., Bouffard, S., Rizzi, B., Jaszczyszyn, Y., et al. (2017). Postembryonic fish brain proliferation zones exhibit neuroepithelial-type gene expression profile. *Stem Cells* 35, 1505–1518. doi: 10.1002/stem.2588

Daponte, V., Tylzanowski, P., and Forlino, A. (2021). Appendage Regeneration in Vertebrates: What Makes This Possible? *Cells* 10:242. doi: 10.3390/cells10020242

Darehzereshki, A., Rubin, N., Gamba, L., Kim, J., Fraser, J., Huang, Y., et al. (2015). Differential regenerative capacity of neonatal mouse hearts after cryoinjury. *Dev. Biol.* 399, 91–99. doi: 10.1016/j.ydbio.2014.12.018

Darnet, S., Dragalzew, A. C., Amaral, D. B., Sousa, J. F., Thompson, A. W., Cass, A. N., et al. (2019). Deep evolutionary origin of limb and fin regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 116, 15106–15115. doi: 10.1073/pnas.1900475116

Dasyani, M., Tan, W. H., Sundaram, S., Imangali, N., Centanin, L., Wittbrodt, J., et al. (2019). Lineage tracing of col10a1 cells identifies distinct progenitor populations for osteoblasts and joint cells in the regenerating fin of medaka (*Oryzias latipes*). *Dev. Biol.* 455, 85–99. doi: 10.1016/j.ydbio.2019.07.012

Davidson, A. E., Balciunas, D., Mohn, D., Shaffer, J., Hermanson, S., Sivasubbu, S., et al. (2003). Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon. *Dev. Biol.* 263, 191–202. doi: 10.1016/j.ydbio.2003.07.013

de Koning, C., Beekhuijzen, M., Tobor-Kaplon, M., de Vries-Buitenweg, S., Schoutsen, D., Leeijen, N., et al. (2015). Visualizing Compound Distribution during Zebrafish Embryo Development: the Effects of Lipophilicity and DMSO. *Birth Defects Res. B. Dev. Reprod. Toxicol.* 104, 253–272. doi: 10.1002/bdrb.21166

de Preux Charles, A. S., Bise, T., Baier, F., Marro, J., and Jazwinska, A. (2016). Distinct effects of inflammation on preconditioning and regeneration of the adult zebrafish heart. *Open Biol.* 6:160102. doi: 10.1098/rsob.160102

Deguchi, T., Fujimori, K. E., Kawasaki, T., Maruyama, K., and Yuba, S. (2012). *In vivo* visualization of the lymphatic vessels in pFLT4-EGFP transgenic medaka. *Genesis* 50, 625–634. doi: 10.1002/dvg.22018

DeLaurier, A., Eames, B. F., Blanco-Sanchez, B., Peng, G., He, X., Swartz, M. E., et al. (2010). Zebrafish sp7:EGFP: a transgenic for studying otic vesicle formation, skeletogenesis, and bone regeneration. *Genesis* 48, 505–511. doi: 10.1002/dvg.20639

DiCicco, R. M., Bell, B. A., Kaul, C., Hollyfield, J. G., Anand-Apte, B., Perkins, B. D., et al. (2014). Retinal regeneration following OCT-guided laser injury in zebrafish. *Invest. Ophthalmol. Vis. Sci.* 55, 6281–6288. doi: 10.1167/iovs.14-14724

Diep, C. Q., Ma, D., Deo, R. C., Holm, T. M., Naylor, R. W., Arora, N., et al. (2011). Identification of adult nephron progenitors capable of kidney regeneration in zebrafish. *Nature* 470, 95–100. doi: 10.1038/nature09669

Diotel, N., Lubke, L., Strahle, U., and Rastegar, S. (2020). Common and Distinct Features of Adult Neurogenesis and Regeneration in the Telencephalon of Zebrafish and Mammals. *Front. Neurosci.* 14:568930. doi: 10.3389/fnins.2020.568930

Doyon, Y., McCammon, J. M., Miller, J. C., Faraji, F., Ngo, C., Katibah, G. E., et al. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* 26, 702–708. doi: 10.1038/nbt1409

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123, 37–46.

Du, S. J., and Dienhart, M. (2001). Zebrafish tiggy-winkle hedgehog promoter directs notochord and floor plate green fluorescence protein expression in transgenic zebrafish embryos. *Dev. Dyn.* 222, 655–666. doi: 10.1002/dvdy.1219

Dwaraka, V. B., and Voss, S. R. (2021). Towards comparative analyses of salamander limb regeneration. *J. Exp. Zool. B Mol. Dev. Evol.* 336, 129–144. doi: 10.1002/jez.b.22902

Dyck, P. K. V., Hockaden, N., Nelson, E. C., Koch, A. R., Hester, K. L., Pillai, N., et al. (2020). Cauterization as a simple method for regeneration studies in the zebrafish heart. *J. Cardiovasc. Dev. Dis.* 7:41. doi: 10.3390/jcdd7040041

Elbaz, I., Yelin-Bekerman, L., Nicenboim, J., Vatine, G., and Appelbaum, L. (2012). Genetic ablation of hypocretin neurons alters behavioral state transitions in zebrafish. *J. Neurosci.* 32, 12961–12972. doi: 10.1523/JNEUROSCI.1284-12.2012

Elchaninov, A., Sukhikh, G., and Fatkhudinov, T. (2021). Evolution of regeneration in animals: a tangled story. *Front. Ecol. Evol.* 9:621686. doi: 10.3389/fevo.2021.621686

Elhelaly, W. M., Lam, N. T., Hamza, M., Xia, S., and Sadek, H. A. (2016). Redox regulation of heart regeneration: an evolutionary Tradeoff. *Front. Cell Dev. Biol.* 4:137. doi: 10.3389/fcell.2016.00137

Ellett, F., Pase, L., Hayman, J., Andrianopoulos, A., and Lieschke, G. (2011). mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood* 117, E49–E56. doi: 10.1182/blood-2010-10-314120

Emelyanov, A., Gao, Y., Naqvi, N. I., and Parinov, S. (2006). Trans-kingdom transposition of the maize dissociation element. *Genetics* 174, 1095–1104. doi: 10.1534/genetics.106.061184

Eschenhagen, T., Bolli, R., Braun, T., Field, L. J., Fleischmann, B. K., Frisén, J., et al. (2017). Cardiomyocyte regeneration: a consensus statement. *Circulation* 136, 680–686. doi: 10.1161/circulationaha.117.029343

Fadool, J. M. (2003). Development of a rod photoreceptor mosaic revealed in transgenic zebrafish. *Dev. Biol.* 258, 277–290. doi: 10.1016/s0012-1606(03)00125-8

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10887–10890. doi: 10.1073/pnas.93.20.10887

Felker, A., and Mosimann, C. (2016). "Chapter 11 - Contemporary zebrafish transgenesis with Tol2 and application for Cre/lox recombination experiments," in *Methods in Cell Biology*, eds H. William Detrich, M. Westerfield, and L. I. Zon (Amsterdam: Elsevier), 219–244. doi: 10.1016/bs.mcb.2016.01.009

Ferguson, H., Kongtorp, R., Taksdal, T., Graham, D., and Falk, K. (2005). An outbreak of disease resembling heart and skeletal muscle inflammation in Scottish farmed salmon, *Salmo salar* L., with observations on myocardial regeneration. *J. Fish Dis.* 28, 119–123. doi: 10.1111/j.1365-2761.2004.00602.x

Fimbel, S. M., Montgomery, J. E., Burket, C. T., and Hyde, D. R. (2007). Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. *J. Neurosci.* 27, 1712–1724. doi: 10.1523/JNEUROSCI.5317-06.2007

Fox, S. F., and McCoy, J. K. (2000). The effects of tail loss on survival, growth, reproduction, and sex ratio of offspring in the lizard Uta stansburiana in the field. *Oecologia* 122, 327–334. doi: 10.1007/s004420050038

Froschauer, A., Sprott, D., Gerwien, F., Henker, Y., Rudolph, F., Pfennig, F., et al. (2012). Effective generation of transgenic reporter and gene trap lines of the medaka (*Oryzias latipes*) using the Ac/Ds transposon system. *Transgenic Res.* 21, 149–162. doi: 10.1007/s11248-011-9514-x

Fuhrmann, J. F., Buono, L., Adelmann, L., Martinez-Morales, J. R., and Centanin, L. (2020). Genetic developmental timing revealed by inter-species transplantations in fish. *Development* 147:dev192500. doi: 10.1242/dev.192500

Furutani-Seiki, M., Sasado, T., Morinaga, C., Suwa, H., Niwa, K., Yoda, H., et al. (2004). A systematic genome-wide screen for mutations affecting organogenesis in Medaka, *Oryzias latipes*. *Mech. Dev.* 121, 647–658. doi: 10.1016/j.mod.2004.04.016

Furutani-Seiki, M., and Wittbrodt, J. (2004). Medaka and zebrafish, an evolutionary twin study. *Mech. Dev.* 121, 629–637. doi: 10.1016/j.mod.2004.05.010

Galis, F., Wagner, G. P., and Jockusch, E. L. (2003). Why is limb regeneration possible in amphibians but not in reptiles, birds, and mammals? *Evol. Dev.* 5, 208–220. doi: 10.1046/j.1525-142x.2003.03028.x

Garcia, J., Bagwell, J., Njaine, B., Norman, J., Levic, D. S., Wopat, S., et al. (2017). Sheath cell invasion and trans-differentiation repair mechanical damage caused by Loss of Caveolae in the Zebrafish Notochord. *Curr. Biol.* 27, 1982–1989.e3. doi: 10.1016/j.cub.2017.05.035

Gemberling, M., Bailey, T. J., Hyde, D. R., and Poss, K. D. (2013). The zebrafish as a model for complex tissue regeneration. *Trends Genet.* 29, 611–620. doi: 10.1016/j.tig.2013.07.003

Gemberling, M., Karra, R., Dickson, A. L., and Poss, K. D. (2015). Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *eLife* 4:e05871. doi: 10.7554/eLife.05871

Géraudie, J., Brulfert, A., Monnot, M. J., and Ferretti, P. (1994). Teratogenic and morphogenetic effects of retinoic acid on the regenerating pectoral fin in zebrafish. *J. Exp. Zool.* 269, 12–22.

Gerlach, G. F., Schrader, L. N., and Wingert, R. A. (2011). Dissection of the adult zebrafish kidney. *J. Vis. Exp.* 54:e2839. doi: 10.3791/2839

Ghosh, S., and Hui, S. P. (2018). Axonal regeneration in zebrafish spinal cord. *Regeneration* 5, 43–60. doi: 10.1002/reg2.99

Godoy, R., Noble, S., Yoon, K., Anisman, H., and Ekker, M. (2015). Chemogenetic ablation of dopaminergic neurons leads to transient locomotor impairments in zebrafish larvae. *J. Neurochem.* 135, 249–260. doi: 10.1111/jnc.13214

Godwin, J. W., Pinto, A. R., and Rosenthal, N. A. (2013). Macrophages are required for adult salamander limb regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9415–9420. doi: 10.1073/pnas.1300290110

Godwin, J. W., and Rosenthal, N. (2014). Scar-free wound healing and regeneration in amphibians: immunological influences on regenerative success. *Differentiation* 87, 66–75. doi: 10.1016/j.diff.2014.02.002

Goldman, D. (2014). Muller glial cell reprogramming and retina regeneration. *Nat. Rev. Neurosci.* 15, 431–442. doi: 10.1038/nrn3723

Goldman, D., Hankin, M., Li, Z., Dai, X., and Ding, J. (2001). Transgenic zebrafish for studying nervous system development and regeneration. *Transgenic Res.* 10, 21–33. doi: 10.1023/a:1008998832552

Goldstein, A. M., and Fishman, M. C. (1998). Notochord regulates cardiac lineage in zebrafish embryos. *Dev. Biol.* 201, 247–252. doi: 10.1006/dbio.1998.8976

Gonzalez-Rosa, J. M., Martín, V., Peralta, M., Torres, M., and Mercader, N. (2011). Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* 138, 1663–1674. doi: 10.1242/dev.060897

González-Rosa, J. M., Peralta, M., and Mercader, N. (2012). Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. *Dev. Biol.* 370, 173–186. doi: 10.1016/j.ydbio.2012.07.007

Gordon, K., Schulte, D., Brice, G., Simpson, M. A., Roukens, M. G., Van Impel, A., et al. (2013). Mutation in vascular endothelial growth factor-C, a ligand for vascular endothelial growth factor receptor-3, is associated with autosomal dominant milroy-like primary lymphedema. *Circ. Res.* 112, 956–960. doi: 10.1161/CIRCRESAHA.113.300350

Gorsuch, R. A., and Hyde, D. R. (2014). Regulation of muller glial dependent neuronal regeneration in the damaged adult zebrafish retina. *Exp. Eye Res.* 123, 131–140. doi: 10.1016/j.exer.2013.07.012

Gorsuch, R. A., Lahne, M., Yarka, C. E., Petravick, M. E., Li, J., and Hyde, D. R. (2017). Sox2 regulates muller glia reprogramming and proliferation in the regenerating zebrafish retina via Lin28 and Ascl1a. *Exp. Eye Res.* 161, 174–192. doi: 10.1016/j.exer.2017.05.012

Grabher, C., Cliffe, A., Miura, K., Hayflick, J., Pepperkok, R., Rorth, P., et al. (2007). Birth and life of tissue macrophages and their migration in embryogenesis and inflammation in medaka. *J. Leukoc. Biol.* 81, 263–271. doi: 10.1189/jlb.0806526

Grabher, C., Henrich, T., Sasado, T., Arenz, A., Wittbrodt, J., and Furutani-Seiki, M. (2003). Transposon-mediated enhancer trapping in medaka. *Gene* 322, 57–66. doi: 10.1016/j.gene.2003.09.009

Grabher, C., Joly, J.-S., and Wittbrodt, J. (2004). Highly efficient zebrafish transgenesis mediated by the meganuclease I-SceI. *Methods Cell Biol.* 77, 381–401. doi: 10.1016/s0091-679x(04)77021-1

Grabher, C., and Wittbrodt, J. (2004). Efficient activation of gene expression using a heat-shock inducible Gal4/Vp16-UAS system in medaka. *BMC Biotechnol.* 4:26. doi: 10.1186/1472-6750-4-26

Grabher, C., and Wittbrodt, J. (2007). Meganuclease and transposon mediated transgenesis in medaka. *Genome Biol.* 8, (Suppl 1):S10.

Grabher, C., and Wittbrodt, J. (2008). Recent advances in meganuclease-and transposon-mediated transgenesis of medaka and zebrafish. *Methods Mol. Biol.* 461, 521–539. doi: 10.1007/978-1-60327-483-8_36

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., and Brand, M. (2006). Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. *Dev. Biol.* 295, 263–277. doi: 10.1016/j.ydbio.2006.03.040

Grandel, H., and Schulte-Merker, S. (1998). The development of the paired fins in the zebrafish (*Danio rerio*). *Mech. Dev.* 79, 99–120. doi: 10.1016/s0925-4773(98)00176-2

Grivas, J., Haag, M., Johnson, A., Manalo, T., Roell, J., Das, T. L., et al. (2014). Cardiac repair and regenerative potential in the goldfish (*Carassius auratus*) heart. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 163, 14–23. doi: 10.1016/j.cbpc.2014.02.002

Gulías, P., Guerra-Varela, J., Gonzalez-Aparicio, M., Ricobaraza, A., Vales, A., Gonzalez-Aseguinolaza, G., et al. (2019). Danio rerio as model organism for adenoviral vector evaluation. *Genes* 10:1053. doi: 10.3390/genes10121053

Gupta, T., and Mullins, M. C. (2010). Dissection of organs from the adult zebrafish. *J. Vis. Exp.* 37:1717. doi: 10.3791/1717

Hamada, K., Tamaki, K., Sasado, T., Watai, Y., Kani, S., Wakamatsu, Y., et al. (1998). Usefulness of the medaka beta-actin promoter investigated using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). *Mol. Mar. Biol. Biotechnol.* 7, 173–180.

Hanovice, N. J., Leach, L. L., Slater, K., Gabriel, A. E., Romanovicz, D., Shao, E., et al. (2019). Regeneration of the zebrafish retinal pigment epithelium after widespread genetic ablation. *PLoS Genet.* 15:e1007939. doi: 10.1371/journal.pgen.1007939

Hans, S., Freudenreich, D., Geffarth, M., Kaslin, J., Machate, A., and Brand, M. (2011). Generation of a non-leaky heat shock-inducible Cre line for conditional Cre/lox strategies in zebrafish. *Dev. Dyn.* 240, 108–115. doi: 10.1002/dvdy.22497

Hans, S., Kaslin, J., Freudenreich, D., and Brand, M. (2009). Temporally-controlled site-specific recombination in zebrafish. *PLoS One* 4:e4640. doi: 10.1371/journal.pone.0004640

Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W., and Rubel, E. W. (2003). Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). *J. Assoc. Res. Otolaryngol.* 4, 219–234. doi: 10.1007/s10162-002-3022-x

Haubner, B. J., Adamowicz-Brice, M., Khadayate, S., Tiefenthaler, V., Metzler, B., Aitman, T., et al. (2012). Complete cardiac regeneration in a mouse model of myocardial infarction. *Aging* 4, 966–977. doi: 10.18632/aging.100526

Haubner, B. J., Schneider, J., Schweigmann, U., Schuetz, T., Dichtl, W., Velik-Salchner, C., et al. (2016). Functional recovery of a human neonatal heart after severe myocardial infarction. *Circ. Res.* 118, 216–221. doi: 10.1161/CIRCRESAHA.115.307017

Hawkins, M. B., Henke, K., and Harris, M. P. (2021). Latent developmental potential to form limb-like skeletal structures in zebrafish. *Cell* 184, 899–911.e13. doi: 10.1016/j.cell.2021.01.003

Her, G. M., Yeh, Y. H., and Wu, J. L. (2003). 435-bp liver regulatory sequence in the liver fatty acid binding protein (L-FABP) gene is sufficient to modulate liver regional expression in transgenic zebrafish. *Dev. Dyn.* 227, 347–356. doi: 10.1002/dvdy.10324

Higashijima, S.-I., Okamoto, H., Ueno, N., Hotta, Y., and Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev. Biol.* 192, 289–299. doi: 10.1006/dbio.1997.8779

Hilgers, L., and Schwarzer, J. (2019). The natural history of model organisms: the untapped potential of medaka and its wild relatives. *eLife* 8:e46994. doi: 10.7554/eLife.46994

Hirose, K., Payumo, A. Y., Cutie, S., Hoang, A., Zhang, H., Guyot, R., et al. (2019). Evidence for hormonal control of heart regenerative capacity during endothermy acquisition. *Science* 364, 184–188. doi: 10.1126/science.aar2038

Hoang, T., Wang, J., Boyd, P., Wang, F., Santiago, C., Jiang, L., et al. (2020). Gene regulatory networks controlling vertebrate retinal regeneration. *Science* 370:eabb8598. doi: 10.1126/science.abb8598

Hoeppner, L. H., Sinha, S., Wang, Y., Bhattacharya, R., Dutta, S., Gong, X., et al. (2015). RhoC maintains vascular homeostasis by regulating VEGF-induced signaling in endothelial cells. *J. Cell Sci.* 128, 3556–3568.

Hogan, B. M., Bos, F. L., Bussmann, J., Witte, M., Chi, N. C., Duckers, H. J., et al. (2009). Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. *Nat. Genet.* 41, 396–398. doi: 10.1038/ng.321

Hong, N., Chen, S., Ge, R., Song, J., Yi, M., and Hong, Y. (2012). Interordinal chimera formation between medaka and zebrafish for analyzing stem cell differentiation. *Stem Cells Dev.* 21, 2333–2341. doi: 10.1089/scd.2011.0630

Honkoop, H., de Bakker, D. E., Aharonov, A., Kruse, F., Shakked, A., Nguyen, P. D., et al. (2019). Single-cell analysis uncovers that metabolic reprogramming by ErbB2 signaling is essential for cardiomyocyte proliferation in the regenerating heart. *eLife* 8:e50163. doi: 10.7554/eLife.50163

Hosoya, O., Chung, M., Ansai, S., Takeuchi, H., and Miyaji, M. (2021). A modified Tet-ON system minimizing leaky expression for cell-type specific gene induction in medaka fish. *Dev. Growth Differ.* 63, 397–405. doi: 10.1111/dgd.12743

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., et al. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496, 498–503. doi: 10.1038/nature12111

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., et al. (2013). Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* 140, 4982–4987. doi: 10.1242/dev.099085

Huang, C. J., Tu, C. T., Hsiao, C. D., Hsieh, F. J., and Tsai, H. J. (2003). Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev. Dyn.* 228, 30–40. doi: 10.1002/dvdy.10356

Huang, H., Vogel, S. S., Liu, N., Melton, D. A., and Lin, S. (2001). Analysis of pancreatic development in living transgenic zebrafish embryos. *Mol. Cell Endocrinol.* 177, 117–124. doi: 10.1016/s0303-7207(01)00408-7

Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., and Zhang, B. (2011). Heritable gene targeting in zebrafish using customized TALENs. *Nat. Biotechnol.* 29, 699–700. doi: 10.1038/nbt.1939

Hui, S. P., Sheng, D. Z., Sugimoto, K., Gonzalez-Rajal, A., Nakagawa, S., Hesselson, D., et al. (2017). Zebrafish regulatory T cells mediate organ-specific regenerative programs. *Dev. Cell* 43, 659–672.e5. doi: 10.1016/j.devcel.2017.11.010

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., et al. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* 31, 227–229. doi: 10.1038/nbt.2501

Ichikawa, K., Tomioka, S., Suzuki, Y., Nakamura, R., Doi, K., Yoshimura, J., et al. (2017). Centromere evolution and CpG methylation during vertebrate speciation. *Nat. Commun.* 8:1833. doi: 10.1038/s41467-017-01982-7

Iismaa, S. E., Kaidonis, X., Nicks, A. M., Bogush, N., Kikuchi, K., Naqvi, N., et al. (2018). Comparative regenerative mechanisms across different mammalian tissues. *NPJ Regen. Med.* 3:6. doi: 10.1038/s41536-018-0044-5

Inoue, D., and Wittbrodt, J. (2011). One for all—a highly efficient and versatile method for fluorescent immunostaining in fish embryos. *PLoS One* 6:e19713. doi: 10.1371/journal.pone.0019713

Ishikawa, Y. (2000). Medakafish as a model system for vertebrate developmental genetics. *Bioessays* 22, 487–495. doi: 10.1002/(SICI)1521-1878(200005)22:5<487::AID-BIES11>3.0.CO;2-8

Ito, K., Morioka, M., Kimura, S., Tasaki, M., Inohaya, K., and Kudo, A. (2014). Differential reparative phenotypes between zebrafish and medaka after cardiac injury. *Dev. Dyn.* 243, 1106–1115. doi: 10.1002/dvdy.24154

Ito, Y., Tanaka, H., Okamoto, H., and Ohshima, T. (2010). Characterization of neural stem cells and their progeny in the adult zebrafish optic tectum. *Dev. Biol.* 342, 26–38. doi: 10.1016/j.ydbio.2010.03.008

Jaźwińska, A., Badakov, R., and Keating, M. T. (2007). Activin-βA signaling is required for zebrafish fin regeneration. *Curr. Biol.* 17, 1390–1395. doi: 10.1016/j.cub.2007.07.019

Jaźwińska, A., and Blanchoud, S. (2020). Towards deciphering variations of heart regeneration in fish. *Curr. Opin. Physiol.* 14, 21–26. doi: 10.1016/j.cophys.2019.11.007

Jessen, J. R., Jessen, T. N., Vogel, S. S., and Lin, S. (2001). Concurrent expression of recombination activating genes 1 and 2 in zebrafish olfactory sensory neurons. *Genesis* 29, 156–162. doi: 10.1002/gene.1019

Jessen, J. R., Willett, C. E., and Lin, S. (1999). Artificial chromosome transgenesis reveals long-distance negative regulation of rag1 in zebrafish. *Nat. Genet.* 23, 15–16. doi: 10.1038/12609

Jh, B. (1947). Regeneration and transplantation of fin rays in the goldfish. *Anat. Rec.* 99, 648–648.

Jimenez, E., Slevin, C. C., Colón-Cruz, L., and Burgess, S. M. (2021). Vestibular and auditory hair cell regeneration following targeted ablation of hair cells with diphtheria toxin in zebrafish. *bioRxiv* [Preprint]. doi: 10.1101/2021.06.08.447587

Jopling, C., Boue, S., and Izpisua Belmonte, J. C. (2011). Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. *Nat. Rev. Mol. Cell Biol.* 12, 79–89. doi: 10.1038/nrm3043

Ju, B., Chong, S. W., He, J., Wang, X., Xu, Y., Wan, H., et al. (2003). Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter. *Dev. Dyn.* 227, 14–26. doi: 10.1002/dvdy.10273

Julier, Z., Park, A. J., Briquez, P. S., and Martino, M. M. (2017). Promoting tissue regeneration by modulating the immune system. *Acta Biomater.* 53, 13–28. doi: 10.1016/j.actbio.2017.01.056

Jung, H. M., Castranova, D., Swift, M. R., Pham, V. N., Venero Galanternik, M., Isogai, S., et al. (2017). Development of the larval lymphatic system in zebrafish. *Development* 144, 2070–2081. doi: 10.1242/dev.145755

Jung, H. M., Isogai, S., Kamei, M., Castranova, D., Gore, A. V., and Weinstein, B. M. (2016). Imaging blood vessels and lymphatic vessels in the zebrafish. *Methods Cell Biol.* 133, 69–103. doi: 10.1016/bs.mcb.2016.03.023

Jung, S., and Kleinheinz, J. (2013). "Angiogenesis—the key to regeneration," in *Regenerative Medicine and Tissue Engineering*, ed. J. A. Andrades (London: IntechOpen). doi: 10.5772/55542

Jungke, P., Hammer, J., Hans, S., and Brand, M. (2015). Isolation of novel CreERT2-driver lines in zebrafish using an unbiased gene trap approach. *PLoS One* 10:e0129072. doi: 10.1371/journal.pone.0129072

Jungke, P., Hans, S., and Brand, M. (2013). The zebrafish CreZoo: an easy-to-handle database for novel CreERT2-driver lines. *Zebrafish* 10, 259–263. doi: 10.1089/zeb.2012.0834

Kanagaraj, P., Chen, J. Y., Skaggs, K., Qadeer, Y., Conner, M., Cutler, N., et al. (2020). Microglia Stimulate Zebrafish Brain Repair Via a Specific Inflammatory Cascade. *bioRxiv* [Preprint]. doi: 10.1101/2020.10.08.330662

Kang, J., Hu, J., Karra, R., Dickson, A. L., Tornini, V. A., Nachtrab, G., et al. (2016). Modulation of tissue repair by regeneration enhancer elements. *Nature* 532, 201–206. doi: 10.1038/nature17644

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., et al. (2007). The medaka draft genome and insights into vertebrate genome evolution. *Nature* 447, 714–719. doi: 10.1038/nature05846

Katogi, R., Nakatani, Y., Shin-i, T., Kohara, Y., Inohaya, K., and Kudo, A. (2004). Large-scale analysis of the genes involved in fin regeneration and blastema formation in the medaka, Oryzias latipes. *Mech. Dev.* 121, 861–872. doi: 10.1016/j.mod.2004.03.015

Kawakami, K. (2007). Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol.* 8(Suppl. 1):S7. doi: 10.1186/gb-2007-8-s1-s7

Kawakami, K., and Shima, A. (1999). Identification of the Tol2 transposase of the medaka fish Oryzias latipes that catalyzes excision of a nonautonomous Tol2 element in zebrafish *Danio rerio*. *Gene* 240, 239–244. doi: 10.1016/s0378-1119(99)00444-8

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Marti, M., Dubova, I., et al. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. *Genes. Dev.* 20, 3232–3237. doi: 10.1101/gad.1475106

Kawasaki, T., Kurauchi, K., Higashihata, A., Deguchi, T., Ishikawa, Y., Yamauchi, M., et al. (2012). Transgenic medaka fish which mimic the endogenous expression of neuronal kinesin, KIF5A. *Brain Res.* 1480, 12–21. doi: 10.1016/j.brainres.2012.08.047

Kettleborough, R. N., Busch-Nentwich, E. M., Harvey, S. A., Dooley, C. M., de Bruijn, E., van Eeden, F., et al. (2013). A systematic genome-wide analysis of zebrafish protein-coding gene function. *Nature* 496, 494–497. doi: 10.1038/nature11992

Khyeam, S., Lee, S., and Huang, G. N. (2021). Genetic, epigenetic, and post-transcriptional basis of divergent tissue regenerative capacities among vertebrates. *Adv. Genet.* 7:e10042. doi: 10.1002/ggn2.10042

Kikuchi, K., Holdway, J. E., Major, R. J., Blum, N., Dahn, R. D., Begemann, G., et al. (2011b). Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell* 20, 397–404. doi: 10.1016/j.devcel.2011.01.010

Kikuchi, K., Gupta, V., Wang, J., Holdway, J. E., Wills, A. A., Fang, Y., et al. (2011a). tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* 138, 2895–2902. doi: 10.1242/dev.067041

Kikuchi, K., Holdway, J. E., Werdich, A. A., Anderson, R. M., Fang, Y., Egnaczyk, G. F., et al. (2010). Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* 464, 601–605. doi: 10.1038/nature08804

Kimura, Y., Hisano, Y., Kawahara, A., and Higashijima, S. (2014). Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. *Sci. Rep.* 4:6545. doi: 10.1038/srep06545

Kinkel, M. D., Eames, S. C., Philipson, L. H., and Prince, V. E. (2010). Intraperitoneal injection into adult zebrafish. *J. Vis. Exp.* 42:2126. doi: 10.3791/2126

Kinoshita, M., Kani, S., Ozato, K., and Wakamatsu, Y. (2000). Activity of the medaka translation elongation factor 1α-A promoter examined using the GFP gene as a reporter. *Dev. Growth Differ.* 42, 469–478. doi: 10.1046/j.1440-169x.2000.00530.x

Kinoshita, M., Murata, K., Naruse, K., and Tanaka, M. (2009). *Medaka: Biology, Management, and Experimental Protocols*. Honoken, NJ: John Wiley & Sons.

Kirchmaier, S., Höckendorf, B., Möller, E. K., Bornhorst, D., Spitz, F., and Wittbrodt, J. (2013). Efficient site-specific transgenesis and enhancer activity tests in medaka using PhiC31 integrase. *Development* 140, 4287–4295. doi: 10.1242/dev.096081

Kirchmaier, S., Naruse, K., Wittbrodt, J., and Loosli, F. (2015). The genomic and genetic toolbox of the teleost medaka (*Oryzias latipes*). *Genetics* 199, 905–918. doi: 10.1534/genetics.114.173849

Kishimoto, N., Shimizu, K., and Sawamoto, K. (2012). Neuronal regeneration in a zebrafish model of adult brain injury. *Dis. Model. Mech.* 5, 200–209. doi: 10.1242/dmm.007336

Kizil, C., and Brand, M. (2011). Cerebroventricular microinjection (CVMI) into adult zebrafish brain is an efficient misexpression method for forebrain ventricular cells. *PLoS One* 6:e27395. doi: 10.1371/journal.pone.0027395

Knopf, F., Hammond, C., Chekuru, A., Kurth, T., Hans, S., Weber, C. W., et al. (2011). Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Dev. Cell* 20, 713–724. doi: 10.1016/j.devcel.2011.04.014

Knopf, F., Schnabel, K., Haase, C., Pfeifer, K., Anastassiadis, K., and Weidinger, G. (2010). Dually inducible TetON systems for tissue-specific conditional gene expression in zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19933–19938. doi: 10.1073/pnas.1007799107

Kobayashi, D., and Takeda, H. (2008). Medaka genome project. *Brief Funct. Genomic. Proteomic.* 7, 415–426. doi: 10.1093/bfgp/eln044

Koga, A., Hori, H., and Sakaizumi, M. (2002). Gene transfer and cloning of flanking chromosomal regions using the medaka fish Tol2 transposable element. *Mar. Biotechnol.* 4, 6–11. doi: 10.1007/s10126-001-0086-2

Kong, P., Christia, P., and Frangogiannis, N. G. (2014). The pathogenesis of cardiac fibrosis. *Cell. Mol. Life Sci.* 71, 549–574.

Kroehne, V., Freudenreich, D., Hans, S., Kaslin, J., and Brand, M. (2011). Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. *Development* 138, 4831–4841. doi: 10.1242/dev.072587

Kucenas, S., Takada, N., Park, H. C., Woodruff, E., Broadie, K., and Appel, B. (2008). CNS-derived glia ensheath peripheral nerves and mediate motor root development. *Nat. Neurosci.* 11, 143–151. doi: 10.1038/nn2025

Kumar, S., Stecher, G., Suleski, M., and Hedges, S. B. (2017). TimeTree: a resource for timelines, timetrees, and divergence times. *Mol. Biol. Evol.* 34, 1812–1819. doi: 10.1093/molbev/msx116

Kurita, R., Sagara, H., Aoki, Y., Link, B. A., Arai, K.-I., and Watanabe, S. (2003). Suppression of lens growth by αA-crystallin promoter-driven expression of diphtheria toxin results in disruption of retinal cell organization in zebrafish. *Dev. Biol.* 255, 113–127. doi: 10.1016/s0012-1606(02)00079-9

Kuroyanagi, Y., Okuyama, T., Suehiro, Y., Imada, H., Shimada, A., Naruse, K., et al. (2010). Proliferation zones in adult medaka (*Oryzias latipes*) brain. *Brain Res.* 1323, 33–40. doi: 10.1016/j.brainres.2010.01.045

Kyritsis, N., Kizil, C., Zocher, S., Kroehne, V., Kaslin, J., Freudenreich, D., et al. (2012). Acute inflammation initiates the regenerative response in the adult zebrafish brain. *Science* 338, 1353–1356. doi: 10.1126/science.1228773

Labusch, M., Mancini, L., Morizet, D., and Bally-Cuif, L. (2020). Conserved and Divergent Features of Adult Neurogenesis in Zebrafish. *Front. Cell Dev. Biol.* 8:525. doi: 10.3389/fcell.2020.00525

Lafontant, P. J., Burns, A. R., Grivas, J. A., Lesch, M. A., Lala, T. D., Reuter, S. P., et al. (2012). The giant danio (D. aequipinnatus) as a model of cardiac remodeling and regeneration. *Anat. Rec.* 295, 234–248. doi: 10.1002/ar.21492

Lai, S.-L., Marín-Juez, R., Moura, P. L., Kuenne, C., Lai, J. K. H., Tsedeke, A. T., et al. (2017). Reciprocal analyses in zebrafish and medaka reveal that harnessing the immune response promotes cardiac regeneration. *eLife* 6:e25605. doi: 10.7554/eLife.25605

Langenau, D. M., Ferrando, A. A., Traver, D., Kutok, J. L., Hezel, J. P., Kanki, J. P., et al. (2004). *In vivo* tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* 101, 7369–7374. doi: 10.1073/pnas.0402248101

Laube, F., Heister, M., Scholz, C., Borchardt, T., and Braun, T. (2006). Re-programming of newt cardiomyocytes is induced by tissue regeneration. *J. Cell Sci.* 119(Pt 22), 4719–4729. doi: 10.1242/jcs.03252

Lawson, N. D., and Weinstein, B. M. (2002). *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* 248, 307–318. doi: 10.1006/dbio.2002.0711

Le, X., Langenau, D. M., Keefe, M. D., Kutok, J. L., Neuberg, D. S., and Zon, L. I. (2007). Heat shock-inducible Cre/Lox approaches to induce diverse types of tumors and hyperplasia in transgenic zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9410–9415. doi: 10.1073/pnas.0611302104

Leach, L. L., Hanovice, N. J., George, S. M., Gabriel, A. E., and Gross, J. M. (2021). The immune response is a critical regulator of zebrafish retinal pigment epithelium regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 118, e2017198118. doi: 10.1073/pnas.2017198118

Lenkowski, J. R., and Raymond, P. A. (2014). Müller glia: stem cells for generation and regeneration of retinal neurons in teleost fish. *Prog. Retin. Eye Res.* 40, 94–123. doi: 10.1016/j.preteyeres.2013.12.007

Letunic, I., and Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301

Li, J., Iwanami, N., Hoa, V. Q., Furutani-Seiki, M., and Takahama, Y. (2007). Noninvasive intravital imaging of thymocyte dynamics in medaka. *J. Immunol.* 179, 1605–1615. doi: 10.4049/jimmunol.179.3.1605

Li, L., Yan, B., Shi, Y. Q., Zhang, W. Q., and Wen, Z. L. (2012). Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration. *J. Biol. Chem.* 287, 25353–25360. doi: 10.1074/jbc.M112.349126

Li, Y., Liu, Y., Yang, H., Zhang, T., Naruse, K., and Tu, Q. (2020). Dynamic transcriptional and chromatin accessibility landscape of medaka embryogenesis. *Genome Res.* 30, 924–937. doi: 10.1101/gr.258871.119

Lin, H.-F., Traver, D., Zhu, H., Dooley, K., Paw, B. H., Zon, L. I., et al. (2005). Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood* 106, 3803–3810. doi: 10.1182/blood-2005-01-0179

Lin, J.-W., Chen, Y.-R., Wang, Y.-H., Hung, K.-C., and Lin, S.-M. (2017). Tail regeneration after autotomy revives survival: a case from a long-term monitored lizard population under avian predation. *Proc. R. Soc. B Biol. Sci.* 284:20162538. doi: 10.1098/rspb.2016.2538

Lindmark, D. G., and Müller, M. (1976). Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. *Antimicrob. Agents Chemother.* 10, 476–482. doi: 10.1128/AAC.10.3.476

Lindsey, B. W., Aitken, G. E., Tang, J. K., Khabooshan, M., Douek, A. M., Vandestadt, C., et al. (2019). Midbrain tectal stem cells display diverse regenerative capacities in zebrafish. *Sci. Rep.* 9:4420. doi: 10.1038/s41598-019-40734-z

Liu, K., Petree, C., Requena, T., Varshney, P., and Varshney, G. K. (2019). Expanding the CRISPR Toolbox in Zebrafish for studying development and disease. *Front. Cell Dev. Biol.* 7:13. doi: 10.3389/fcell.2019.00013

Liu, X., Li, Y.-S., Shinton, S. A., Rhodes, J., Tang, L., Feng, H., et al. (2017). Zebrafish B cell development without a pre–B cell stage, revealed by CD79 fluorescence reporter transgenes. *J. Immunol.* 199, 1706–1715. doi: 10.4049/jimmunol.1700552

Long, D. W., Webb, C. H., and Wang, Y. (2019). Persistent fibrosis and decreased cardiac function following cardiac injury in the *Ctenopharyngodon idella* (grass carp). *bioRxiv* [Preprint]. doi: 10.1101/627752

Loosli, F., Koster, R. W., Carl, M., Kuhnlein, R., Henrich, T., Mucke, M., et al. (2000). A genetic screen for mutations affecting embryonic development in medaka fish (*Oryzias latipes*). *Mech. Dev.* 97, 133–139. doi: 10.1016/s0925-4773(00)00406-8

Lopez-Baez, J. C., Simpson, D. J., LLeras Forero, L., Zeng, Z., Brunsdon, H., Salzano, A., et al. (2018). Wilms Tumor 1b defines a wound-specific sheath cell subpopulation associated with notochord repair. *eLife* 7:e30657. doi: 10.7554/eLife.30657

Lush, M. E., and Piotrowski, T. (2014). Sensory hair cell regeneration in the zebrafish lateral line. *Dev. Dyn.* 243, 1187–1202. doi: 10.1002/dvdy.24167

Lust, K., and Wittbrodt, J. (2018). Activating the regenerative potential of Müller glia cells in a regeneration-deficient retina. *eLife* 7:e32319. doi: 10.7554/eLife.32319

Maekawa, S., Chiang, Y.-A., Hikima, J.-I., Sakai, M., Lo, C.-F., Wang, H.-C., et al. (2016). Expression and biological activity of two types of interferon genes in medaka (*Oryzias latipes*). *Fish Shellfish Immunol.* 48, 20–29. doi: 10.1016/j.fsi.2015.11.036

Maginnis, T. L. (2006). The costs of autotomy and regeneration in animals: a review and framework for future research. *Behav. Ecol.* 17, 857–872. doi: 10.1093/beheco/arl010

Maheras, A. L., Dix, B., Carmo, O. M., Young, A. E., Gill, V. N., Sun, J. L., et al. (2018). Genetic pathways of neuroregeneration in a novel mild traumatic brain injury model in adult zebrafish. *Eneuro* 5:ENEURO.0208-17.2017. doi: 10.1523/ENEURO.0208-17

Marín-Juez, R., El-Sammak, H., Helker, C. S., Kamezaki, A., Mullapuli, S. T., Bibli, S.-I., et al. (2019). Coronary revascularization during heart regeneration is regulated by epicardial and endocardial cues and forms a scaffold for cardiomyocyte repopulation. *Dev. Cell* 51, 503–515.e4. doi: 10.1016/j.devcel.2019.10.019

Marín-Juez, R., Marass, M., Gauvrit, S., Rossi, A., Lai, S.-L., Materna, S. C., et al. (2016). Fast revascularization of the injured area is essential to support zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11237–11242. doi: 10.1073/pnas.1605431113

Marletaz, F., Firbas, P. N., Maeso, I., Tena, J. J., Bogdanovic, O., Perry, M., et al. (2018). Amphioxus functional genomics and the origins of vertebrate gene regulation. *Nature* 564, 64–70. doi: 10.1038/s41586-018-0734-6

Marques, I. J., Lupi, E., and Mercader, N. (2019). Model systems for regeneration: zebrafish. *Development* 146:dev167692. doi: 10.1242/dev.167692

Martinez-Morales, J. R., Rembold, M., Greger, K., Simpson, J. C., Brown, K. E., Quiring, R., et al. (2009). Ojoplano-mediated basal constriction is essential for optic cup morphogenesis. *Development* 136, 2165–2175. doi: 10.1242/dev.033563

Martins, R. R., Ellis, P. S., MacDonald, R. B., Richardson, R. J., and Henriques, C. M. (2019). Resident immunity in tissue repair and maintenance: the zebrafish model coming of age. *Front. Cell Dev. Biol.* 7:12. doi: 10.3389/fcell.2019.00012

Marz, M., Schmidt, R., Rastegar, S., and Strahle, U. (2011). Regenerative response following stab injury in the adult zebrafish telencephalon. *Dev. Dyn.* 240, 2221–2231. doi: 10.1002/dvdy.22710

Matias Santos, D., Rita, A. M., Casanellas, I., Brito Ova, A., Araujo, I. M., Power, D., et al. (2016). Ear wound regeneration in the African spiny mouse *Acomys cahirinus*. *Regeneration* 3, 52–61. doi: 10.1002/reg2.50

McDonald, B., Pittman, K., Menezes, G. B., Hirota, S. A., Slaba, I., Waterhouse, C. C., et al. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 330, 362–366. doi: 10.1126/science.1195491

Mekdara, P. J., Schwalbe, M. A., Coughlin, L. L., and Tytell, E. D. (2018). The effects of lateral line ablation and regeneration in schooling giant danios. *J. Exp. Biol.* 221:jeb175166. doi: 10.1242/jeb.175166

Meng, X., Noyes, M. B., Zhu, L. J., Lawson, N. D., and Wolfe, S. A. (2008). Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat. Biotechnol.* 26, 695–701. doi: 10.1038/nbt1398

Mescher, A. L., and Neff, A. W. (2005). "Regenerative capacity and the developing immune system," in *Regenerative Medicine I: Theories, Models and Methods*, ed. I. V. Yannas (Berlin: Springer), 39–66. doi: 10.1007/b99966

Metzger, D., Clifford, J., Chiba, H., and Chambon, P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6991–6995. doi: 10.1073/pnas.92.15.6991

Mierzwa, A. S., Nguyen, F., Xue, M., and Jonz, M. G. (2020). Regeneration of the gill filaments and replacement of serotonergic neuroepithelial cells in adult zebrafish (*Danio rerio*). *Respir. Physiol. Neurobiol.* 274:103366. doi: 10.1016/j.resp.2019.103366

Miskey, C., Izsvák, Z., Plasterk, R. H., and Ivics, Z. (2003). The Frog Prince: a reconstructed transposon from Rana pipiens with high transpositional activity in vertebrate cells. *Nucleic Acids Res.* 31, 6873–6881. doi: 10.1093/nar/gkg910

Mitchell, D. M., Sun, C., Hunter, S. S., New, D. D., and Stenkamp, D. L. (2019). Regeneration associated transcriptional signature of retinal microglia and macrophages. *Sci. Rep.* 9:4768. doi: 10.1038/s41598-019-41298-8

Moens, C. B., Donn, T. M., Wolf-Saxon, E. R., and Ma, T. P. (2008). Reverse genetics in zebrafish by TILLING. *Brief Funct. Genomic Proteomic* 7, 454–459. doi: 10.1093/bfgp/eln046

Mollova, M., Bersell, K., Walsh, S., Savla, J., Das, L. T., Park, S. Y., et al. (2013). Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc. Natl. Acad. Sci. U.S.A.* 110, 1446–1451. doi: 10.1073/pnas.1214608110

Moreno, R. L., Josey, M., and Ribera, A. B. (2017). Zebrafish in Situ spinal cord preparation for electrophysiological recordings from spinal sensory and Motor Neurons. *J. Vis. Exp.* 122:e55507. doi: 10.3791/55507

Moreno-Mateos, M. A., Vejnar, C. E., Beaudoin, J. D., Fernandez, J. P., Mis, E. K., Khokha, M. K., et al. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting *in vivo*. *Nat. Methods* 12, 982–988. doi: 10.1038/nmeth.3543

Morgan, T. H. (1901). Regeneration and liability to injury. *Science* 14, 235–248. doi: 10.1126/science.14.346.235

Moriyama, A., Inohaya, K., Maruyama, K., and Kudo, A. (2010). Bef medaka mutant reveals the essential role of c-myb in both primitive and definitive hematopoiesis. *Dev. Biol.* 345, 133–143. doi: 10.1016/j.ydbio.2010.06.031

Mosimann, C., Kaufman, C. K., Li, P., Pugach, E. K., Tamplin, O. J., and Zon, L. I. (2011). Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish. *Development* 138, 169–177. doi: 10.1242/dev.059345

Mosimann, C., Puller, A. C., Lawson, K. L., Tschopp, P., Amsterdam, A., and Zon, L. I. (2013). Site-directed zebrafish transgenesis into single landing sites with the phiC31 integrase system. *Dev. Dyn.* 242, 949–963. doi: 10.1002/dvdy.23989

Moss, J. B., Koustubhan, P., Greenman, M., Parsons, M. J., Walter, I., and Moss, L. G. (2009). Regeneration of the pancreas in adult zebrafish. *Diabetes* 58, 1844–1851. doi: 10.2337/db08-0628

Motoike, T., Loughna, S., Perens, E., Roman, B. L., Liao, W., Chau, T. C., et al. (2000). Universal GFP reporter for the study of vascular development. *Genesis* 28, 75–81. doi: 10.1002/1526-968x(200010)28:2<75::aid-gene50>3.0.co;2-s

Mukherjee, K., and Liao, E. C. (2018). Generation and characterization of a zebrafish muscle specific inducible Cre line. *Transgenic Res.* 27, 559–569. doi: 10.1007/s11248-018-0098-6

Murakami, Y., Ansai, S., Yonemura, A., and Kinoshita, M. (2017). An efficient system for homology-dependent targeted gene integration in medaka (*Oryzias latipes*). *Zool. Lett.* 3:10. doi: 10.1186/s40851-017-0071-x

Murata, K., Kinoshita, M., Naruse, K., Tanaka, M., and Kamei, Y. (2019). *Medaka: Biology, Management, and Experimental Protocols*. Honoken, NJ: John Wiley & Sons, Incorporated.

Nakamura, R., Tsukahara, T., Qu, W., Ichikawa, K., Otsuka, T., Ogoshi, K., et al. (2014). Large hypomethylated domains serve as strong repressive machinery for key developmental genes in vertebrates. *Development* 141, 2568–2580. doi: 10.1242/dev.108548

Nakamura, S., Saito, D., and Tanaka, M. (2008). Generation of transgenic medaka using modified bacterial artificial chromosome. *Dev. Growth Differ.* 50, 415–419. doi: 10.1111/j.1440-169X.2008.01027.x

Nakatani, Y., Kawakami, A., and Kudo, A. (2007). Cellular and molecular processes of regeneration, with special emphasis on fish fins. *Dev. Growth Differ.* 49, 145–154. doi: 10.1111/j.1440-169X.2007.00917.x

Nakatani, Y., Nishidate, M., Fujita, M., Kawakami, A., and Kudo, A. (2008). Migration of mesenchymal cell fated to blastema is necessary for fish fin regeneration. *Dev. Growth Differ.* 50, 71–83. doi: 10.1111/j.1440-169X.2007.00977.x

Nasevicius, A., and Ekker, S. C. (2000). Effective targeted gene 'knockdown'in zebrafish. *Nat. Genet.* 26, 216–220. doi: 10.1038/79951

Ng, G. H. B., and Gong, Z. (2011). Maize Ac/Ds transposon system leads to highly efficient germline transmission of transgenes in medaka (*Oryzias latipes*). *Biochimie* 93, 1858–1864. doi: 10.1016/j.biochi.2011.07.006

Nguyen-Chi, M., Laplace-Builhe, B., Travnickova, J., Luz-Crawford, P., Tejedor, G., Phan, Q. T., et al. (2015). Identification of polarized macrophage subsets in zebrafish. *eLife* 4:e07288. doi: 10.7554/eLife.07288

Nieto-Arellano, R., and Sanchez-Iranzo, H. (2019). zfRegeneration: a database for gene expression profiling during regeneration. *Bioinformatics* 35, 703–705. doi: 10.1093/bioinformatics/bty659

Offen, N., Blum, N., Meyer, A., and Begemann, G. (2008). Fgfr1 signalling in the development of a sexually selected trait in vertebrates, the sword of swordtail fish. *BMC Dev. Biol.* 8:98. doi: 10.1186/1471-213X-8-98

Okuda, K. S., Astin, J. W., Misa, J. P., Flores, M. V., Crosier, K. E., and Crosier, P. S. (2012). lyve1 expression reveals novel lymphatic vessels and new mechanisms for lymphatic vessel development in zebrafish. *Development* 139, 2381–2391. doi: 10.1242/dev.077701

Okuyama, T., Isoe, Y., Hoki, M., Suehiro, Y., Yamagishi, G., Naruse, K., et al. (2013). Controlled Cre/loxP site-specific recombination in the developing brain in medaka fish, *Oryzias latipes*. *PLoS One* 8:e66597. doi: 10.1371/journal.pone.0066597

Otsuka, T., and Takeda, H. (2017). Targeted Ablation of Pancreatic beta Cells in Medaka. *Zool. Sci.* 34, 179–184. doi: 10.2108/zs170004

Otsuka, T., Tsukahara, T., and Takeda, H. (2015). Development of the pancreas in medaka, *Oryzias latipes*, from embryo to adult. *Dev. Growth Differ.* 57, 557–569. doi: 10.1111/dgd.12237

Ozato, K., Kondoh, H., Inohara, H., Iwamatsu, T., Wakamatsu, Y., and Okada, T. S. (1986). Production of transgenic fish: introduction and expression of chicken delta-crystallin gene in medaka embryos. *Cell Differ.* 19, 237–244. doi: 10.1016/0045-6039(86)90100-4

Page, D. M., Wittamer, V., Bertrand, J. Y., Lewis, K. L., Pratt, D. N., Delgado, N., et al. (2013). An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood J. Am. Soc. Hematol.* 122, e1–e11. doi: 10.1182/blood-2012-12-471029

Park, H. C., Kim, C. H., Bae, Y. K., Yeo, S. Y., Kim, S. H., Hong, S. K., et al. (2000). Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev. Biol.* 227, 279–293. doi: 10.1006/dbio.2000.9898

Perner, B., Englert, C., and Bollig, F. (2007). The Wilms tumor genes wt1a and wt1b control different steps during formation of the zebrafish pronephros. *Dev. Biol.* 309, 87–96. doi: 10.1016/j.ydbio.2007.06.022

Petrie, T. A., Strand, N. S., Yang, C. T., Rabinowitz, J. S., and Moon, R. T. (2014). Macrophages modulate adult zebrafish tail fin regeneration. *Development* 141, 2581–2591. doi: 10.1242/dev.098459

Pfefferli, C., and Jazwinska, A. (2015). The art of fin regeneration in zebrafish. *Regeneration* 2, 72–83. doi: 10.1002/reg2.33

Pfefferli, C., and Jaźwińska, A. (2017). The careg element reveals a common regulation of regeneration in the zebrafish myocardium and fin. *Nat. Commun.* 8:15151. doi: 10.1038/ncomms15151

Phan, Q. T., Tan, W. H., Liu, R., Sundaram, S., Buettner, A., Kneitz, S., et al. (2020). Cxcl9l and Cxcr3.2 regulate recruitment of osteoclast progenitors to bone matrix in a medaka osteoporosis model. *Proc. Natl. Acad. Sci. U.S.A.* 117, 19276–19286. doi: 10.1073/pnas.2006093117

Pisharath, H., Rhee, J. M., Swanson, M. A., Leach, S. D., and Parsons, M. J. (2007). Targeted ablation of beta cells in the embryonic zebrafish pancreas using E. coli nitroreductase. *Mech. Dev.* 124, 218–229. doi: 10.1016/j.mod.2006.11.005

Poleo, G., Brown, C. W., Laforest, L., and Akimenko, M. A. (2001). Cell proliferation and movement during early fin regeneration in zebrafish. *Dev. Dyn.* 221, 380–390. doi: 10.1002/dvdy.1152

Polizzotti, B. D., Ganapathy, B., Haubner, B. J., Penninger, J. M., and Kühn, B. (2016). A cryoinjury model in neonatal mice for cardiac translational and regeneration research. *Nat. Protoc.* 11, 542–552. doi: 10.1038/nprot.2016.031

Porazinski, S. R., Wang, H., and Furutani-Seiki, M. (2010b). Microinjection of medaka embryos for use as a model genetic organism. *J. Vis. Exp.* 46:e1937. doi: 10.3791/1937

Porazinski, S. R., Wang, H., and Furutani-Seiki, M. (2010a). Dechorionation of medaka embryos and cell transplantation for the generation of chimeras. *J. Vis. Exp.* 46:e2055. doi: 10.3791/2055

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., et al. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* 331, 1078–1080. doi: 10.1126/science.1200708

Poss, K. D., Keating, M. T., and Nechiporuk, A. (2003). Tales of regeneration in zebrafish. *Dev. Dyn.* 226, 202–210. doi: 10.1002/dvdy.10220

Poss, K. D., Wilson, L. G., and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* 298, 2188–2190. doi: 10.1126/science.1077857

Potts, H. G., Stockdale, W. T., and Mommersteeg, M. T. M. (2021). Unlocking the secrets of the regenerating fish heart: comparing regenerative models to shed light on successful regeneration. *J. Cardiovasc. Dev. Dis.* 8:10004. doi: 10.3390/jcdd8010004

Powell, C., Cornblath, E., Elsaeidi, F., Wan, J., and Goldman, D. (2016). Zebrafish Müller glia-derived progenitors are multipotent, exhibit proliferative biases and regenerate excess neurons. *Sci. Rep.* 6:24851. doi: 10.1038/srep24851

Provost, E., Rhee, J., and Leach, S. D. (2007). Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis* 45, 625–629. doi: 10.1002/dvg.20338

Pugach, E. K., Li, P., White, R., and Zon, L. (2009). Retro-orbital injection in adult zebrafish. *J. Vis. Exp.* 34:1645. doi: 10.3791/1645

Qi, Y., Dasa, O., Maden, M., Vohra, R., Batra, A., Walter, G., et al. (2021). Functional heart recovery in an adult mammal, the spiny mouse. *Int. J. Cardiol.* 338, 196–203. doi: 10.1016/j.ijcard.2021.06.015

Ramachandran, R., Fausett, B. V., and Goldman, D. (2010a). Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nat. Cell Biol.* 12, 1101–1107. doi: 10.1038/ncb2115

Ramachandran, R., Reifler, A., Parent, J. M., and Goldman, D. (2010b). Conditional gene expression and lineage tracing of tuba1a expressing cells during zebrafish development and retina regeneration. *J. Comp. Neurol.* 518, 4196–4212. doi: 10.1002/cne.22448

Ramel, M.-C., Progatzky, F., Rydlova, A., Wane, M., Schymeinsky, J., Williams, C., et al. (2021). Dynamics of repair and regeneration of adult zebrafish respiratory gill tissue after cryoinjury. *bioRxiv* [Preprint]. doi: 10.1101/2021.05.27.445469

Raymond, P. A., Barthel, L. K., Bernardos, R. L., and Perkowski, J. J. (2006). Molecular characterization of retinal stem cells and their niches in adult zebrafish. *BMC Dev. Biol.* 6:36. doi: 10.1186/1471-213X-6-36

Raymond, P. A., Reifler, M. J., and Rivlin, P. K. (1988). Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. *J. Neurobiol.* 19, 431–463. doi: 10.1002/neu.480190504

Redd, M. J., Kelly, G., Dunn, G., Way, M., and Martin, P. (2006). Imaging macrophage chemotaxis in vivo: studies of microtubule function in zebrafish wound inflammation. *Cell Motil. Cytoskeleton* 63, 415–422. doi: 10.1002/cm.20133

Reddien, P. W., and Alvarado, A. S. (2004). Fundamentals of planarian regeneration. *Annu. Rev. Cell Dev. Biol.* 20, 725–757. doi: 10.1146/annurev.cellbio.20.010403.095114

Reddy, P. C., Gungi, A., and Unni, M. (2019). Cellular and molecular mechanisms of Hydra regeneration. *Results Probl. Cell Differ.* 68, 259–290. doi: 10.1007/978-3-030-23459-1_12

Reimschuessel, R., and Williams, D. (1995). Development of new nephrons in adult kidneys following gentamicin-induced nephrotoxicity. *Ren. Fail.* 17, 101–106. doi: 10.3109/08860229509026246

Reinhardt, R., Centanin, L., Tavhelidse, T., Inoue, D., Wittbrodt, B., Concordet, J. P., et al. (2015). Sox2, Tlx, Gli3, and Her9 converge on Rx2 to define retinal stem cells *in vivo*. *EMBO J.* 34, 1572–1588. doi: 10.15252/embj.201490706

Renn, J., Büttner, A., To, T. T., Chan, S. J. H., and Winkler, C. (2013). A col10a1: nlGFP transgenic line displays putative osteoblast precursors at the medaka notochordal sheath prior to mineralization. *Dev. Biol.* 381, 134–143. doi: 10.1016/j.ydbio.2013.05.030

Renn, J., and Winkler, C. (2009). Osterix-mCherry transgenic medaka for *in vivo* imaging of bone formation. *Dev. Dyn.* 238, 241–248. doi: 10.1002/dvdy.21836

Renshaw, S. A., Loynes, C. A., Trushell, D. M., Elworthy, S., Ingham, P. W., and Whyte, M. K. (2006). A transgenic zebrafish model of neutrophilic inflammation. *Blood* 108, 3976–3978. doi: 10.1182/blood-2006-05-024075

Richardson, R., Slanchev, K., Kraus, C., Knyphausen, P., Eming, S., and Hammerschmidt, M. (2013). Adult zebrafish as a model system for cutaneous

wound-healing research. *J. Invest. Dermatol.* 133, 1655–1665. doi: 10.1038/jid.2013.16

Robert, J., and Ohta, Y. (2009). Comparative and developmental study of the immune system in Xenopus. *Dev. Dyn.* 238, 1249–1270. doi: 10.1002/dvdy.21891

Roh-Johnson, M., Shah, A. N., Stonick, J. A., Poudel, K. R., Kargl, J., Yang, G. H., et al. (2017). Macrophage-dependent cytoplasmic transfer during melanoma invasion *in vivo*. *Dev. Cell* 43, 549–562.e6. doi: 10.1016/j.devcel.2017.11.003

Rottbauer, W., Saurin, A. J., Lickert, H., Shen, X., Burns, C. G., Wo, Z. G., et al. (2002). Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell* 111, 661–672. doi: 10.1016/s0092-8674(02)01112-1

Ruyra, A., Cano-Sarabia, M., Garcia-Valtanen, P., Yero, D., Gibert, I., Mackenzie, S. A., et al. (2014). Targeting and stimulation of the zebrafish (*Danio rerio*) innate immune system with LPS/dsRNA-loaded nanoliposomes. *Vaccine* 32, 3955–3962. doi: 10.1016/j.vaccine.2014.05.010

Ruzicka, L., Howe, D. G., Ramachandran, S., Toro, S., Van Slyke, C. E., Bradford, Y. M., et al. (2019). The Zebrafish information network: new support for non-coding genes, richer gene ontology annotations and the alliance of genome resources. *Nucleic Acids Res.* 47, D867–D873. doi: 10.1093/nar/gky1090

Sadler, K. C., Krahn, K. N., Gaur, N. A., and Ukomadu, C. (2007). Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1570–1575. doi: 10.1073/pnas.0610774104

Sanchez-Iranzo, H., Galardi-Castilla, M., Sanz-Morejon, A., Gonzalez-Rosa, J. M., Costa, R., Ernst, A., et al. (2018). Transient fibrosis resolves via fibroblast inactivation in the regenerating zebrafish heart. *Proc. Natl. Acad. Sci. U.S.A.* 115, 4188–4193. doi: 10.1073/pnas.1716713115

Sander, J. D., Cade, L., Khayter, C., Reyon, D., Peterson, R. T., Joung, J. K., et al. (2011). Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat. Biotechnol.* 29, 697–698. doi: 10.1038/nbt.1934

Sanderson, L. E., Chien, A.-T., Astin, J. W., Crosier, K. E., Crosier, P. S., and Hall, C. J. (2015). An inducible transgene reports activation of macrophages in live zebrafish larvae. *Dev. Comp. Immunol.* 53, 63–69. doi: 10.1016/j.dci.2015.06.013

Sano, S., Takashima, S., Niwa, H., Yokoi, H., Shimada, A., Arenz, A., et al. (2009). Characterization of teleost Mdga1 using a gene-trap approach in medaka (*Oryzias latipes*). *Genesis* 47, 505–513. doi: 10.1002/dvg.20528

Sanz-Morejon, A., Garcia-Redondo, A. B., Reuter, H., Marques, I. J., Bates, T., Galardi-Castilla, M., et al. (2019). Wilms Tumor 1b Expression Defines a Pro-regenerative Macrophage Subtype and Is Required for Organ Regeneration in the Zebrafish. *Cell Rep.* 28, 1296–1306.e6. doi: 10.1016/j.celrep.2019.06.091

Sasado, T., Tanaka, M., Kobayashi, K., Sato, T., Sakaizumi, M., and Naruse, K. (2010). The National BioResource Project Medaka (NBRP Medaka): an integrated bioresource for biological and biomedical sciences. *Exp. Anim.* 59, 13–23. doi: 10.1538/expanim.59.13

Sassen, W. A., and Köster, R. W. (2015). A molecular toolbox for genetic manipulation of zebrafish. *Adv. Genomics Genet.* 5, 151–163. doi: 10.2147/AGG.S57585

Schaafhausen, M. K., Yang, W.-J., Centanin, L., Wittbrodt, J., Bosserhoff, A., Fischer, A., et al. (2013). Tumor angiogenesis is caused by single melanoma cells in a reactive oxygen species and NF-κB dependent manner. *J. Cell Sci.* 126, 3862–3872. doi: 10.1242/jcs.125021

Schall, K. A., Holoyda, K. A., Grant, C. N., Levin, D. E., Torres, E. R., Maxwell, A., et al. (2015). Adult zebrafish intestine resection: a novel model of short bowel syndrome, adaptation, and intestinal stem cell regeneration. *Am. J. Physiol. Gastrointest. Liver Physiol.* 309, G135–G145. doi: 10.1152/ajpgi.00311.2014

Schmidt, R., Beil, T., Strähle, U., and Rastegar, S. (2014). Stab wound injury of the zebrafish adult telencephalon: a method to investigate vertebrate brain neurogenesis and regeneration. *J. Vis. Exp.* 90:e51753. doi: 10.3791/51753

Schnabel, K., Wu, C. C., Kurth, T., and Weidinger, G. (2011). Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS One* 6:e18503. doi: 10.1371/journal.pone.0018503

Schuck, J. B., and Smith, M. E. (2009). Cell proliferation follows acoustically-induced hair cell bundle loss in the zebrafish saccule. *Hear. Res.* 253, 67–76. doi: 10.1016/j.heares.2009.03.008

Seifert, A. W., Kiama, S. G., Seifert, M. G., Goheen, J. R., Palmer, T. M., and Maden, M. (2012). Skin shedding and tissue regeneration in African spiny mice (Acomys). *Nature* 489, 561–565. doi: 10.1038/nature11499

Seleit, A., Gross, K., Onistschenko, J., Hoang, O. P., Theelke, J., and Centanin, L. (2022). Local tissue interactions govern pLL patterning in medaka. *Dev. Biol.* 481, 1–13. doi: 10.1016/j.ydbio.2021.09.002

Seleit, A., Gross, K., Onistschenko, J., Woelk, M., Autorino, C., and Centanin, L. (2020). Development and regeneration dynamics of the Medaka notochord. *Dev. Biol.* 463, 11–25. doi: 10.1016/j.ydbio.2020.03.001

Seleit, A., Krämer, I., Riebesehl, B. F., Ambrosio, E. M., Stolper, J. S., Lischik, C. Q., et al. (2017b). Neural stem cells induce the formation of their physical niche during organogenesis. *eLife* 6:e29173. doi: 10.7554/eLife.29173

Seleit, A., Kramer, I., Ambrosio, E., Dross, N., Engel, U., and Centanin, L. (2017a). Sequential organogenesis sets two parallel sensory lines in medaka. *Development* 144, 687–697. doi: 10.1242/dev.142752

Senut, M.-C., Gulati-Leekha, A., and Goldman, D. (2004). An element in the α1-tubulin promoter is necessary for retinal expression during optic nerve regeneration but not after eye injury in the adult zebrafish. *J. Neurosci.* 24, 7663–7673. doi: 10.1523/JNEUROSCI.2281-04.2004

Sherpa, T., Fimbel, S. M., Mallory, D. E., Maaswinkel, H., Spritzer, S. D., Sand, J. A., et al. (2008). Ganglion cell regeneration following whole-retina destruction in zebrafish. *Dev. Neurobiol.* 68, 166–181. doi: 10.1002/dneu.20568

Shimizu, Y., Ito, Y., Tanaka, H., and Ohshima, T. (2015). Radial glial cell-specific ablation in the adult Z ebrafish brain. *Genesis* 53, 431–439. doi: 10.1002/dvg.22865

Shimizu, Y., and Kawasaki, T. (2021). Differential regenerative capacity of the optic Tectum of Adult Medaka and Zebrafish. *Front. Cell Dev. Biol.* 9:686755. doi: 10.3389/fcell.2021.686755

Shimizu, Y., Ueda, Y., and Ohshima, T. (2018). Wnt signaling regulates proliferation and differentiation of radial glia in regenerative processes after stab injury in the optic tectum of adult zebrafish. *Glia* 66, 1382–1394. doi: 10.1002/glia.23311

Simkin, J., Gawriluk, T. R., Gensel, J. C., and Seifert, A. W. (2017). Macrophages are necessary for epimorphic regeneration in African spiny mice. *eLife* 6:e24623. doi: 10.7554/eLife.24623

Simoes, F. C., Cahill, T. J., Kenyon, A., Gavriouchkina, D., Vieira, J. M., Sun, X., et al. (2020). Macrophages directly contribute collagen to scar formation during zebrafish heart regeneration and mouse heart repair. *Nat. Commun.* 11:600. doi: 10.1038/s41467-019-14263-2

Simon, A., and Tanaka, E. M. (2013). Limb regeneration. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 291–300. doi: 10.1002/wdev.73

Singh, S. P., Holdway, J. E., and Poss, K. D. (2012). Regeneration of amputated zebrafish fin rays from de novo osteoblasts. *Dev. Cell* 22, 879–886. doi: 10.1016/j.devcel.2012.03.006

Soehnlein, O., and Lindbom, L. (2010). Phagocyte partnership during the onset and resolution of inflammation. *Nat. Rev. Immunol.* 10, 427–439. doi: 10.1038/nri2779

Sorlien, E. L., Witucki, M. A., and Ogas, J. (2018). Efficient Production and Identification of CRISPR/Cas9-generated Gene Knockouts in the Model System *Danio rerio*. *J. Vis. Exp.* 138:56969. doi: 10.3791/56969

Steinhauser, M. L., and Lee, R. T. (2011). Regeneration of the heart. *EMBO Mol. Med.* 3, 701–712.

Stemmer, M., Thumberger, T., Del Sol, Keyer, M., Wittbrodt, J., and Mateo, J. L. (2015). CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PLoS One* 10:e0124633. doi: 10.1371/journal.pone.0124633

Stockdale, W. T., Lemieux, M. E., Killen, A. C., Zhao, J., Hu, Z., Riepsaame, J., et al. (2018). Heart regeneration in the Mexican Cavefish. *Cell Rep.* 25, 1997–2007.e7. doi: 10.1016/j.celrep.2018.10.072

Stolper, J., Ambrosio, E. M., Danciu, D.-P., Buono, L., Elliott, D. A., Naruse, K., et al. (2019). Stem cell topography splits growth and homeostatic functions in the fish gill. *eLife* 8:e43747. doi: 10.7554/eLife.43747

Straube, W. L., and Tanaka, E. M. (2006). Reversibility of the differentiated state: regeneration in amphibians. *Artif. Organs* 30, 743–755. doi: 10.1111/j.1525-1594.2006.00296.x

Streisinger, G., Singer, F., Walker, C., Knauber, D., and Dower, N. (1986). Segregation analyses and gene-centromere distances in zebrafish. *Genetics* 112, 311–319. doi: 10.1093/genetics/112.2.311

Streisinger, G., Walker, C., Dower, N., Knauber, D., and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* 291, 293–296. doi: 10.1038/291293a0

Strungs, E. G., Ongstad, E. L., O'Quinn, M. P., Palatinus, J. A., Jourdan, L. J., and Gourdie, R. G. (2013). Cryoinjury models of the adult and neonatal mouse heart for studies of scarring and regeneration. *Methods Mol. Biol.* 1037, 343–353. doi: 10.1007/978-1-62703-505-7_20

Suehiro, Y., Kinoshita, M., Okuyama, T., Shimada, A., Naruse, K., Takeda, H., et al. (2010). Transient and permanent gene transfer into the brain of the teleost fish medaka (Oryzias latipes) using human adenovirus and the Cre-loxP system. *FEBS Lett.* 584, 3545–3549. doi: 10.1016/j.febslet.2010.06.047

Suster, M. L., Abe, G., Schouw, A., and Kawakami, K. (2011). Transposon-mediated BAC transgenesis in zebrafish. *Nat. Protoc.* 6, 1998–2021. doi: 10.1038/nprot.2011.416

Sveen, L. R., Timmerhaus, G., Krasnov, A., Takle, H., Handeland, S., and Ytteborg, E. (2019). Wound healing in post-smolt Atlantic salmon (*Salmo salar* L.). *Sci. Rep.* 9:3565. doi: 10.1038/s41598-019-39080-x

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872. doi: 10.1016/j.cell.2007.11.019

Takanezawa, S., Saitou, T., and Imamura, T. (2021). Wide field light-sheet microscopy with lens-axicon controlled two-photon Bessel beam illumination. *Nat. Commun.* 12:2979. doi: 10.1038/s41467-021-23249-y

Takechi, M., Hamaoka, T., and Kawamura, S. (2003). Fluorescence visualization of ultraviolet-sensitive cone photoreceptor development in living zebrafish. *FEBS Lett.* 553, 90–94. doi: 10.1016/s0014-5793(03)00977-3

Takeuchi, A., and Okubo, K. (2013). Post-proliferative immature radial glial cells female-specifically express aromatase in the medaka optic tectum. *PLoS One* 8:e73663. doi: 10.1371/journal.pone.0073663

Taneda, Y., Konno, S., Makino, S., Morioka, M., Fukuda, K., Imai, Y., et al. (2010). Epigenetic control of cardiomyocyte production in response to a stress during the medaka heart development. *Dev. Biol.* 340, 30–40. doi: 10.1016/j.ydbio.2010.01.014

Taniguchi, Y., Takeda, S., Furutani-Seiki, M., Kamei, Y., Todo, T., Sasado, T., et al. (2006). Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol.* 7:R116. doi: 10.1186/gb-2006-7-12-r116

Tena, J. J., Gonzalez-Aguilera, C., Fernandez-Minan, A., Vazquez-Marin, J., Parra-Acero, H., Cross, J. W., et al. (2014). Comparative epigenomics in distantly related teleost species identifies conserved cis-regulatory nodes active during the vertebrate phylotypic period. *Genome Res.* 24, 1075–1085. doi: 10.1101/gr.163915.113

Terzibasi, E., Valenzano, D. R., and Cellerino, A. (2007). The short-lived fish Nothobranchius furzeri as a new model system for aging studies. *Exp. Gerontol.* 42, 81–89. doi: 10.1016/j.exger.2006.06.039

Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J., et al. (2002). I-SceI meganuclease mediates highly efficient transgenesis in fish. *Mech. Dev.* 118, 91–98. doi: 10.1016/s0925-4773(02)00218-6

Thummel, R., Burket, C. T., Brewer, J. L., Sarras, M. P. Jr., Li, L., Perry, M., et al. (2005). Cre-mediated site-specific recombination in zebrafish embryos. *Dev. Dyn.* 233, 1366–1377. doi: 10.1002/dvdy.20475

Thummel, R., Kassen, S. C., Enright, J. M., Nelson, C. M., Montgomery, J. E., and Hyde, D. R. (2008). Characterization of Müller glia and neuronal progenitors during adult zebrafish retinal regeneration. *Exp. Eye Res.* 87, 433–444. doi: 10.1016/j.exer.2008.07.009

To, T. T., Witten, P. E., Renn, J., Bhattacharya, D., Huysseune, A., and Winkler, C. (2012). Rankl-induced osteoclastogenesis leads to loss of mineralization in a medaka osteoporosis model. *Development* 139, 141–150. doi: 10.1242/dev.071035

Tong, S. K., Mouriec, K., Kuo, M. W., Pellegrini, E., Gueguen, M. M., Brion, F., et al. (2009). A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. *Genesis* 47, 67–73. doi: 10.1002/dvg.20459

Traver, D., Paw, B. H., Poss, K. D., Penberthy, W. T., Lin, S., and Zon, L. I. (2003). Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat. Immunol.* 4, 1238–1246. doi: 10.1038/ni1007

Trede, N. S., Langenau, D. M., Traver, D., Look, A. T., and Zon, L. I. (2004). The use of zebrafish to understand immunity. *Immunity* 20, 367–379. doi: 10.1016/s1074-7613(04)00084-6

Ueno, T., Yasumasu, S., Hayashi, S., and Iuchi, I. (2004). Identification of choriogenin cis-regulatory elements and production of estrogen-inducible, liver-specific transgenic Medaka. *Mech. Dev.* 121, 803–815. doi: 10.1016/j.mod.2004.03.027

Uesaka, M., Kuratani, S., Takeda, H., and Irie, N. (2019). Recapitulation-like developmental transitions of chromatin accessibility in vertebrates. *Zool. Lett.* 5:33. doi: 10.1186/s40851-019-0148-9

Underwood, J. L., Hestand, R. S. III, and Thompson, B. Z. (1986). Gonad regeneration in grass carp following bilateral gonadectomy. *Prog. Fish Cult.* 48, 54–56. doi: 10.1577/1548-8640(1986)48<54:grigcf>2.0.co;2

Van Houcke, J., Mariën, V., Zandecki, C., Vanhunsel, S., Moons, L., Ayana, R., et al. (2021). Aging impairs the essential contributions of non-glial progenitors to neurorepair in the dorsal telencephalon of the Killifish Nothobranchius furzeri. *Aging Cell* 20:e13464. doi: 10.1111/acel.13464

Van Wettere, A. J., Kullman, S. W., Hinton, D. E., and Law, J. M. (2013). Immunohistochemical characterization of the hepatic progenitor cell compartment in medaka (*Oryzias latipes*) following hepatic injury. *J. Comp. Pathol.* 149, 434–445. doi: 10.1016/j.jcpa.2013.03.008

Var, S. R., and Byrd-Jacobs, C. A. (2020). Role of macrophages and microglia in zebrafish regeneration. *Int. J. Mol. Sci.* 21:4768. doi: 10.3390/ijms21134768

Vihtelic, T. S., and Hyde, D. R. (2000). Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (Danio rerio) retina. *J. Neurobiol.* 44, 289–307. doi: 10.1002/1097-4695(20000905)44:3<289::aid-neu1>3.0.co;2-h

Vivien, C. J., Hudson, J. E., and Porrello, E. R. (2016). Evolution, comparative biology and ontogeny of vertebrate heart regeneration. *NPJ Regen. Med.* 1:16012. doi: 10.1038/npjregenmed.2016.12

Walton, E. M., Cronan, M. R., Beerman, R. W., and Tobin, D. M. (2015). The macrophage-specific promoter mfap4 allows live, long-term analysis of macrophage behavior during Mycobacterial Infection in Zebrafish. *PLoS One* 10:e0138949. doi: 10.1371/journal.pone.0138949

Wan, H., Korzh, S., Li, Z., Mudumana, S. P., Korzh, V., Jiang, Y.-J., et al. (2006). Analyses of pancreas development by generation of gfp transgenic zebrafish using an exocrine pancreas-specific elastaseA gene promoter. *Exp. Cell Res.* 312, 1526–1539. doi: 10.1016/j.yexcr.2006.01.016

Wang, J., Karra, R., Dickson, A. L., and Poss, K. D. (2013). Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration. *Dev. Biol.* 382, 427–435. doi: 10.1016/j.ydbio.2013.08.012

Wang, J., Panakova, D., Kikuchi, K., Holdway, J. E., Gemberling, M., Burris, J. S., et al. (2011). The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development* 138, 3421–3430. doi: 10.1242/dev.068601

Wang, R., Li, Z., Wang, Y., and Gui, J.-F. (2011). An *Apo-14* promoter-driven transgenic zebrafish that marks liver organogenesis. *PLoS One* 6:e22555. doi: 10.1371/journal.pone.0022555

Wang, Z., Cui, M., Shah, A. M., Tan, W., Liu, N., Bassel-Duby, R., et al. (2020). Cell-type-specific gene regulatory networks underlying murine neonatal heart regeneration at single-cell resolution. *Cell Rep.* 33:108472.

Wang, W., Hu, C. K., Zeng, A., Alegre, D., Hu, D., Gotting, K., et al. (2020). Changes in regeneration-responsive enhancers shape regenerative capacities in vertebrates. *Science* 369:eaaz3090. doi: 10.1126/science.aaz3090

Wang, Y., Kaiser, M. S., Larson, J. D., Nasevicius, A., Clark, K. J., Wadman, S. A., et al. (2010). Moesin1 and Ve-cadherin are required in endothelial cells during *in vivo* tubulogenesis. *Development* 137, 3119–3128. doi: 10.1242/dev.048785

Watakabe, I., Hashimoto, H., Kimura, Y., Yokoi, S., Naruse, K., and Higashijima, S. I. (2018). Highly efficient generation of knock-in transgenic medaka by CRISPR/Cas9-mediated genome engineering. *Zool. Lett.* 4:3. doi: 10.1186/s40851-017-0086-3

Watanabe, N., Kato, M., Suzuki, N., Inoue, C., Fedorova, S., Hashimoto, H., et al. (2009). Kidney regeneration through nephron neogenesis in medaka. *Dev. Growth Differ.* 51, 135–143. doi: 10.1111/j.1440-169X.2009.01090.x

Wehner, D., Becker, T., and Becker, C. G. (2018). Restoration of anatomical continuity after spinal cord transection depends on Wnt/β-catenin signaling in larval zebrafish. *Data Brief* 16, 65–70. doi: 10.1016/j.dib.2017.10.068

Wendler, S., Hartmann, N., Hoppe, B., and Englert, C. (2015). Age-dependent decline in fin regenerative capacity in the short-lived fish Nothobranchius furzeri. *Aging Cell* 14, 857–866. doi: 10.1111/acel.12367

White, R. M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., et al. (2008). Transparent adult zebrafish as a tool for *in vivo* transplantation analysis. *Cell Stem Cell* 2, 183–189. doi: 10.1016/j.stem.2007.11.002

Wienholds, E., Van Eeden, F., Kosters, M., Mudde, J., Plasterk, R. H., and Cuppen, E. (2003). Efficient target-selected mutagenesis in zebrafish. *Genome Res.* 13, 2700–2707. doi: 10.1101/gr.1725103

Wierson, W. A., Welker, J. M., Almeida, M. P., Mann, C. M., Webster, D. A., Torrie, M. E., et al. (2020). Efficient targeted integration directed by short homology in zebrafish and mammalian cells. *eLife* 9:e53968. doi: 10.7554/eLife.53968

Willems, B., Buttner, A., Huysseune, A., Renn, J., Witten, P. E., and Winkler, C. (2012). Conditional ablation of osteoblasts in medaka. *Dev. Biol.* 364, 128–137. doi: 10.1016/j.ydbio.2012.01.023

Wittamer, V., Bertrand, J. Y., Gutschow, P. W., and Traver, D. (2011). Characterization of the mononuclear phagocyte system in zebrafish. *Blood J. Am. Soc. Hematol.* 117, 7126–7135. doi: 10.1182/blood-2010-11-321448

Wittbrodt, J., Shima, A., and Schartl, M. (2002). Medaka—a model organism from the far East. *Nat. Rev. Genet.* 3, 53–64. doi: 10.1038/nrg704

Xiao, C., Wang, F., Hou, J., Zhu, X., Luo, Y., and Xiong, J. W. (2018). Nanoparticle-mediated siRNA gene-silencing in adult zebrafish heart. *J. Vis. Exp.* 137:58054. doi: 10.3791/58054

Xiao, T., Roeser, T., Staub, W., and Baier, H. (2005). A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. doi: 10.1242/dev.01861

Yamamoto, T. (1975). *Medaka (killifish): Biology and Strains*. Tokyo: Keigaku Pub. Co.

Yang, K., and Kang, J. (2019). Tissue regeneration enhancer elements: a way to unlock endogenous healing power. *Dev. Dyn.* 248, 34–42. doi: 10.1002/dvdy.24676

Yano, T., and Tamura, K. (2013). The making of differences between fins and limbs. *J. Anat.* 222, 100–113. doi: 10.1111/j.1469-7580.2012.01491.x

Yi, Ni Lam, E., Chau, J. Y., Kalev-Zylinska, M. L., Fountaine, T. M., Mead, R. S., et al. (2009). Zebrafish runx1 promoter-EGFP transgenics mark discrete sites of definitive blood progenitors. *Blood J. Am. Soc. Hematol.* 113, 1241–1249. doi: 10.1182/blood-2008-04-149898

Yokoyama, H., Ogino, H., Stoick-Cooper, C. L., Grainger, R. M., and Moon, R. T. (2007). Wnt/β-catenin signaling has an essential role in the initiation of limb regeneration. *Dev. Biol.* 306, 170–178.

Yoshinari, N., Ando, K., Kudo, A., Kinoshita, M., and Kawakami, A. (2012). Colored medaka and zebrafish: transgenics with ubiquitous and strong transgene expression driven by the medaka beta-actin promoter. *Dev. Growth Differ.* 54, 818–828. doi: 10.1111/dgd.12013

You, M. S., Jiang, Y. J., Yuh, C. H., Wang, C. M., Tang, C. H., Chuang, Y. J., et al. (2016). A Sketch of the Taiwan Zebrafish Core Facility. *Zebrafish* 13(Suppl. 1), S24–S29. doi: 10.1089/zeb.2015.1208

Yun, M. H. (2015). Changes in regenerative capacity through lifespan. *Int. J. Mol. Sci.* 16, 25392–25432. doi: 10.3390/ijms161025392

Zattara, E. E., Fernandez-Alvarez, F. A., Hiebert, T. C., Bely, A. E., and Norenburg, J. L. (2019). A phylum-wide survey reveals multiple independent gains of head regeneration in Nemertea. *Proc. Biol. Sci.* 286:20182524. doi: 10.1098/rspb.2018.2524

Zecchin, E., Filippi, A., Biemar, F., Tiso, N., Pauls, S., Ellertsdottir, E., et al. (2007). Distinct delta and jagged genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish. *Dev. Biol.* 301, 192–204. doi: 10.1016/j.ydbio.2006.09.041

Zeng, Z., Liu, X., Seebah, S., and Gong, Z. (2005). Faithful expression of living color reporter genes in transgenic medaka under two tissue-specific zebrafish promoters. *Dev. Dyn.* 234, 387–392. doi: 10.1002/dvdy.20491

Zhang, R., Han, P., Yang, H., Ouyang, K., Lee, D., Lin, Y. F., et al. (2013). *In vivo* cardiac reprogramming contributes to zebrafish heart regeneration. *Nature* 498, 497–501. doi: 10.1038/nature12322

Zhao, L., Ben-Yair, R., Burns, C. E., and Burns, C. G. (2019). Endocardial Notch signaling promotes cardiomyocyte proliferation in the regenerating zebrafish heart through Wnt pathway antagonism. *Cell Rep.* 26, 546–554.e5. doi: 10.1016/j.celrep.2018.12.048

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 10 February 2022
doi: 10.3389/fcell.2022.813314

Brain Regeneration Resembles Brain Cancer at Its Early Wound Healing Stage and Diverges From Cancer Later at Its Proliferation and Differentiation Stages

Yeliz Demirci[1,2,3], *Guillaume Heger*[4], *Esra Katkat*[1,2], *Irene Papatheodorou*[5], *Alvis Brazma*[5] *and Gunes Ozhan*[1,2]*

[1]*Izmir Biomedicine and Genome Center (IBG), Dokuz Eylul University Health Campus, Inciralti-Balcova, Izmir, Turkey,* [2]*Izmir International Biomedicine and Genome Institute (IBG-Izmir), Dokuz Eylul University, Inciralti-Balcova, Izmir, Turkey,* [3]*Wellcome Sanger Institute, Hinxton, United Kingdom,* [4]*École Centrale de Nantes, Nantes, France,* [5]*European Molecular Biology Laboratory–European Bioinformatics Institute (EMBL-EBI), Cambridge, United Kingdom*

OPEN ACCESS

Edited by:
Stefano Tiozzo,
Université Paris-Sorbonne, France

Reviewed by:
David D. Eisenstat,
Murdoch Childrens Research Institute, Australia
Ilaria Decimo,
University of Verona, Italy

***Correspondence:**
Gunes Ozhan
gunes.ozhan@ibg.edu.tr

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *11 November 2021*
Accepted: *18 January 2022*
Published: *10 February 2022*

Citation:
Demirci Y, Heger G, Katkat E, Papatheodorou I, Brazma A and Ozhan G (2022) Brain Regeneration Resembles Brain Cancer at Its Early Wound Healing Stage and Diverges From Cancer Later at Its Proliferation and Differentiation Stages. Front. Cell Dev. Biol. 10:813314. doi: 10.3389/fcell.2022.813314

Gliomas are the most frequent type of brain cancers and characterized by continuous proliferation, inflammation, angiogenesis, invasion and dedifferentiation, which are also among the initiator and sustaining factors of brain regeneration during restoration of tissue integrity and function. Thus, brain regeneration and brain cancer should share more molecular mechanisms at early stages of regeneration where cell proliferation dominates. However, the mechanisms could diverge later when the regenerative response terminates, while cancer cells sustain proliferation. To test this hypothesis, we exploited the adult zebrafish that, in contrast to the mammals, can efficiently regenerate the brain in response to injury. By comparing transcriptome profiles of the regenerating zebrafish telencephalon at its three different stages, i.e., 1 day post-lesion (dpl)-early wound healing stage, 3 dpl-early proliferative stage and 14 dpl-differentiation stage, to those of two brain cancers, i.e., low-grade glioma (LGG) and glioblastoma (GBM), we reveal the common and distinct molecular mechanisms of brain regeneration and brain cancer. While the transcriptomes of 1 dpl and 3 dpl harbor unique gene modules and gene expression profiles that are more divergent from the control, the transcriptome of 14 dpl converges to that of the control. Next, by functional analysis of the transcriptomes of brain regeneration stages to LGG and GBM, we reveal the common and distinct molecular pathways in regeneration and cancer. 1 dpl and LGG and GBM resemble with regard to signaling pathways related to metabolism and neurogenesis, while 3 dpl and LGG and GBM share pathways that control cell proliferation and differentiation. On the other hand, 14 dpl and LGG and GBM converge with respect to developmental and morphogenetic processes. Finally, our global comparison of gene expression profiles of three brain regeneration stages, LGG and GBM exhibit that 1 dpl is the most similar stage to LGG and GBM while 14 dpl is the most distant stage to both brain cancers. Therefore, early convergence and later divergence of brain regeneration and brain cancer constitutes a key starting point in comparative

understanding of cellular and molecular events between the two phenomena and development of relevant targeted therapies for brain cancers.

Keywords: wound healing, proliferation, differentiation, zebrafish, low-grade glioma (LGG), glioblastoma, comparative transcriptome analysis

INTRODUCTION

Despite decades of research, primary brain tumors are still the most difficult-to-treat and deadliest types of cancer. They can occur due to the continual uncontrolled proliferation of brain cells including neurons and glial cells. About 240,000 cases of brain and nervous system-related cancers are diagnosed worldwide every year (Boffetta et al., 2014). Among these, gliomas, arising from glial tissue, are the most frequently occurring type of tumors in the central nervous system (CNS) and responsible for 80% of all malignant primary brain and CNS cancers (Schwartzbaum et al., 2006; Agnihotri et al., 2013; Ostrom et al., 2013; Boffetta et al., 2014; Messali et al., 2014; Hanif et al., 2017). Gliomas are classified into grade I to IV by WHO according to their histopathological and immunohistochemical similarities to the putative cell of origin. Whereas grade I gliomas are less aggressive and slow-growing, grades II to IV are more aggressive, malignant and invasive (Louis et al., 2016; Hanif et al., 2017; Louis et al., 2021). Grade IV gliomas, also known as glioblastoma (GBM), are the most aggressive diffuse forms of all gliomas and account for more than 50% of adults diagnosed with glioma (Louis et al., 2016; Tamimi and Juweid, 2017; Louis et al., 2021). Genetic and environmental factors including age, gender, ethnicity, inherited susceptibility, immune factors and prior radiation have been associated with the risk of developing glioma (Bondy et al., 2008; Prasad and Haas-Kogan, 2009; Ostrom et al., 2013; Tamimi and Juweid, 2017; Ladomersky et al., 2019). While some types of gliomas such as pilocytic astrocytoma are more prevalent in children and young adults, the incidence of GBM increases with advancing age (Merchant et al., 2010; Das and Kumar, 2017; Jones et al., 2017; Ladomersky et al., 2019). In addition to common mutations in the genes *isocitrate dehydrogenase* (*IDH*) *1* and *2*, and *telomerase reverse transcriptase* (*TERT*), progression of glioma has been associated with alterations in various pathways that are crucial to today's treatments for glioma/glioblastoma (Idilli et al., 2017): 1) alterations in the PI3K-PTEN-Akt-mTOR signaling pathway regulated by epidermal growth factor (EGF) and its receptor EGFR (Zoncu et al., 2011; Li et al., 2016), 2) mutations in the p53 pathway that promote excessive cell cycle progression and prevent apoptosis (Ohgaki and Kleihues, 2007; Viotti et al., 2014; Speidel, 2015), 3) mutations in *NF1, BRAF, RAF1, MEK, PDGFR* and *RTK* genes that affect RAS/MAPK signaling pathways (Venkatesan et al., 2016; Nasser and Mehdipour, 2018) and 4) changes in the genes regulating cell cycle and cell growth such as *retinoblastoma protein* (*pRB*), *cyclin-dependent kinase 4 (cdk4)* and *cyclin-dependent kinase inhibitor 2A* (*cdkn2A*) (Mao et al., 2012; Idilli et al., 2017; Nasser and Mehdipour, 2018).

Despite the modern therapies, curing brain tumors is still a considerable challenge due to the tumor heterogeneity, presence of blood-brain barrier (BBB) and missing pieces in the underlying molecular mechanisms. Among these tumors, GBM remains one of the deadliest cancer types, having a very poor prognosis with a median survival of about 15 months from the diagnosis and a 5-year survival rate of only 5% in adults (Ohka et al., 2012; Fernandes et al., 2017; Tamimi and Juweid, 2017; Wang et al., 2021). GBM treatment consists of a complex multidisciplinary approach including maximal surgical resection followed by radiation therapy and chemotherapy. After resection, applying radiotherapy together with temozolomide (TMZ) is the most effective combinatorial treatment that has been shown to extend survival (Stupp et al., 2005; Fernandes et al., 2017). Combinations of conventional therapies and new approaches targeting several molecular events, such as triggering of apoptosis and suppression of angiogenesis, can improve the prognosis of patients with GBM (Fernandes et al., 2017; Tamimi and Juweid, 2017). Nevertheless, for over 4 decades, the outcomes of GBM treatment have remained stable, necessitating rapid development of new therapeutic approaches.

Cancer and regeneration have been historically linked as both processes are triggered with the same biological phenomenon, i.e. cell proliferation. Historically, cell proliferation had first been proposed as a mechanistic link between development, regeneration and cancer by Waddington in the early 1930s (Waddington 1935; Stern, 2000). Due to the cellular similarities between tumor stroma and granulation tissue, which forms at the wound site, cancers have long been described as wounds that do not heal (Haddow, 1972; Dvorak, 1986; Schafer and Werner, 2008). A proper regeneration process is terminated in a controlled manner so that the regenerating tissue does not transform into a mass of cells that undergo uncontrolled proliferation. If regeneration cannot be processed or terminated properly, the tissue might -as in the case of cancer-undertake continuous proliferation due to chronic injury, hypoxia and inflammation and cannot re-establish tissue integrity (Dvorak, 1986; Coussens and Werb, 2002; Beachy et al., 2004; Gurtner et al., 2008; Schafer and Werner, 2008; Oviedo and Beane, 2009; Verkhratsky and Butt, 2013). In contrast to the limited ability of the human brain to regenerate, the non-mammalian vertebrate zebrafish can regenerate the CNS throughout its life (Grandel et al., 2006; Diotel et al., 2020). This ability of the adult zebrafish brain is maintained by the existence of stem/progenitor cells that can continuously proliferate and a permissive environment for neurogenesis (Kizil et al., 2012b). While mammalian adult neurogenesis is restricted to only two regions of the forebrain, i.e., the subventricular zone (SVZ) of the lateral ventricles in the telencephalon and the subgranular zone

(SGZ) of the dentate gyrus in the hippocampus, zebrafish has sixteen distinct proliferative niches located in the ventricular zone and deeper in the brain parenchyma with self-renewing neural progenitors (Kriegstein and Alvarez-Buylla, 2009; Bonfanti and Peretto, 2011; Kaslin et al., 2017; Zambusi and Ninkovic, 2020). Thus, this high regenerative capacity of the zebrafish brain constitutes a unique platform to compare the transcriptome of a healing brain at its different stages with that of continuously growing/metastasizing brain tumors. To address this striking issue, we have first set out to identify the genes that are differentially expressed in the adult zebrafish telencephalon at the following three stages of brain regeneration in response to stab wound injury: the early wound healing stage at 1 day post-lesion (dpl), the early proliferative stage at 3 dpl and the late differentiation stage at 14 dpl. We have identified 6,123, 4,662 and 1954 differentially expressed genes (DEGs) at 1, 3 and 14 dpl, respectively. A vast majority of the DEGs identified at all three stages were upregulated. Using Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, we have identified that neurogenesis-related genes were prominent among DEGs at 1 dpl. While 3 dpl was marked by the genes related to immune response, cell proliferation and apoptosis, genes with key roles in neuronal differentiation and the Notch pathway were abundant among DEGs at 14 dpl. Weighted gene co-expression network analysis (WGCNA) of three regeneration stages revealed twelve distinct co-expression modules, nine of which were specific to a particular stage. Moreover, gene modules and gene expression profiles at 1 dpl and 3 dpl were unique, while those at 14 dpl are rather similar to the control group. Next, we have compared the whole transcriptomes of the regenerating brain at the three stages to those of the human adult brain tumors low-grade glioma (LGG) and glioblastoma (GBM). The early wound healing stage was similar to brain cancer with respect to activation of metabolic responses and neurogenesis-related signaling pathways. The early proliferative stage and brain cancers shared DEGs related to cell proliferation. The differentiation stage was similar to cancer with respect to activation of developmental and morphogenetic processes. Finally, our comparative transcriptomics and functional analyses of the genes that are differentially expressed in at least one stage of brain regeneration and shared with at least one type of brain cancer have revealed that the stage that most resembled the brain cancer was the early wound healing stage (1 dpl) and that the similarity decreased at the later stages of brain regeneration. Overall, by revealing the stage-dependent similarities and discrepancies between brain regeneration and brain cancer, our study paves the way to test the potential of specific molecular mechanisms of regeneration to stop cancer.

MATERIALS AND METHODS

Stab Wound Assay and Sample Collection

Stab injury was performed in 6–10 month-old wild-type (wt) AB zebrafish as previously described (Kroehne et al., 2011; Baumgart et al., 2012). Before generating a lesion, fish were anaesthetized with 0.02% (w/v) of tricaine methanesulphonate (Supelco, PA, United States) (Schmidt et al., 2014). Stab wound injury was generated by inserting a 30-gauge needle through the left nostril up to the caudal end of the telencephalon (**Figure 1A**). Following injury, the fish were transferred into a tank of freshwater. At 1, 3 or 14 dpl of stab injury, zebrafish were re-anaesthetized with 0.02% (w/v) of tricaine solution and euthanized by submersion in ice water for 5 min (Schmidt et al., 2014). After extracting the whole telencephalon tissue, lesioned (left) hemispheres were dissected and collected individually in RNAprotect tissue reagent (Qiagen, Germany) to prevent RNA degradation. The left hemispheres of healthy zebrafish telencephalons were used as control samples. All stab lesions were performed on the same day, and fish were sacrificed at corresponding time points from that moment (1, 3 or 14 dpl). Control fish were sacrificed on the day of the stab lesion. Experiments were carried out in quadruplets for each group. Zebrafish were raised and handled in accordance with the guidelines of the Izmir Biomedicine and Genome Center's Animal Care and Use Committee. Animal experiments were inspected and approved by the Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC).

RNA Isolation and cDNA Preparation

Following removal of RNAprotect, 700 µL of Qiazol reagent (Qiagen, Germany) was added on the brain tissues and the tissues were homogenized by using a sterile disposable pestle. Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Germany) according to the manufacturer's instructions and quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, United States). RNA integrity and quality was measured by using an Agilent RNA 6000 Pico kit in a 2,100 Bioanalyzer (Agilent Technologies, CA, United States) following the manufacturer's instructions.

Library Construction and RNA Sequencing (RNA-Seq)

The samples with an RNA Integrity Number (RIN) ≥ 8 were selected for RNA sequencing (RNA-seq). RNA quality was further tested by performing quantitative reverse transcription PCR (RT-PCR) with a primer pair producing an 812-bp product for zebrafish *beta actin 1* (*actb1*) as a housekeeping gene. To work with equal amounts, RNA samples were adjusted to 100 ng. Samples that passed the quality control steps were sent to the Genomics Core Facility (GeneCore, EMBL Heidelberg, Germany) for library preparation and RNA-seq. Libraries were prepared with an Illumina TruSeq RNA Library Preparation Kit v2 (Illumina, San Diego, CA, United States) according to the manufacturer's instructions. 500 ng of cDNA was used for each reaction. A paired-end, strand-specific sequencing platform was used on an Illumina NextSeq 500 (Illumina, CA, United States) with a read length of 75 bp.

Quantitative Polymerase Chain Reaction

To validate the differentially expressed genes obtained via RNA-seq analysis within the original RNA samples, RNA was

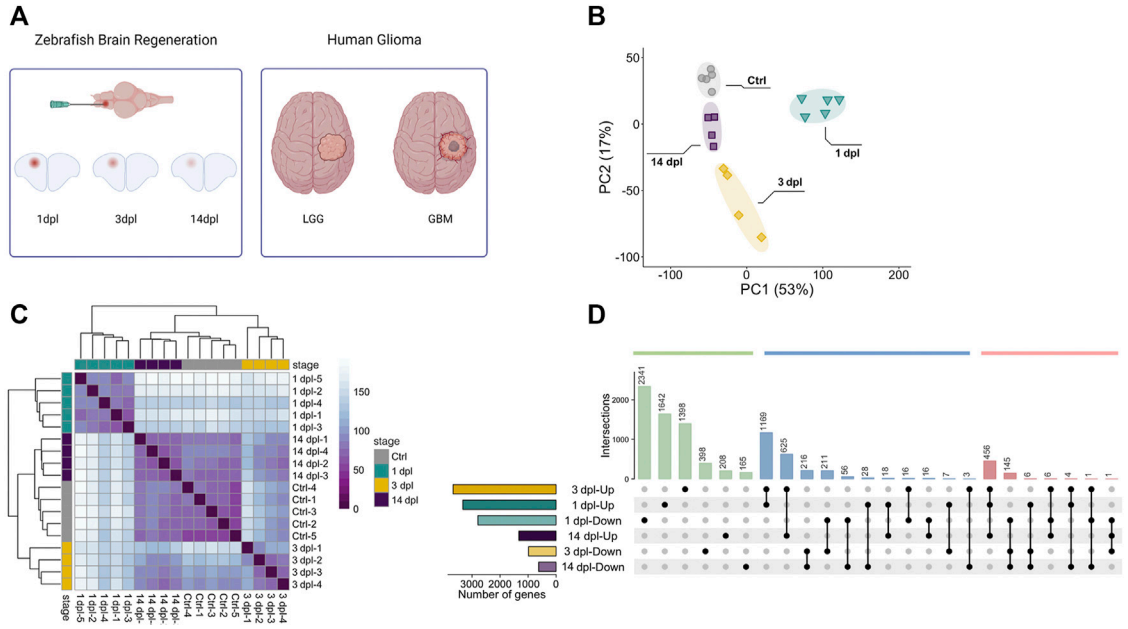


FIGURE 1 | Sample preparation from three different stages of zebrafish brain regeneration and initial analyses of the transcriptome data. **(A)** Generation of the stab lesion and preparation of the RNA samples from lesioned hemispheres at 1, 3 and 14 dpl. Transcriptomes of the regenerating brain were compared to those of human adult LGG and GBM. **(B)** Principal component analysis (PCA) of three brain regeneration stages and their controls. Different colors of circle, square or rectangle dots represent the four groups of samples. Four or five dots with the same color refer to the biological replicates of a sample group. Four sample groups were well clustered among their replicates and well separated from other sample groups. **(C)** Sample-to-sample distance heatmap generated by using normalized counts for overall gene expression patterns for three stages of brain regeneration and control brain samples generated by the DESeq2 package. Different colors of dots represent the four groups of samples. **(D)** UpSet plot shows the comparison of DEG sets between regeneration stages. Total number of DEGs as Up or Down and time points are shown on *x* and *y* axes, respectively. Green bars represent the genes unique to a time point, blue bars the intersection of genes between two different time points and red bars the intersection of genes between three different time points. Black dots connected by lines correspond to the time point and Up/Down state. Numbers of overlapping genes are shown above each bar. dpl: days post-lesion, ctrl: control, LGG: low-grade glioma, GBM: glioblastoma, Up: upregulated, Down: downregulated.

converted to cDNA by using the ProtoScript II First Strand cDNA Synthesis kit (New England Biolabs, MA, United States). qPCR was performed in triplicates by using GoTaq qPCR Master Mix (Promega, WI, United States) in an Applied Biosystems 7,500 Fast Real-Time PCR machine (Thermo Fisher Scientific, MA, United States). Expression values of each sample were normalized to *Danio rerio ribosomal protein L13a (rpl13a)*. The efficiency of each primer pair was assessed by using the standard curve assay according to the relevant program of the machine. Standard curve with C_T values were generated by using the ABI software and a correlation coefficient (R^2) was calculated for each primer pair. Primer pairs with the R^2 values equal to or greater than 0.99 and an efficiency falling in the acceptable range (90–110%) were used in the qPCR reactions. Data were analyzed with the GraphPad Prism 8 software (Graphpad Software Inc., CA, United States). The values are indicated as mean ± SEM (Standard Error of Mean) of triplicates. Primer sequences for the tested zebrafish genes are provided in **Supplementary Table S1**.

Transcriptomic Analyses of Zebrafish Brain Regeneration and Human Brain Cancers

Read quality control of each zebrafish brain RNA-seq sample was initially performed by using the FastQC tool (Andrews, 2010). The reads were aligned to the zebrafish reference genome GRCz11 (danRer11) using HISAT2 (version 2.1.0) (Kim et al., 2015). After mapping, transcripts were counted with HTSeq 0.6.0 tool by using the annotation file *Danio*_rerio.GRCz11.93. gtf obtained from the Ensembl (Anders et al., 2015). Normalization and transformation (vst) of the read counts, as well as differential expression analysis, were performed by using DESeq2 package (version 1.28.1) of Bioconductor (Love et al., 2014). Principal component analysis (PCA) and sample-to-sample distance analysis were conducted to check data and plots were visualized by using ggplot2 (version 3.3.2) and pheatmap package (version 1.0.12) (Kolde, 2012; Wickham, 2016) (**Figures 1B,C**). To find differentially expressed genes (DEGs), Wald tests were performed on DESeq2 for the following comparisons: 1) 1 dpl lesioned hemisphere vs. control, 2) 3 dpl lesioned hemisphere vs. control, and 3) 14 dpl lesioned hemisphere vs. control. Secondly, to analyze human brain cancer data, a count matrix was generated using the count data of low-grade glioma (LGG) and glioblastoma (GBM) samples downloaded from The *Cancer* Genome Atlas (TCGA). To identify DEGs, the samples of TCGA-LGG and/or TCGA-GBM projects were compared with the normal tissue samples (control) of the same project. Genes were tested for differential expression using a Wald test with DESeq2 for the following comparisons: 1) TCGA-LGG vs. control and 2) TCGA-GBM vs. control. For all comparisons, genes were marked as upregulated

for the fold change >1.5 and downregulated for the fold change <0.67 (= 1/1.5) and for Benjamini–Hochberg adjusted p-value (FDR) < 0.05, which will thereafter be referred to as "FC > 1.5 in either directions".

Weighted Gene Co-Expression Network Analysis

We ran WGCNA on a filtered and transformed expression matrix of the zebrafish brain regeneration dataset. Raw counts were transformed using the variance-stabilizing transformation (vst) of the R package DESeq2 (Love et al., 2014) as recommended by the WGCNA manual (Langfelder and Horvath, 2008). Genes with less than 10 counts in more than 90% of the samples were filtered for subsequent analysis. After this filtering, 22,853 genes were fed to WGCNA for the regeneration dataset. Network was constructed using unsigned co-expression similarities between genes. As opposed to signed co-expression, unsigned co-expression conserves similarity between highly correlated genes, even in the case of negative correlation. Unsigned co-expression similarity between two genes i and j is defined as the absolute value of their sample correlation: $s_{i,j} = |cor(x_i, x_j)|$. A soft threshold (also called power) of 9 was picked due to the sample size (n = 18) accepted as small according in order to construct a co-expression network. The soft threshold β expresses the way the co-expression similarity translates into an adjacency weight in the network: $a_{i,j} = s_{i,j}^{\beta}$. The higher the soft threshold, the further weak co-expressions are pushed towards 0, although without being made equal to 0, i.e., soft thresholding. For zebrafish brain regeneration data, a power of 9 was chosen by default, due to the sample size (n = 18) accepted as small according to the WGCNA manual (Langfelder and Horvath, 2008). A weighted co-expression network was constructed using these parameters. Gene modules were then delineated from the clustering using the dynamic hybrid tree cut algorithm with a deep split parameter of 2 and a minimum cluster size of 100. In other words, modules are defined by pruning the hierarchical clustering dendrogram and grouping the genes that fall in the same branch together. Depending on the parameters, WGCNA merges modules that show similar patterns.

Collection of Brain Cancer Samples' Data from The Cancer Genome Atlas

RNA-seq data of adult human GBM and LGG samples were obtained from the TCGA data portal (National Cancer Institute, 2020). TCGA defines LGG as tumors of grades II and III based on standards set by the World Health Organization (WHO). The "Level 3" gene expression data for all TCGA-LGG (529 LGG samples and 4 control samples) and TCGA-GBM (165 GBM samples and 5 control samples) samples were downloaded from the TCGA database.

Ortholog Conversion

To compare the events measured in the zebrafish and human models, a table of unambiguous orthologous genes was generated between *Homo sapiens* and *Danio rerio* by using BioMart annotations (Smedley et al., 2009). The orthology table obtained from BioMart was first filtered to keep only the pairs of genes indicated with high confidence or with similarities in genes names. The resulting table was further filtered to resolve ambiguities so that each zebrafish gene is assigned a unique human ortholog. For a given zebrafish gene that does not have a human ortholog with the same gene name, a unique human ortholog is selected by ranking the orthology metrics with the following order of priority: gene order conservation score, whole genome alignment coverage, percentage of identity of zebrafish gene to human gene, percentage of identity of human gene to zebrafish gene. Finally, to reduce the number of human genes matched to multiple zebrafish genes, only high confidence pairs were retained (**Supplementary Table S7**).

Functional Annotation

The lists of significantly altered genes acquired from individual comparisons were used as inputs of functional analyses for the database for Annotation, Visualization and Integrated Discovery (DAVID version 6.8) (Huang da et al., 2009). When comparing stages of zebrafish brain regeneration and human cancers, lists of shared or exclusive genes were built using human orthologs of zebrafish genes (**Supplementary Table S7**). For comparisons within the zebrafish model, the original gene identifiers were used. For functional enrichment, the ease score, a modified one-tailed Fisher's exact test, was used to determine the enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by means of a user-defined gene list for each annotated DAVID GO term and KEGG pathway. Functional enrichment was performed according to biological domains of GO terms with respect to three aspects: biological process (BP), molecular function (MF), and cellular component (CC). Gene lists obtained from the AmiGO database (Carbon et al., 2009) and manually curated as related to the selected functions (**Figure 2A**) were plotted using the R package pheatmap (version 1.0.12) (Kolde, 2012). Gene lists related to the selected KEGG pathways were obtained from the KEGG database and plotted using the GOplot package (Walter et al., 2015). Significantly enriched GO terms and KEGG pathways were plotted using the package ggplot2 (Wickham, 2016).

RESULTS

Transcriptome Profiling of Brain Regeneration During Early Wound Healing, Proliferation and Differentiation Stages

Brain regeneration has been analyzed at the transcriptional level in the zebrafish traumatic brain injury model at 5 dpl (Gourain et al., 2021). Moreover, we have recently conducted a comparative transcriptomic profiling of the regenerating zebrafish telencephalon at two early stages of regeneration (Demirci et al., 2020). However, there exists no study that compares the gene expression profiles at the early and late stages of regeneration. Thus, we set out to unravel the dynamic

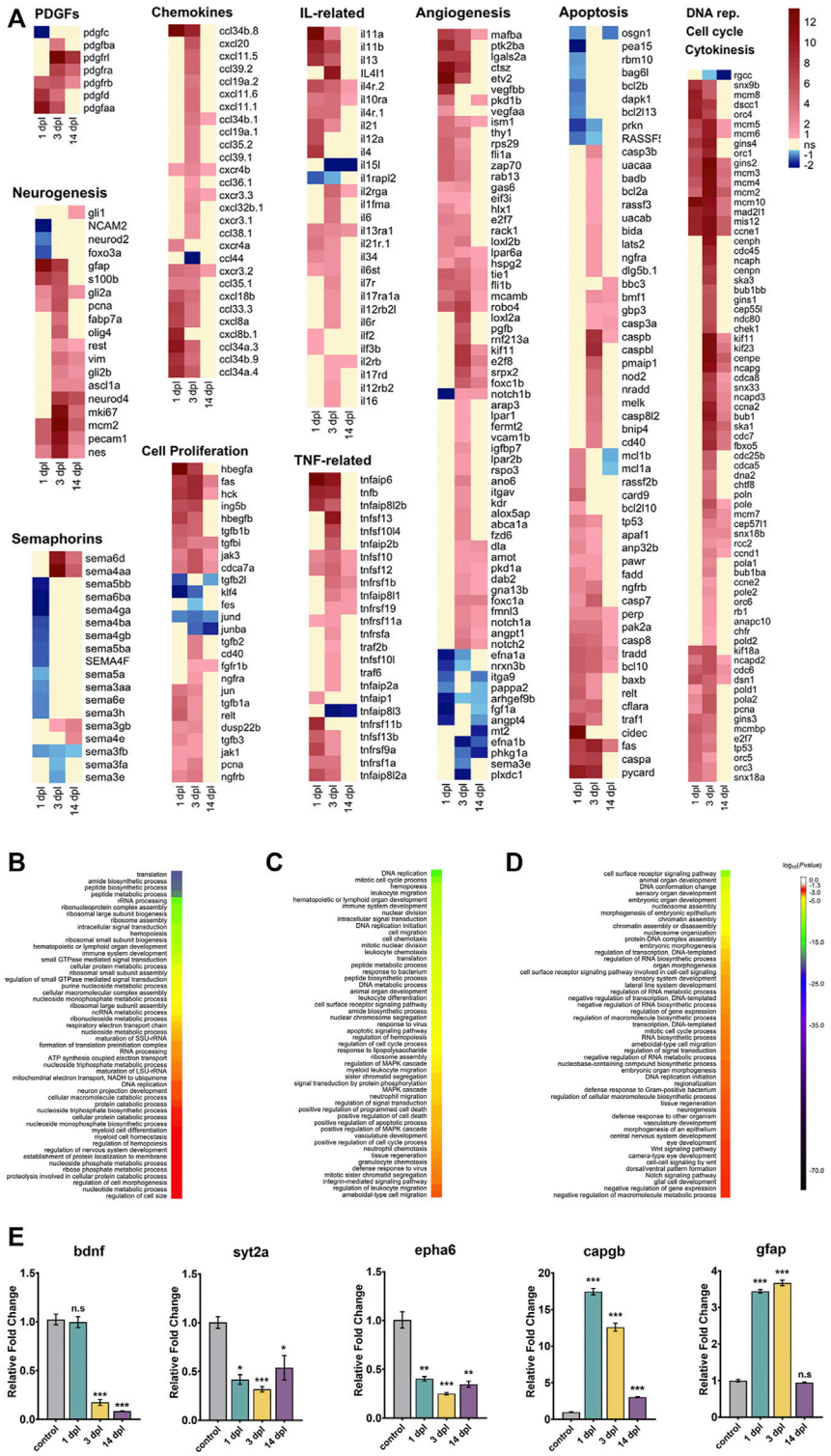


FIGURE 2 | Transcriptome profiling and functional annotation of the telencephalon during early wound healing (1 dpl), early proliferative (3 dpl) and differentiation (14 dpl) stages of zebrafish brain regeneration. **(A)** Heatmaps of $\log_2$ fold changes of selected genes across three stages of brain regeneration. Each column represents a time point and each row shows a single gene. The scale bar shows red for Up, blue for Down, yellow for weak regulation (FC < 1.5 in either direction) or statistically non-significant (Benjamini–Hochberg adjusted *p*-value (FDR) > 0.1). **(B–D)** GO-BP terms enriched at 1 dpl **(B)**, 3 dpl **(C)** and 14 dpl **(D)** by using all DEGs. DAVID was

(*Continued*)

FIGURE 2 | used to show the most significantly enriched GO-BP terms. All DEGs (1 dpl: 6,123, 3 dpl: 4,662, 14 dpl: 1954) were used for the analyses. The heatmap scale shows $\log_{10}$ of the ease *p*-values for the most significantly enriched GO terms. **(E)** Relative expression levels of genes that are Down or Up at different stages of regeneration. *bdnf* is Down at 3 dpl and 14 dpl, while *syt2a* and *epha6* are Down at all stages. *capgb* is Up at all stages, while *gfap* is Up at 1 dpl and 3 dpl. Statistical significance was evaluated using unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. ns: non-significant. Error bars represent ±standard error of mean (SEM, $n = 3$). Up: upregulated, Down: downregulated, dpl: days post-lesion, DAVID: database for Annotation, Visualization and Integrated Discovery, GO: Gene Ontology, BP: Biological Process.

alterations in gene expression that occur from the early wound healing stage (1 dpl), through the proliferative stage (3 dpl) to the late differentiation stage (14 dpl) of brain regeneration (Kroehne et al., 2011; Lindsey et al., 2017; Demirci et al., 2020). To this purpose, we dissected the lesioned (left) hemispheres of the injured zebrafish brain at 1, 3 and 14 dpl, and compared with the equivalent hemispheres of the uninjured control brains. PCA showed clear separation of the samples between control and regeneration stages, which clustered in distinct zones of the principal plane of variance (**Figure 1B**). The sample-to-sample distance heatmap further supported that the samples exactly matched the main ramifications of the hierarchical clustering (**Figure 1C**). Among the regeneration stages, samples of 14 dpl positioned most closely to the control samples in both analyses, suggesting that the transcriptome of the late differentiation stage converged to that of the control. Next, we performed differential gene expression analysis. We have detected 6,123 genes (3,330 upregulated [Up] and 2,793 downregulated [Down]), 4,662 genes (3,678 Up, 984 Down) and 1954 genes (1,330 Up, 624 Down) that were differentially expressed in response to injury at 1, 3 and 14 dpl, respectively (**Supplementary Figures S1A,C**). Differential expression at all stages was asymmetrical in favor of Up genes, with 1 dpl having the highest number of DEGs (**Supplementary Table S2**). 3,983 DEGs (1,642 Up, 2,341 Down) were unique to 1 dpl (**Figure 1D**). Heatmaps of selected genes undertaking specific roles during regeneration showed that the Down group at 1 dpl consisted of several neurogenesis-related genes such as *neurod2, olig1, notch3, foxo3a*, *amigo1* and a large number of *semaphorin* genes, encoding for a family of secreted and membrane proteins involved in axonal growth (**Supplementary Table S2**, **Figure 2A**). Interestingly, several neural stem/progenitor cell markers including *gfap, nes* and *s100b* were Up, as a sign of reactive neurogenesis (**Supplementary Table S2**, **Figure 2A**). At 3 dpl, 1796 DEGs (1,398 Up, 398 Down) were unique (**Figure 1D**) and mostly consisted of genes related to regulation of apoptosis, cell cycle and cell proliferation (**Supplementary Table S2**, **Figure 2A**). Genes related to immune response, chemotaxis and angiogenesis as well as markers of neurogenesis such as *gfap*, *s100b*, *fabp7a*, *neurod4*, *olig4* and *gli* were prominently Up at 3 dpl (**Supplementary Table S2**, **Figure 2A**). At 14 dpl, the number of unique DEGs decreased dramatically to 373 (208 Up, 165 Down) (**Figure 1D**), including the Up neuronal differentiation genes *gli1*, *foxd3*, *her4.2, otpb*, *fzd1* and *fzd4* (**Supplementary Table S2**, **Figure 2A**). Strikingly, several members of Notch signaling including *notch1a, notch1b, notch2, notchl, her15.1, dla, dlb, dlc, dld, jag1a,* and *jag1b*, were Up at 14 dpl while being Down at 1 dpl (**Supplementary Table S2**), in accordance with the key roles of Notch signaling in regulation of neuronal differentiation (Imayoshi and Kageyama, 2011).

To investigate the function of the DEGs, we performed GO term enrichment analysis for all three regeneration stages (**Supplementary Table S3**, **Figures 2B–D**). At 1 dpl, biosynthetic processes, immune system development and regulation of nervous system development were in the top 50 GO-BP terms (**Supplementary Table S3**, **Figure 2B**). KEGG pathways at 1 dpl were also enriched mainly in biosynthetic metabolic pathways as well as several signaling pathways such as mTOR and MAPK (**Supplementary Table S4**, **Supplementary Figure S2**). At 3 dpl, top 50 GO-BP terms were enriched mainly in cell cycle, activation of immune response and apoptosis (**Supplementary Table S3**, **Figure 2C**). KEGG pathways were likewise enriched in cell cycle, apoptosis, cytokine activation, apoptosis-related p53 signaling and immune response-related JAK-STAT pathway (**Supplementary Table S4**, **Supplementary Figure S2**). At 14 dpl, most prominent GO-BP terms were related to organ morphogenesis, neurogenesis, CNS development and vasculogenesis as well as Wnt and Notch signaling pathways (**Supplementary Table S3**, **Figure 2D**), which were also enriched in the KEGG pathways (**Supplementary Table S4**, **Supplementary Figure S2**). To validate differential gene expression, we selected DEGs that are related to neurogenesis and regulated differently at 1, 3 and 14 dpl. *bdnf*, encoding for a neurotrophic factor, was strongly and selectively Down at 3 dpl and 14 dpl, while the synaptic vesicle protein encoding *syt2a* and ephrin receptor gene *epha6* were Down at all three stages (**Figure 2E**). On the other hand, regeneration-related capgb was Up at all stages, whereas the glial marker gfap was selectively Up at 1 dpl and 3 dpl (**Figure 2E**). These results were collectively compatible with the RNA-seq results (**Supplementary Table S2**).

Gene Co-Expression Network Analysis Reveals Divergence from Control in Early Stages of Brain Regeneration and Convergence to Control at Late Stages

Next, to explore the co-expression relationship between different gene sets, we performed weighted gene co-expression network analysis (WGCNA) on 1, 3 and 14 dpl samples and identified twelve distinct groups of co-expressed genes, the so-called modules (**Supplementary Figure S3**, **Supplementary Table S5**). Expression of the genes clustered in nine modules (M1-M5 and M7-M10) showed a stage-specific component (**Figure 3A**, **Supplementary Figure S3B**), i.e., the genes in these modules revealed expression patterns that distinguished one stage of regeneration from the others, indicating a grouped response peaking at that particular stage. Notably, GO term enrichment analyses performed by using the genes clustered in

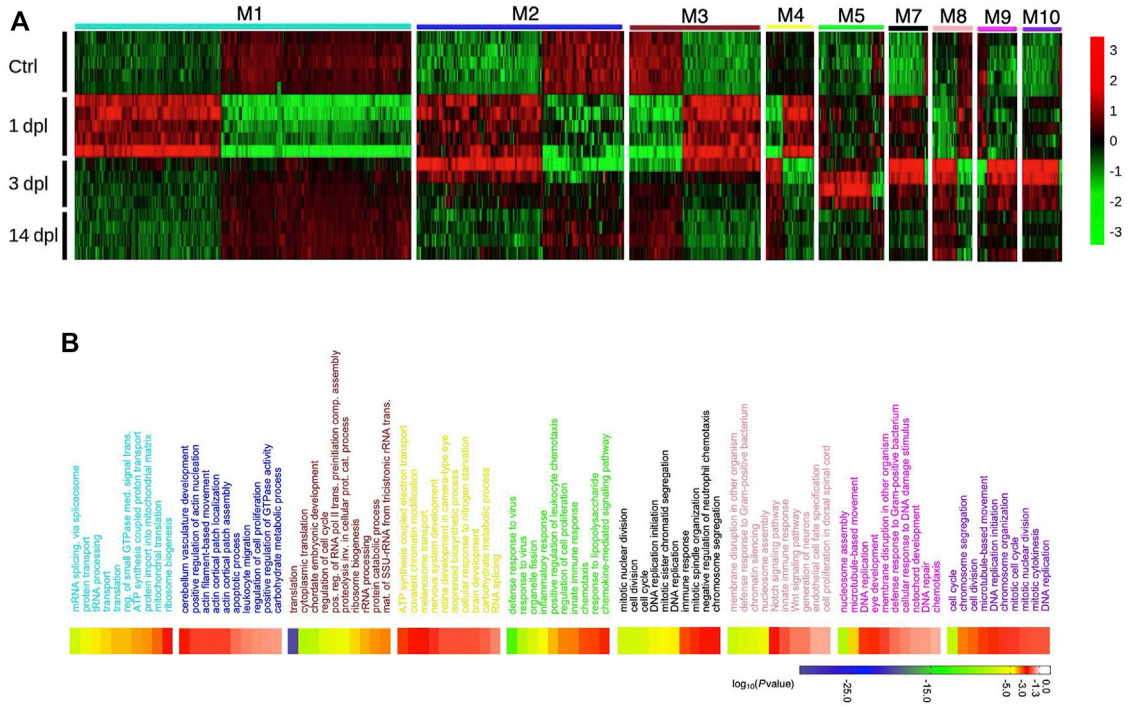


FIGURE 3 | Network analysis of zebrafish brain regeneration at three stages reveals stage-specific modules. **(A)** Heatmap representing relative expression (z-score) of genes that are enriched in each module for the three stages of the adult zebrafish brain regeneration. Each row represents a sample, and each column shows a single gene. Red and green shades show high or low relative expressions, respectively. **(B)** DAVID was used to show the most significantly enriched GO-BP terms based on the transcriptional changes of each significant module and their associated enrichment *p*-values for Top 10 GO-BP terms. The heatmap scale shows $\log_{10}$ of the ease *p*-values for the most significantly enriched GO terms. dpl: days post-lesion, ctrl: control.

these nine modules showed a similar pattern with that performed by using the DEGs for each stage in BP category (**Figure 3B**, **Supplementary Table S6**). For example, genes enriched in M1 (turquoise) and M3 (brown) showed an expression pattern specific to 1 dpl (**Figure 3A**). GO terms of these two modules were associated with translation and ribosome biogenesis, similar to GO-BP terms obtained from analysis of all DEGs at 1 dpl (compare **Figure 3B** to **Figure 2B**). Genes enriched in M5 (green) and M10 (purple) likewise showed a pattern specific to 3 dpl (**Figure 3A**) and had GO terms enriched in immune response and cell cycle that are compatible with the GO-BP terms generated from all DEGs at 3 dpl (compare **Figure 3B** to **Figure 2C**). Genes that were affected at both 1 dpl and 3 dpl were enriched in M2 (blue) and M7 (black) (**Figure 3A**) and consisted of GO terms related with immune response, cell cycle and apoptosis, which were significantly enriched in GO terms and KEGG pathways performed with genes differentially expressed at one of these stages (compare **Figure 3B** to **Figures 2B,C** and **Supplementary Figure S2**). Interestingly, there was no module specific to 14 dpl, which mostly displayed modules similar to control. Moreover, clustering of regeneration-related GO-BP terms enriched at all three stages further supports that biological events occurring during adult brain regeneration display stage-specific patterns (**Supplementary Figure S4**).

To understand the changes in gene expression profiles during adult brain regeneration at a global level, we drew heatmap plots using all DEGs (9,136 genes, **Supplementary Table S7**) identified at three stages by using variance-stabilized counts normalized as z-scores for all samples (**Supplementary Figure S5**). Control samples showed the lowest variability. Samples of 1 dpl and 3 dpl displayed a high variability, probably due to activation of intense regeneration events such as reactive proliferation, which can vary significantly between individuals. In contrast, the variability decreased in samples of 14 dpl and gene expression patterns became similar to the control, most likely because neuronal circuits are partially re-established at this stage (Kroehne et al., 2011). These data collectively indicate that while 1 dpl and 3 dpl were unique with respect to their gene modules and gene expression profiles, 14 dpl is rather similar to the control group, suggesting that gene expression patterns in later stages of regeneration converge to those of the uninjured state.

The Early Wound Healing Stage of Brain Regeneration is More Similar to Glioblastoma than to Low-Grade Glioma in Terms of Activation of Metabolic and Neurogenic Pathways

Due to the growing evidence that bridge the mechanisms of regeneration and cancer, we hypothesize that regeneration and cancer must share some molecular mechanisms at the early stages of regeneration where proliferation is the prominent event. However, the mechanisms must diverge later when the

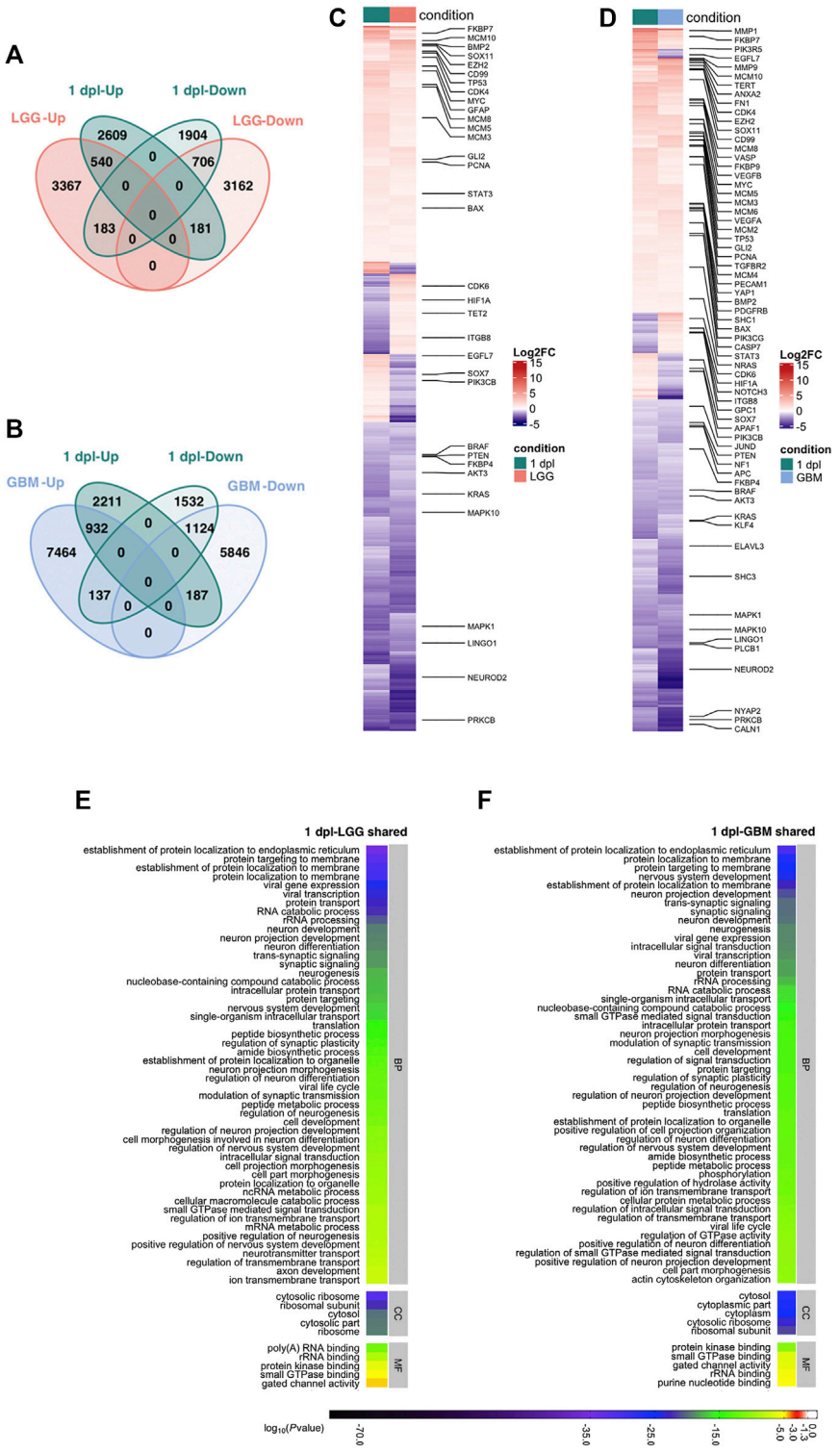


FIGURE 4 | Early wound healing stage (1 dpl) of brain regeneration is similar to brain cancer with respect to induction of metabolism- and neurogenesis-related signaling responses. **(A, B)** Venn diagrams showing the number of upregulated (Up) or downregulated (Down) DEGs and the overlap between 1 dpl (turquoise) and **(A)** LGG (pink) and **(B)** GBM (blue). **(C, D)** Heatmaps show the expression of genes shared between 1 dpl (turquoise) and **(C)** LGG (pink) and **(D)** GBM (blue). Each column represents a condition (1 dpl, LGG or GBM) and each row shows a single gene. The scale bar shows $\log_2$ fold changes from high to low regulation, represented by a color gradient from red to purple, respectively. **(E, F)** DAVID was used to show the most significantly enriched GO-BP (top 50), CC (top 5), and MF (top 5) terms based on transcriptional changes in comparison of 1 dpl with **(E)** LGG and **(F)** GBM by using human identifiers of shared DEGs. The heatmap scale shows $\log_{10}$ of the ease *p*-values for the most significantly enriched GO terms. dpl: days post-lesion, DAVID: database for Annotation, Visualization and Integrated Discovery, GO: Gene Ontology, BP: Biological Process, MF: Molecular Function, CC: Cellular Component, dpl: days post lesion, LGG: low-grade glioma, GBM: glioblastoma.

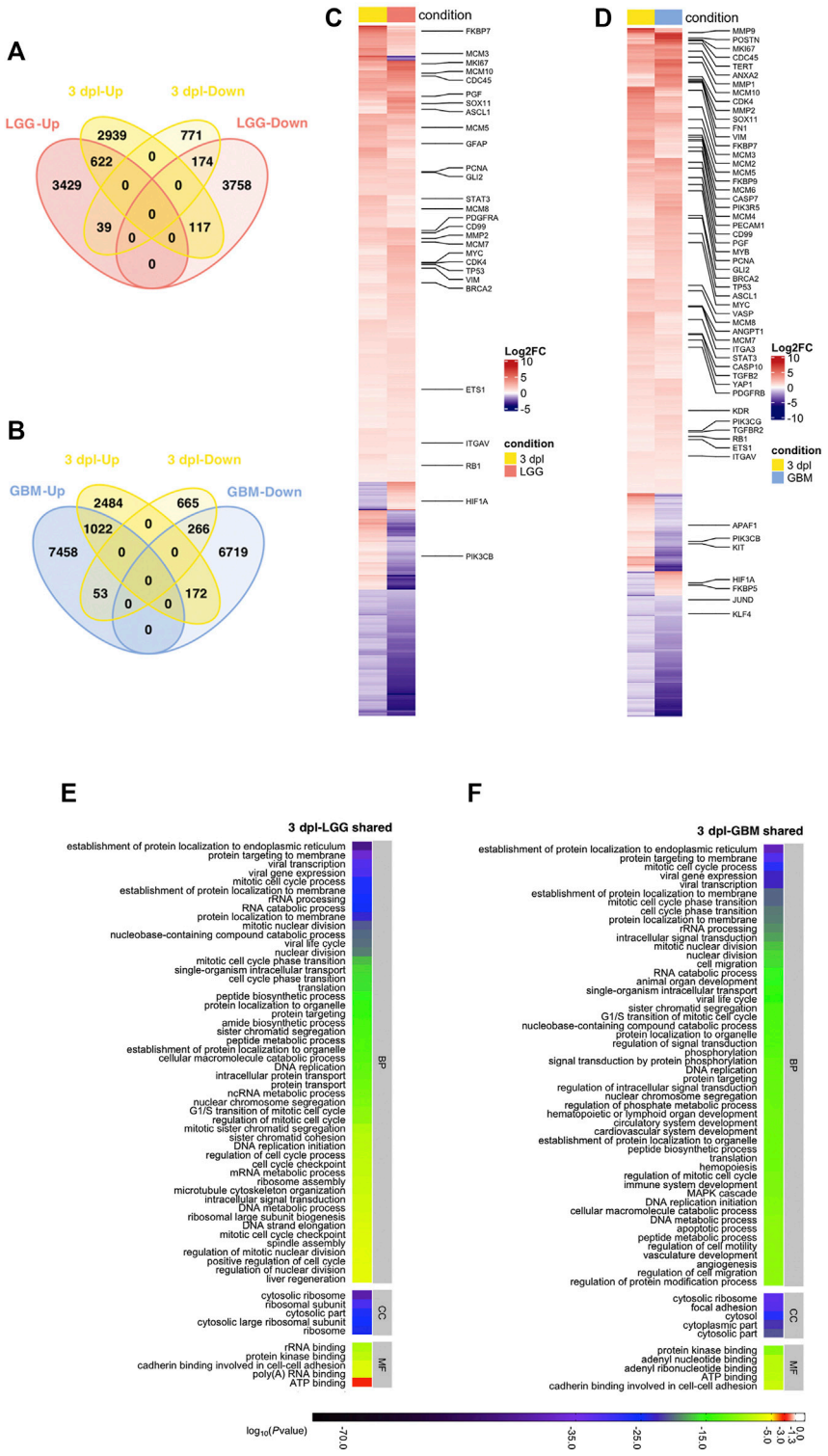


FIGURE 5 | Early proliferative stage (3 dpl) of brain regeneration resembles brain cancer with regard to activation of cell proliferation. **(A,B)** Venn diagrams showing the number of upregulated (Up) or downregulated (Down) DEGs and the overlap between 3 dpl (yellow) and **(A)** LGG (pink) and **(B)** GBM (blue). **(C,D)** Heatmaps showing the expression of genes shared between 3 dpl (yellow) and **(C)** LGG (pink) and **(D)** GBM (blue). Each column represents a condition (3 dpl, LGG or GBM) and each row shows a single gene. The scale bar shows $\log_2$ fold changes from high to low regulation, represented by a color gradient from red to purple, respectively. **(E,F)** DAVID was used to show the most significantly enriched GO-BP (top 50), CC (top 5), and MF (top 5) terms based on transcriptional changes in comparison of 3 dpl with **(E)** LGG and **(F)** GBM by using human identifiers of shared DEGs. The heatmap scale shows $\log_{10}$ of the ease *p*-values for the most significantly enriched GO terms. dpl: days post-lesion, DAVID: database for Annotation, Visualization and Integrated Discovery, GO: Gene Ontology, BP: Biological Process, MF: Molecular Function, CC: Cellular Component, dpl: days post lesion, LGG: low-grade glioma, GBM: glioblastoma.

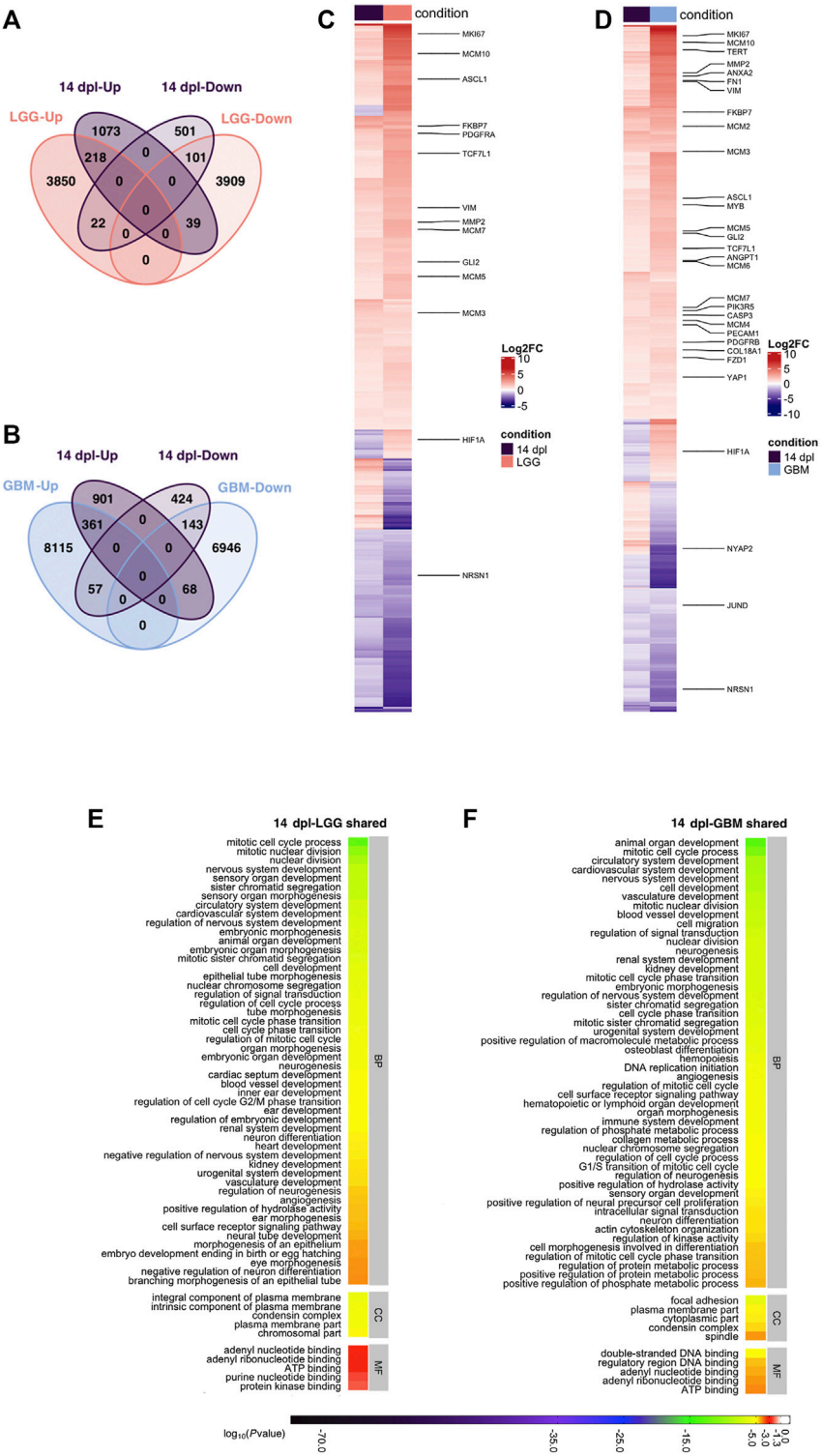
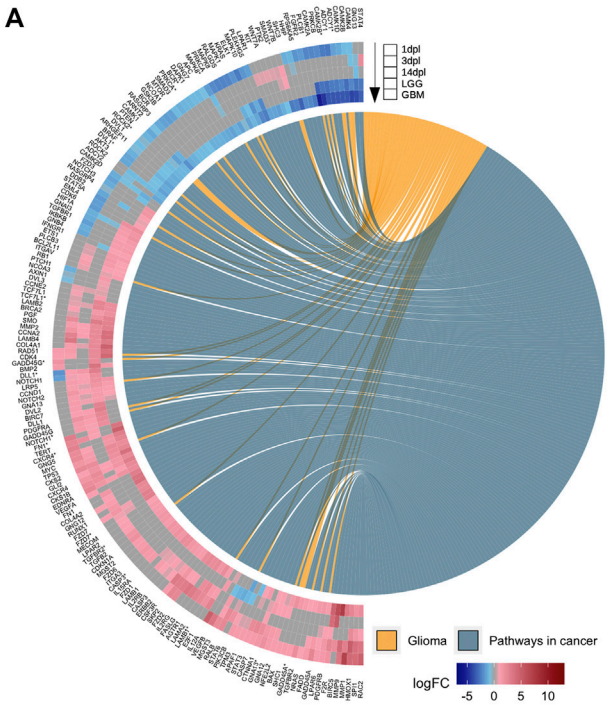


FIGURE 6 | Differentiation stage (14 dpl) of brain regeneration and brain cancer share mechanisms related to developmental and morphogenetic processes. **(A,B)** Venn diagrams showing the number of upregulated (Up) or downregulated (Down) DEGs and the overlap between 14 dpl (purple) and **(A)** LGG (pink) and **(B)** GBM (blue). **(C,D)** Heatmaps showing the expression of genes shared between 14 dpl (purple), and **(C)** LGG (pink), and **(D)** GBM (blue). Each column represents a condition (14 dpl, LGG or GBM) and each row shows a single gene. The scale bar shows their $\log_2$ fold changes from high to low regulation, represented by a color gradient from red to purple, respectively. **(E,F)** DAVID was used to show the most significantly enriched GO-BP (top 50), CC (top 5), and MF (top 5) terms based on transcriptional changes in comparison of 14 dpl with **(E)** LGG and **(F)** GBM by using human identifiers of shared DEGs. The heatmap scale shows $\log_{10}$ of the ease *p*-values for the most significantly enriched GO terms. dpl: days post-lesion, DAVID: database for Annotation, Visualization and Integrated Discovery, GO: Gene Ontology, BP: Biological Process, MF: Molecular Function, CC: Cellular Component, dpl: days post lesion, LGG: low-grade glioma, GBM: glioblastoma.



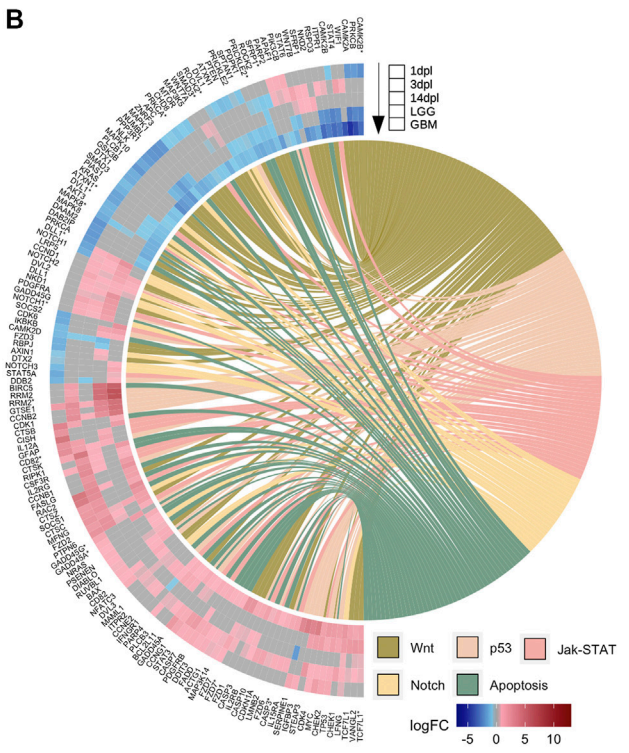


FIGURE 7 | Early wound healing stage of brain regeneration is more similar to LGG and GBM than the proliferation and differentiation stages. **(A,B)** GOChord plots show log_2 fold changes of the genes annotated in selected KEGG pathways **(A)** "Glioma" and "Pathways in cancer" and **(B)** "Wnt", "p53", "Jak-STAT", "Notch" and "Apoptosis" for three stages of the zebrafish brain regeneration and two types of human brain cancers. The genes are linked to their assigned pathways by ribbons and ordered according to their log_2 fold change values from high to low regulation, represented by a color gradient from blue to red, respectively. log_2 fold changes are shown from the outer to the inner annulus in the following order: 1, 3, 14 dpl, LGG and GBM. An asterisk was appended to human genes associated as orthologs to several zebrafish genes in the list. dpl: days post-lesion, LGG: low-grade glioma, GBM: glioblastoma.

(*Continued*)

regenerative response terminates precisely, while cancer cells keep proliferating. To test whether this hypothesis holds true for the brain, we set out to compare the transcriptome of the regenerating adult brain to that of the brain with cancer. As a first step, we compared LGG/GBM samples from TCGA with normal tissue to identify the DEGs. Expression of 7,992 genes (4,036 Up, 3,956 Down) and 15,469 genes (8,451 Up, 7,018 Down) were significantly altered in LGG and GBM, respectively (**Supplementary Figures S6A,B**, **Supplementary Table S2**).

To investigate the shared genes between early wound healing stage of brain regeneration with LGG and GBM, we intersected unique human orthologs of DEGs at 1 dpl with the DEGs in LGG and GBM. Out of the 6,123 genes that were differentially expressed at 1 dpl, 1,610 genes were shared with LGG and 1,246 of them were altered in the same direction, i.e., both Up or both Down (**Figure 4A**, **Supplementary Table S7**). Among shared genes, *tp53*, *gfap*, and *pcna* were Up, while *neurod2, braf, kras, pten* and *akt3* were Down (**Figure 4C**, **Supplementary Table S7**). Interestingly, 4,513 genes (2,609 Up, 1904 Down) were unique to 1 dpl. Between 1 dpl and GBM, the number of shared genes increased to 2,380, 2056 of which were regulated in the same direction and included majority of the genes shared between 1 dpl and LGG (**Figures 4B,D**; **Supplementary Table S7**). Here, 3,743 genes (2,211 Up, 1,532 Down) were unique to 1 dpl (**Figure 4B**). Thus, early wound healing stage of regeneration is more similar to GBM than to LGG at the transcriptional level, most likely due to the high number and variation of DEGs detected in GBM.

Next, we performed functional annotation of shared genes by using human gene identifiers (**Figures 4E,F**, **Supplementary Figure S7A**; **Supplementary Tables S7–S9**). 39 terms were shared between top 50 GO-BP terms enriched in the comparisons of shared DEGs in 1 dpl-LGG and 1 dpl-GBM (**Figures 4E,F**, **Supplementary Table S8**). These terms included various processes related to protein metabolism and neurogenesis. Furthermore, KEGG pathway enrichment of shared DEGs showed that various neurogenesis-related pathways including mTOR, ErbB, MAPK and oxytocin signaling as well several synapse and axonal pathways were shared between 1 dpl and LGG (**Supplementary Figure S7A**, **Supplementary Table S9**). Strikingly, glioma was enriched in shared DEGs of 1 dpl with both LGG and GBM (**Supplementary Figure S7A**, **Supplementary Table S9**). To identify KEGG pathways that were specific to the very early stage of brain regeneration, we exploited the DEGs unique to 1 dpl with respect to LGG or GBM. Among the unique top 30 KEGG pathways, apoptosis and the JAK-STAT signaling pathway were prominent (**Supplementary Figure S7B**, **Supplementary Table S10**). In summary, our results indicate that the early wound

healing stage of brain regeneration is similar to brain cancer with respect to induction of metabolism- and neurogenesis-related signaling responses and different from cancer mainly via induction of apoptosis during early regeneration.

The Early Proliferative Stage of Brain Regeneration is Similar to Low-Grade Glioma/Glioblastoma with Respect to Active Proliferation

Next, to reveal the shared genes between the early proliferative stage of brain regeneration and brain cancer, we overlapped human orthologs of DEGs at 3 dpl with the DEGs in LGG and GBM. 952 out of 4,662 DEGs determined at 3 dpl were shared with LGG and 796 out of 952 were Up/Down in both 3 dpl and LGG (**Figure 5A**, **Supplementary Table S7**). Shared genes involved the proliferation and glial markers *mki67, pcna*, several *mcm* genes and *gfap*, which were all Up (**Figure 5C**, **Supplementary Table S7**). The percentage of unique genes at 3 dpl were greater than that at 1 dpl and reached a total number of 3,710 (2,939 Up, 771 Down) (**Figure 5A**). When compared to GBM, 1,513 DEGs were shared with 3 dpl and 1,288 of them were altered in the same direction (**Figure 5B**, **Supplementary Table S7**). Among the shared Up genes were many proliferative and cancer-related genes such as *angpt1, vim, brca2*, *pcna, mcm2,* and *mki67* (**Figure 5D**). Here, we found 3,149 DEGs (2,484 Up, 665 Down) that were unique to 3 dpl (**Figure 5B**).

Functional annotations of shared DEGs revealed that 31 terms out of the top 50 GO-BP terms were mutual between 3 dpl-LGG and 3 dpl-GBM (**Figures 5E,F**, **Supplementary Figure S7A**, **Supplementary Tables S8, S9**). The mutual GO-BP terms contained a number of proliferation-related ones such as various mitotic cell cycle processes, nuclear division processes and DNA replication. GO terms were supported by the KEGG pathway enrichment analysis, which showed that shared DEGs were enriched in various pathways related to proliferation and DNA repair as well as p53, MAPK and calcium signaling pathways (**Figure 7B**, **Supplementary Table S9**). Here, several cancer-related pathways were enriched in shared DEGs of 3 dpl with both LGG and GBM (**Figure 7A**, **Supplementary Table S9**). Next, we determined the KEGG pathways that are specific to the early proliferative stage of brain regeneration and found that DEGs unique to 3 dpl were enriched in immune response-related processes and apoptosis, p53, Toll-like receptor and JAK-STAT signaling pathways, within the top 30 KEGG pathways (**Supplementary Figure S7B**, **Supplementary Table S10**). These data suggest that the early proliferative stage of brain regeneration resembles brain cancer mainly by promotion of cell proliferation, while differing from cancer by the active immune response and apoptosis.

Developmental and Morphogenetic Signaling Pathways are Commonly Activated During the Differentiation Stage of Brain Regeneration and Low-Grade Glioma/Glioblastoma

Next, to compare the differentiation stage of adult brain regeneration with brain cancer, we intersected human orthologs of DEGs at 14 dpl with the DEGs in LGG and GBM. Among 1954 DEGs detected at 14 dpl, 380 were shared with LGG and 319 of the shared DEGs were regulated similarly at 14 dpl and LGG (**Figure 6A**, **Supplementary Table S7**). Shared DEGs contained several *mcm* genes and differentiation-related genes (**Figure 6C**, **Supplementary Table S7**). 1,574 genes (1,073 Up, 501 Down) were unique to 14 dpl (**Figure 6A**). 14 dpl and GBM shared 629 genes, 504 of which were regulated in the same direction and mostly overlapped with those shared between 14 dpl and LGG (**Figures 6B,D**; **Supplementary Table S7**). 1,325 genes (901 Up, 424 Down) were unique to 14 dpl when compared to GBM (**Figure 6B**).

Our functional annotation of genes shared between the differentiation stage of brain regeneration and brain cancer demonstrated that 31 of the top 50 GO-BP terms were shared between 14 dpl-LGG and 14 dpl-GBM (**Figures 6E,F**, S7A, **Supplementary Tables S8, S9**). A number of GO-BP terms related to development and morphogenesis including nervous system development, neuron differentiation and angiogenesis were remarkable. Moreover, Notch, Wnt, Hippo and calcium signaling pathways were enriched in the top 30 KEGG pathways (**Supplementary Figure S7A**). Interestingly, several cancer-related pathways were enriched in DEGs between 14 dpl and LGG/GBM (**Supplementary Figure S7A**, **Supplementary Table S9**). The Wnt signaling pathway was also enriched in the KEGG pathways that are unique to 14 dpl with respect to LGG/GBM along with p53 and Toll-like receptor signaling (**Supplementary Figure S7B**, **Supplementary Table S10**). Thus, signaling pathways that control certain developmental and morphogenetic processes are commonly activated during the differentiation stage of brain regeneration and brain cancer.

The Early Wound Healing Stage of Brain Regeneration is More Similar to Low-Grade Glioma and Glioblastoma than the Proliferation and Differentiation Stages

While individual comparisons of the regenerative stages to LGG and GBM are informative about particular similarities of these stages to gliomas, a global comparison is necessary to reveal which stage of brain regeneration is most comparable to brain cancer. To this purpose, we drew heatmaps of $\log_2$ fold changes of the 3,615 genes that are differentially expressed in at least one stage of brain regeneration and shared with at least one type of brain cancer (**Supplementary Figure S8**). The genes obtained from the KEGG database included a substantial number of genes involved in glioma, pathways in cancer, as well as Wnt, p53, JAK-STAT Notch, apoptosis, RAS, MAPK, mTOR and PI3K-Akt signaling pathways (**Figures 7A,B**, **Supplementary Figure S9**). To compare the changes in gene expression associated with these selected pathways in three regenerative stages and two brain cancers, we intersected the genes annotated in these pathways with the DEG sets. Strikingly, the majority of the DEGs of the early wound healing stage showed an expression pattern that is similar to the both human brain cancers, but mostly to GBM (**Figures 7A,B**, **Supplementary Figure S9**). In general, if a gene is Up at 1 dpl, it is generally Up in LGG/GBM and if a gene is Down

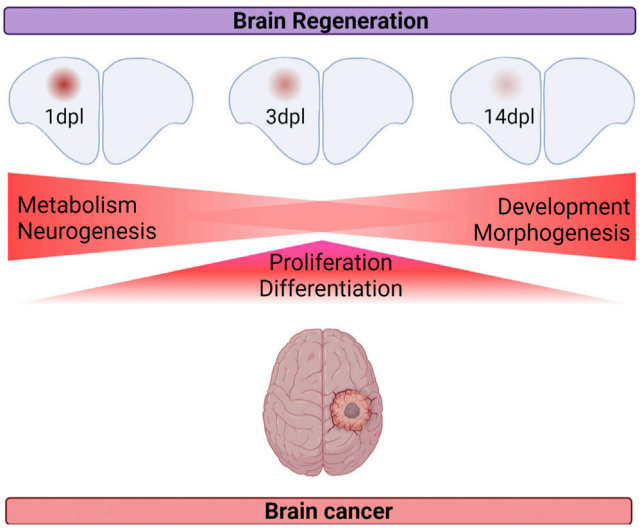


FIGURE 8 | Summary of the shared cellular mechanisms between brain regeneration at three different stages of regeneration and brain cancer.

at 1 dpl, it is likewise Down in LGG/GBM (**Supplementary Figure S8**). The number of significantly altered genes was highest at 1 dpl and decreased at 3 dpl and 14 dpl for all pathways. While most Wnt signaling-related genes were Down or absent across DEG sets, p53 signaling-related genes were mainly Up or absent across DEG sets (**Figure 7B**). Interestingly, the expression of several genes in the KEGG pathway "pathways in cancer", such as *ptk2, kit, lpar1, notch1, rasgrp4, ifngr1, ptch1, apaf1* and *dll1*, and Wnt pathway-related genes, such as *wif1, rspo3, nkd2, sfrp1, smad3, wnt7a, wnt7b* and *axin1*, showed opposite expression patterns between brain regeneration and brain cancers, suggesting that these genes may play key roles in preventing the cells from undergoing carcinogenesis (**Figures 7A,B**). In conclusion, among the three stages of brain regeneration, the early wound healing stage was the most similar one to the brain cancers LGG and GBM with respect to their transcriptomes, while the similarity decreased as regeneration proceeded to the proliferation and differentiation stages.

DISCUSSION

Despite the studies investigating the common and distinct molecular mechanisms underlying regeneration and cancer, how brain regeneration and brain cancer compare with each other at the level of gene expression has been overlooked. This study has two novel aspects. First, it unravels the gene expression profiles of the regenerating adult zebrafish telencephalon at two early (1 dpl and 3 dpl) and one relatively late (14 dpl) stage of regeneration: 1 dpl as the early wound healing stage, 3 dpl as the early proliferative stage and 14 dpl as the differentiation stage. Second, this study is the first that compares gene expression profiles of the three different stages of adult brain regeneration with two different brain cancers: low-grade glioma (LGG) and glioblastoma (GBM). Based on our detailed analyses, we have drawn the following conclusions: 1) the total number of DEGs at 1 dpl are higher than those at 3 dpl and 14 dpl. 65, 38.5 and 19% of the total DEGs are unique to 1, 3 and 14 dpl, respectively. 2) The more distinctive expression pattern of 1 dpl, and to a lesser extent 3 dpl, is further supported by the unique gene modules that are detected within the transcriptomes of 1 dpl and 3 dpl and by the gene expression profiles that are more divergent from the control. In contrast, the transcriptome of 14 dpl is rather similar to the control group and converges to the transcriptome of the uninjured brain. 3) 1 dpl of brain regeneration is similar to LGG/GBM with respect to activation of metabolism- and neurogenesis-related signaling pathways and different from cancer in the way of activating apoptosis (**Figure 8**). 4) 3 dpl and LGG/GBM are similar with regard to elevated cell proliferation and differentiation (**Figure 8**). 5) 14 dpl resembles LGG/GBM because of induced developmental and morphogenetic processes (**Figure 8**). 6) 1 dpl is more similar to LGG/GBM than 3 dpl and 14 dpl are. Thus, brain regeneration and brain cancer appear to share higher number of molecular mechanisms in the early stages of regeneration, while the similarity decreases at its later stages.

The Immune Response is Induced Early After Injury and Starts to Decline After the Proliferative Stage

Tissue damage triggers a cascade of early regenerative processes including initiation of wound closure and activation of immune response that is necessary for clearance of tissue debris and deposition of extracellular matrix (Marques et al., 2019). Because of bleeding and inflammation, the lesion site is infiltrated by platelets and immune cells, which are controlled by numerous signaling molecules (Krafts, 2010; Kroehne et al., 2011; Marques et al., 2019). For example, a variety of cells including fibroblasts, macrophages and monocytes, which are primed by mesenchymal stem/stromal cells, are essential for regeneration and activated by the platelet-derived growth factors (PDGFs) to enhance proliferation, chemotaxis and gene expression (Pierce et al., 1991; Andrae et al., 2008; Krafts, 2010). PDGFs have also been shown to be important for myelin regeneration in CNS by stimulating proliferation, differentiation and survival of the cells in the oligodendroglial lineage (Webster, 1997; Watzlawik et al., 2013). Our data showed significant upregulation of PDGF and PDGF receptor (PDGFR) genes such as *pdgfba* selectively at 3 dpl, *pdgfd* and *pdgfaa* at 1 dpl and 3 dpl, and *pdgfrl, pdgfra* and *pdgfrb* at 3 dpl and 14 dpl. In addition to the growth factors, cytokines secreted by cells of the immune system act as immunomodulators to regulate the acute inflammatory response that is necessary for functional regeneration of the zebrafish CNS after injury (Krafts, 2010; Kyritsis et al., 2012; Elsaeidi et al., 2014; Fuller-Carter et al., 2015; Tsarouchas et al., 2018). We found several anti-inflammatory cytokines and their receptors including *il6st, il11a, il11b, il13, il21, il21r.1* and *il34* to be significantly upregulated at the two early stages (1 dpl and 3 dpl) of brain

regeneration. Moreover, the signature cytokines, including *il12rb2*, *il7r*, *ifng1* and *stat4* (3 dpl) and *il13* and *irf1b* (1 dpl and 3 dpl), for T helper1 (Th1) cell subset are upregulated at the early stages of regeneration (Hamalainen et al., 2001; Duhen et al., 2014; Raphael et al., 2015). Th2 signature cytokines such as *il4* (1 dpl) and *il13* (1 dpl and 3 dpl) were likewise upregulated at the early stages and *ifngr1l* was downregulated at 14 dpl. These findings suggest that both Th1- and Th2-mediated immune responses are activated mainly at the early stages of brain regeneration. Moreover chemokines, a specific type of cytokines, and their receptors play key roles in the activation and infiltration of the immune cells to the injury site in CNS (Jaerve and Muller, 2012). Chemokines have been shown to control immune and progenitor cell homeostasis and thereby regeneration in several zebrafish tissues (Kizil et al., 2012a; Xu et al., 2014; Bussmann and Raz, 2015; Iribarne, 2021). Notably, a number of chemokine and chemokine receptor genes including *cxcl20, cxcl11.5, ccl39.2, cxcl11.6, ccl19a.1* and *ccl36.1* were upregulated at 3 dpl and almost vanished at 14 dpl of brain regeneration. Another group of signaling molecules consists of the members of the tumor necrosis factor superfamily (TNFSF) that are expressed mainly by the immune cells and act as cytokines to regulate neuroinflammation and autoimmunity in the CNS (Sonar and Lal, 2015; Fresegna et al., 2020). Several TNFSF and its corresponding TNFSF receptor superfamily (TNFRSF) genes, such as *tnfb, tnfsf10, tnfsf12, tnfsf13b*, *tnfrsf9a* and *tnfrsf1a*, were significantly upregulated during early regeneration, especially at 3 dpl. The number of altered TNFSF and TNFRSF genes reduced dramatically at 14 dpl. Overall, the parallel activation of PDGFs, cytokines, chemokines and TNF-related factors at the early wound healing stage, their peaking at the proliferative stage and their depletion at the differentiation stage suggest that the immune response is induced early after injury, remains strongly active during establishment of a proliferative response in regeneration and dampens as tissue differentiation starts.

Activation of Apoptosis Is Regulated in Parallel to Proliferation

Apoptosis is another prominent event that is activated in the early phases of brain regeneration for effective wound healing (Wilson et al., 2007; Guerin et al., 2021). Apoptosis has been shown to be activated twice during early regeneration processes in different organisms. For example, *Hydra* and Planaria appear to have the first peak of apoptosis very early after bisection and the second peak at 3 days after the injury (Chera et al., 2009; Pellettieri et al., 2010; Beane et al., 2013). The adult zebrafish fin regeneration follows a similar route in activation of apoptosis at 12 h post-amputation (hpa) and 72 hpa (Gauron et al., 2013). However, in the *Xenopus* tail regeneration, apoptosis is absent during wound healing, activated at 12 hpa and remains active until 48 hpa (Tseng et al., 2007). We noted a significant upregulation of the apoptosis-related genes *tp53, apaf1, caspa, casp7* and *baxb* at both early regenerative stages, 1 dpl and 3 dpl. Strikingly, the number of apoptosis-related genes doubled at 3 dpl. Apoptosis is considered to have a critical role in resolving inflammation by converting the immune response in early stages of tissue repair into a wound healing response (Brown et al., 1997; Wu and Chen, 2014). Besides, multiple studies have proposed that apoptosis can stimulate proliferation within the regenerating tissues of *Hydra*, Planaria, *Xenopus* and zebrafish (Gargioli and Slack, 2004; Jopling et al., 2010; Morata et al., 2011; Diwanji and Bergmann, 2018; Kha et al., 2018; Stocum, 2019; Guerin et al., 2021). Mainly at 3 dpl, we observed strong activation of apoptosis-related gene expression with a concomitant elevation of cell proliferation. Thus, the capability of the zebrafish telencephalon to convert an early inflammatory reaction into a healing capacity could be reinforced by the parallel elevation in expression level of genes associated with apoptosis at the early wound healing and proliferation stages (Demirci et al., 2020).

Angiogenic Activity and Proliferation During Brain Regeneration

Angiogenic sprouting into the wound site has been revealed as another essential event of the regeneration process and observed 15 h after injury during heart regeneration in zebrafish (Marin-Juez et al., 2016). *Vascular endothelial growth factor Aa* (*vegfaa*), which is actively involved in angiogenesis, vasculogenesis and endothelial cell growth, is upregulated during heart regeneration of zebrafish (Marin-Juez et al., 2016). Our results revealed upregulation of *vegfaa* specifically at 1 dpl, suggesting that injury triggers a rapid angiogenic sprouting at early brain regeneration. While angiogenesis was strongly promoted at 1 dpl, a massive rise in the number of angiogenesis-related genes was detected at 3 dpl. *Angiopoietin-1* (*angpt1*) has been shown essential to mouse vasculature during response to injury (Jeansson et al., 2011). We found that *angpt1* was upregulated at 3 dpl and 14 dpl. Angiogenesis has been demonstrated to be activated within 4–7 days after cerebral ischemia and contribute to neuronal remodeling and functional recovery via first providing guidance to the sprouting axons through VEGF signaling and second enhancing proliferation, migration and differentiation of neural stem/progenitor cells (Wang et al., 2007; Ruan et al., 2015; Kanazawa et al., 2017; Hatakeyama et al., 2020). Thus, early activation and continued maintenance of angiogenesis during brain regeneration imply a similar role for angiogenesis in the repair of traumatic brain injury.

Adult zebrafish brain regeneration is achieved by injury-induced proliferation of the radial glial cells (RGCs) that gives rise to new neurons (Ghosh and Hui, 2016). RGCs express the glial fibrillary acidic protein (Gfap), an intermediate filament marker of the mammalian astrocytes (Jurisch-Yaksi et al., 2020). Moreover, proliferating cell nuclear antigen (Pcna), a cell proliferation marker, is released by actively dividing RGCs as an indicator of constitutive neurogenesis (Zacchetti et al., 2003). We identified a remarkable increase in the expression of *gfap* and *pcna* during both early stages of regeneration. Besides, *s100b* and *fabp7a*, enriched in quiescent RGC genes, as well as *mki67* (only at 1 dpl) and *mcm2*, markers of dividing cells, were upregulated at the early stages of brain regeneration (Zhang and Jiao, 2015; Kaslin et al., 2017; Lange et al., 2020).

Brain Regeneration Resembles Brain Cancer at its Earlier Stages and Diverges from Cancer with Regard to Opposite Regulation of Key Cancer-Related Genes

There is growing evidence that associates regeneration with cancer. For example, melanomas have been demonstrated to express genes that have important functions in development of the melanocyte lineage and regeneration of the melanocytes, strongly suggesting that human cancers share features with both development and tissue regeneration (White and Zon, 2008). A previous study in zebrafish has likewise revealed that 40% of the genes that were upregulated during blastema formation in regeneration of the caudal fin are also overexpressed in human melanoma (Hagedorn et al., 2016). However, the underlying mechanistic connection between regeneration and cancer has not been analyzed so far at the molecular level as regard to comparative analysis of the transcriptomes of regenerating brain and brain cancer. The comparison of the three stages of brain regeneration (1, 3 and 14 dpl) with two different brain cancers (LGG and GBM) showed that the number of shared and unique DEGs were the highest in the comparison of 1 dpl with GBM. This is most likely a consequence of the total DEG numbers being highest at 1 dpl and in GBM. Furthermore, the global comparison of the three regeneration stages with two cancers revealed that 1 dpl was the most similar regenerative stage to both LGG and GBM. The DEGs shared between 1 dpl and LGG/GBM were enriched in the KEGG pathway "glioma". The majority of the genes in this pathway were regulated in the same direction (both Up or both Down) at 1 dpl and LGG/GBM. For example, *Camk2* genes have been found to be strongly downregulated in GBM compared to the normal brain tissue (Johansson et al., 2005; Xiong et al., 2019; He and Li, 2021). *Shc3* and *kras* are likewise downregulated in primary cultures and patient samples of GBM, while *shc1*, *gadd45a* and *tgfbr2* are strongly upregulated (Magrassi et al., 2005; Lymbouridou et al., 2009; Guo et al., 2018; Hirakata et al., 2021). Moreover, the tumor suppressors *pten* and *tp53* are frequently mutated and non-functional in GBM (Benitez et al., 2017; Zhang et al., 2018). Strikingly, the expression of those genes did not change significantly at 3 dpl, nor at 14 dpl. This means that while these genes are essential for the early initiation of a regenerative response upon injury, they need to be suppressed later for the regeneration to be terminated precisely and prevent the transformation of a normal cell into a cancer cell. Thus, the fact that expression of glioma-related genes is similarly regulated exclusively in the early stages of regeneration but not in later stages mark them as drug-targetable candidates for GBM treatment.

Among the shared genes between brain regeneration and brain cancer, a wide range of genes that are related with apoptosis, proliferation, angiogenesis and invasion and have been associated with glioma showed opposite directions of expression regulation. For example, the transcription factor SRY-related HMG-box 7 (Sox7), which acts as a tumor suppressor, has been found to be downregulated in a variety of cancers including GBM and its downregulation has been associated with poor prognosis (Katoh, 2002; Stovall et al., 2013; Liu et al., 2014; Zhao et al., 2016; Oh et al., 2017; Kim et al., 2018). Similarly, apoptosis protease-activating factor-1 (Apaf1), a key molecule in the apoptotic pathways, is downregulated in different cancer types (Soengas et al., 2006; Tanase et al., 2015). In accordance with these findings, we observed downregulation of both *sox7* and *apaf1* in LGG/GBM. However, they were both upregulated at 1 dpl and *apaf1* also at 3 dpl of brain regeneration. In contrast, Hypoxia inducible factor 1 (HIF-1), a key regulator of hypoxia, has been demonstrated to promote the migratory and invasive behavior of glioma cells as well as to induce angiogenesis by regulating the expression of VEGF, PDGFs and PDGFRs (Mendez et al., 2010; Peng et al., 2021). The cell cycle regulator cyclin-dependent kinase 6 (Cdk6) is also known to be significantly upregulated in glioma cells, and its elevated expression correlates with the grades of glioma malignancy and glioma resistance to chemotherapy (Lu et al., 2018). While expression of both *hif-1* and *cdk6* increased in both LGG and GBM, we found them to have decreased in at least one stage of brain regeneration. A recent study showed that overexpression of Annexin A2 (Anxa2) increased the expression of Glypican 1 (Gpc1) via c-Myc, creating a positive feedback loop that enhances proliferation of glioma cells (Li et al., 2021). *Anxa2* expression increased during early regeneration and GBM. Interestingly, while being upregulated in GBM, *gpc1* expression was strongly downregulated at 1 dpl, proposing that the feedback loop activated by Gpc1 in cancer cannot be activated during regeneration. Altogether, these findings strongly suggest that while early brain regeneration is more similar to brain cancer than late regeneration, it also diverges from cancer due to important differences with regard to opposite regulation of key genes related to cancer progression and activation of signaling mechanisms that prevent carcinogenesis.

Glioblastoma stem-like cells (GSCs) are a highly tumorigenic cell group in GBMs and mediate cancer progression, resistance to traditional treatment and recurrence of glioma (Hemmati et al., 2003; Singh et al., 2004; Bao et al., 2006; Gilbert and Ross, 2009; Zhou et al., 2009; Chen et al., 2012). The sustainability of GSCs and progression of glioma rely on the gene that encodes for the Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) (Suvà et al., 2009). The transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) is also a key player for propagation and sustainability of multipotency in GSCs (Rahaman et al., 2002; Sherry et al., 2009). EZH2-STAT3 interaction has been shown in GSCs by knockdown of EZH2 using shRNA that causes reduced expression of STAT3 by decreasing H3K27 trimethylation (Kim et al., 2013). EZH2 is also necessary for proliferation of progenitor cells in hippocampal and cortical neurogenesis in mice (Pereira et al., 2010; Zhang et al., 2014). We found that *ezh2* and *stat3* were remarkably elevated during early brain regeneration and LGG/GBM. This suggests that the stem cell characteristics are maintained during early regeneration until cues that direct differentiation are received later.

Semaphorins act as guidance cues during axonal development, and control proliferation, migration and differentiation of neurons during nervous system during development as well as maintenance and function of neuronal circuitries in adult

neurogenesis (Carulli et al., 2021). A wide spectrum of roles have been defined for various Semaphorin molecules from regenerative reinnervation to the control of adult neuronal plasticity. For example, Sema3g is necessary for establishment of neural circuit stability and cognitive functions (Tan et al., 2019). On the other hand, glioma patients who expressed lower levels of Sema3g showed shortened survival (Karayan-Tapon et al., 2008). We observed a parallel pattern in our analysis where *sema3gb* was upregulated at 3 dpl and 14 dpl while being downregulated in GBM. Interestingly, a large number of semaphorin genes were exclusively downregulated at 1 dpl and were not altered at later stages. Several semaphorins including Sema3a, Sema3f, Sema3g and Sema6a have been reported to exert tumor growth-inhibiting activities while several others such as Sema4d and Sema6d have been associated with tumor-promoting functions in various cancer types (Law and Lee, 2012; Angelucci et al., 2019). Thus, detailed functional analyses for individual semaphorins are essential to compare their roles in brain regeneration and brain cancer.

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, our comparative analyses of the transcriptomes of the regenerating zebrafish brain at three different regenerative stages with those of two different brain cancers reveal the common and distinctive mechanisms that operate during regeneration and cancer of the brain. Characterization of cellular signals that ensure timely cessation of proliferation, a key step of regeneration, at the correct and controlled termination of regeneration might indeed be exceptionally helpful to identify candidate signals that can stop abnormal proliferative responses to chronic injury or inflammation, stop tumor growth and, perhaps, even direct tumor cells to a regeneration-like route. At this point, the zebrafish represents an excellent model with its organs that show high homology to those of mammals, regenerate and can be induced to develop cancer. Future studies that compare regeneration and cancer using their zebrafish models will not only contribute to our understanding of differential mechanisms of both phenomena but also open new avenues in development of novel anti-cancer therapies. Moreover, an elegant work has presented a comprehensive approach for the DNA methylation-based classification of central nervous system tumors (Capper et al., 2018). Thus, we believe that identification of the genome-wide DNA methylation profiles of the regenerating zebrafish brain and comparison of these cohorts to the human brain tumor classifiers will reinforce our understanding of regulation of brain regeneration mechanisms.

DATA AVAILABILITY STATEMENT

All datasets have been deposited in ArrayExpress under the link: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11163/ with the accession number "E-MTAB-11163".

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC).

AUTHOR CONTRIBUTIONS

GO and YD designed the experiments. YD and EK performed the molecular and cell biology experiments. YD and GH conducted the bioinformatics analyses. GO, YD and GH wrote the manuscript. All authors contributed to the discussion.

FUNDING

This work was supported by the Scientific and Technological Research Council of Turkey (TUBITAK, grant number 215Z365). GO Lab is funded by EMBO Installation Grant (IG 3024). YD was supported by a TUBITAK 2214-A International Research Fellowship Program for PhD Students. YD and EK were supported by TUBITAK 2211-C Domestic Priority Areas Doctoral Scholarship Program.

ACKNOWLEDGMENTS

We would like to thank Izmir Biomedicine and Genome Center Vivarium-Zebrafish Core Facility, Optical Imaging Core Facility and Histopathology Core Facility for providing zebrafish care, microscope facility support and histopathology service support, respectively. We also thank the Genomics Core Facility (*GeneCore*) of EMBL, Heidelberg.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.813314/full#supplementary-material

REFERENCES

Agnihotri, S., Burrell, K. E., Wolf, A., Jalali, S., Hawkins, C., Rutka, J. T., et al. (2013). Glioblastoma, a Brief Review of History, Molecular Genetics, Animal Models and Novel Therapeutic Strategies. *Arch. Immunol. Ther. Exp.* 61 (1), 25–41. doi:10.1007/s00005-012-0203-0

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq--a Python Framework to Work with High-Throughput Sequencing Data. *Bioinformatics* 31 (2), 166–169. doi:10.1093/bioinformatics/btu638

Andrae, J., Gallini, R., and Betsholtz, C. (2008). Role of Platelet-Derived Growth Factors in Physiology and Medicine. *Genes Dev.* 22 (10), 1276–1312. doi:10.1101/gad.1653708

Andrews, S. (2010). *A Quality Control Tool for High Throughput Sequence Data.*

Angelucci, C., Lama, G., and Sica, G. (2019). Multifaceted Functional Role of Semaphorins in Glioblastoma. *Ijms* 20 (9), 2144. doi:10.3390/ijms20092144

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., et al. (2006). Glioma Stem Cells Promote Radioresistance by Preferential Activation of the DNA Damage Response. *Nature* 444 (7120), 756–760. doi:10.1038/nature05236

Baumgart, E. V., Barbosa, J. S., Bally-Cuif, L., Götz, M., and Ninkovic, J. (2012). Stab Wound Injury of the Zebrafish Telencephalon: a Model for Comparative Analysis of Reactive Gliosis. *Glia* 60 (3), 343–357. doi:10.1002/glia.22269

Beachy, P. A., Karhadkar, S. S., and Berman, D. M. (2004). Tissue Repair and Stem Cell Renewal in Carcinogenesis. *Nature* 432 (7015), 324–331. doi:10.1038/nature03100

Beane, W. S., Morokuma, J., Lemire, J. M., and Levin, M. (2013). Bioelectric Signaling Regulates Head and Organ Size during Planarian Regeneration. *Development (Cambridge, England)* 140 (2), 313–322. doi:10.1242/dev.086900

Benitez, J. A., Ma, J., D'Antonio, M., Boyer, A., Camargo, M. F., Zanca, C., et al. (2017). PTEN Regulates Glioblastoma Oncogenesis through Chromatin-Associated Complexes of DAXX and Histone H3.3. *Nat. Commun.* 8 (1), 15223. doi:10.1038/ncomms15223

Boffetta, P., Boccia, S., and Vecchia, C. L. (2014). "Distribution, Causes and Prevention of Individual Neoplasms," in *A Quick Guide to Cancer Epidemiology. SpringerBriefs in Cancer Research* (Cham: Springer). doi:10.1007/978-3-319-05068-3_4

Bondy, M. L., Scheurer, M. E., Malmer, B., Barnholtz-Sloan, J. S., Davis, F. G., Il'yasova, D., et al. (2008). Brain Tumor Epidemiology: Consensus from the Brain Tumor Epidemiology Consortium. *Cancer* 113 (7 Suppl. l), 1953–1968. doi:10.1002/cncr.23741

Bonfanti, L., and Peretto, P. (2011). Adult Neurogenesis in Mammals - a Theme with many Variations. *Eur. J. Neurosci.* 34 (6), 930–950. doi:10.1111/j.1460-9568.2011.07832.x

Brown, D. L., Kao, W. W.-Y., and Greenhalgh, D. G. (1997). Apoptosis Down-Regulates Inflammation under the Advancing Epithelial Wound Edge: Delayed Patterns in Diabetes and Improvement with Topical Growth Factors. *Surgery* 121 (4), 372–380. doi:10.1016/s0039-6060(97)90306-8

Bussmann, J., and Raz, E. (2015). Chemokine-guided Cell Migration and Motility in Zebrafish Development. *EMBO J.* 34 (10), 1309–1318. doi:10.15252/embj.201490105

Capper, D., Jones, D. T. W., Sill, M., Hovestadt, V., Schrimpf, D., Sturm, D., et al. (2018). DNA Methylation-Based Classification of central Nervous System Tumours. *Nature* 555 (7697), 469–474. doi:10.1038/nature26000

Carbon, S., Ireland, A., Mungall, C. J., Shu, S., Marshall, B., Lewis, S., et al. (2009). AmiGO: Online Access to Ontology and Annotation Data. *Bioinformatics* 25 (2), 288–289. doi:10.1093/bioinformatics/btn615

Carulli, D., de Winter, F., and Verhaagen, J. (2021). Semaphorins in Adult Nervous System Plasticity and Disease. *Front. Synaptic Neurosci.* 13 (20). doi:10.3389/fnsyn.2021.672891

Chen, J., Li, Y., Yu, T.-S., McKay, R. M., Burns, D. K., Kernie, S. G., et al. (2012). A Restricted Cell Population Propagates Glioblastoma Growth after Chemotherapy. *Nature* 488 (7412), 522–526. doi:10.1038/nature11287

Chera, S., Ghila, L., Dobretz, K., Wenger, Y., Bauer, C., Buzgariu, W., et al. (2009). Apoptotic Cells Provide an Unexpected Source of Wnt3 Signaling to Drive hydra Head Regeneration. *Developmental Cell* 17 (2), 279–289. doi:10.1016/j.devcel.2009.07.014

Coussens, L. M., and Werb, Z. (2002). Inflammation and Cancer. *Nature* 420 (6917), 860–867. doi:10.1038/nature01322

Das, K. K., and Kumar, R. (2017). "Pediatric Glioblastoma," in *Glioblastoma*. Editor S. De Vleeschouwer (Brisbane, AU: Codon Publications).

Demirci, Y., Cucun, G., Poyraz, Y. K., Mohammed, S., Heger, G., Papatheodorou, I., et al. (2020). Comparative Transcriptome Analysis of the Regenerating Zebrafish Telencephalon Unravels a Resource with Key Pathways during Two Early Stages and Activation of Wnt/β-Catenin Signaling at the Early Wound Healing Stage. *Front. Cell Dev. Biol.* 8, 584604. doi:10.3389/fcell.2020.584604

Diotel, N., Lübke, L., Strähle, U., and Rastegar, S. (2020). Common and Distinct Features of Adult Neurogenesis and Regeneration in the Telencephalon of Zebrafish and Mammals. *Front. Neurosci.* 14 (957). doi:10.3389/fnins.2020.568930

Diwanji, N., and Bergmann, A. (2018). An Unexpected Friend – ROS in Apoptosis-Induced Compensatory Proliferation: Implications for Regeneration and Cancer. *Semin. Cell Developmental Biol.* 80, 74–82. doi:10.1016/j.semcdb.2017.07.004

Duhen, T., Ni, C., and Campbell, D. (2014). Identification of a Specific Gene Signature in Human Th1/17 Cells (BA13P.126). *J. Immunol.* 192 (1 Suppl. ment), 177112.

Elsaeidi, F., Bemben, M. A., Zhao, X.-F., and Goldman, D. (2014). Jak/Stat Signaling Stimulates Zebrafish Optic Nerve Regeneration and Overcomes the Inhibitory Actions of Socs3 and Sfpq. *J. Neurosci.* 34 (7), 2632–2644. doi:10.1523/JNEUROSCI.3898-13.2014

Fernandes, C., Costa, A., Osorio, L., Lago, R. C., Linhares, P., Carvalho, B., et al. (2017). "Current Standards of Care in Glioblastoma Therapy," in *Glioblastoma*. Editor S. De Vleeschouwer (Brisbane, AU: Codon Publications).

Flier, J. S., Underhill, L. H., and Dvorak, H. F. (1986). Tumors: Wounds that Do Not Heal. *N. Engl. J. Med.* 315 (26), 1650–1659. doi:10.1056/NEJM198612253152606

Fresegna, D., Bullitta, S., Musella, A., Rizzo, F. R., De Vito, F., Guadalupi, L., et al. (2020). Re-Examining the Role of TNF in MS Pathogenesis and Therapy. *Cells* 9 (10), 2290. doi:10.3390/cells9102290

Fuller-Carter, P. I., Carter, K. W., Anderson, D., Harvey, A. R., Giles, K. M., and Rodger, J. (2015). Integrated Analyses of Zebrafish miRNA and mRNA Expression Profiles Identify miR-29b and miR-223 as Potential Regulators of Optic Nerve Regeneration. *BMC Genomics* 16, 591. doi:10.1186/s12864-015-1772-1

Gargioli, C., and Slack, J. M. W. (2004). Cell Lineage Tracing duringXenopustail Regeneration. *Development* 131 (11), 2669–2679. doi:10.1242/dev.01155

Gauron, C., Rampon, C., Bouzaffour, M., Ipendey, E., Teillon, J., Volovitch, M., et al. (2013). Sustained Production of ROS Triggers Compensatory Proliferation and Is Required for Regeneration to Proceed. *Sci. Rep.* 3, 2084. doi:10.1038/srep02084

Ghosh, S., and Hui, S. P. (2016). Regeneration of Zebrafish CNS: Adult Neurogenesis. *Neural Plasticity* 2016, 1–21. doi:10.1155/2016/5815439

Gilbert, C. A., and Ross, A. H. (2009). Cancer Stem Cells: Cell Culture, Markers, and Targets for New Therapies. *J. Cell. Biochem.* 108 (5), 1031–1038. doi:10.1002/jcb.22350

Gourain, V., Armant, O., Lübke, L., Diotel, N., Rastegar, S., and Strähle, U. (2021). Multi-Dimensional Transcriptome Analysis Reveals Modulation of Cholesterol Metabolism as Highly Integrated Response to Brain Injury. *Front. Neurosci.* 15 (543). doi:10.3389/fnins.2021.671249

Grandel, H., Kaslin, J., Ganz, J., Wenzel, I., and Brand, M. (2006). Neural Stem Cells and Neurogenesis in the Adult Zebrafish Brain: Origin, Proliferation Dynamics, Migration and Cell Fate. *Developmental Biol.* 295 (1), 263–277. doi:10.1016/j.ydbio.2006.03.040

Guerin, D. J., Kha, C. X., and Tseng, K. A.-S. (2021). From Cell Death to Regeneration: Rebuilding after Injury. *Front. Cell Dev. Biol.* 9 (547). doi:10.3389/fcell.2021.655048

Guo, S.-K., Shen, M.-F., Yao, H.-W., and Liu, Y.-S. (2018). Enhanced Expression of TGFBI Promotes the Proliferation and Migration of Glioma Cells. *Cell Physiol Biochem* 49 (3), 1138–1150. doi:10.1159/000493293

Gurtner, G. C., Werner, S., Barrandon, Y., and Longaker, M. T. (2008). Wound Repair and Regeneration. *Nature* 453 (7193), 314–321. doi:10.1038/nature07039

Haddow, A. (1972). Molecular Repair, Wound Healing, and Carcinogenesis: Tumor Production a Possible Overhealing? *Adv. Cancer Res.* 16, 181–234. doi:10.1016/s0065-230x(08)60341-3

Hagedorn, M., Siegfried, G., Hooks, K. B., and Khatib, A.-M. (2016). Integration of Zebrafish Fin Regeneration Genes with Expression Data of Human Tumors In Silico Uncovers Potential Novel Melanoma Markers. *Oncotarget* 7 (44), 71567–71579. doi:10.18632/oncotarget.12257

Hamalainen, H., Zhou, H., Chou, W., Hashizume, H., Heller, R., and Lahesmaa, R. (2001). Distinct Gene Expression Profiles of Human Type 1 and Type 2 T Helper Cells. *Genome Biol.* 2 (7), research00221. doi:10.1186/gb-2001-2-7-research0022

Hanif, F., Muzaffar, K., Perveen, K., Malhi, S. M., and Simjee, Sh. U. (2017). Glioblastoma Multiforme: A Review of its Epidemiology and Pathogenesis through Clinical Presentation and Treatment. *Asian Pac. J. Cancer Prev.* 18 (1), 3–9. doi:10.22034/APJCP.2017.18.1.3

He, Q., and Li, Z. (2021). The Dysregulated Expression and Functional Effect of CaMK2 in Cancer. *Cancer Cell Int* 21 (1), 326. doi:10.1186/s12935-021-02030-7

Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., et al. (2003). Cancerous Stem Cells Can Arise from Pediatric Brain Tumors. *Proc. Natl. Acad. Sci.* 100 (25), 15178–15183. doi:10.1073/pnas.2036535100

Hirakata, C., Lima, K., De Almeida, B., De Miranda, L., Florêncio, K., Furtado, L., et al. (2021). Targeting Glioma Cells by Antineoplastic Activity of Reversine. *Oncol. Lett.* 22 (2), 610. doi:10.3892/ol.2021.12871

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009). Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources. *Nat. Protoc.* 4 (1), 44–57. doi:10.1038/nprot.2008.211

Idilli, A., Precazzini, F., Mione, M., and Anelli, V. (2017). Zebrafish in Translational Cancer Research: Insight into Leukemia, Melanoma, Glioma and Endocrine Tumor Biology. *Genes* 8 (9), 236. doi:10.3390/genes8090236

Imayoshi, I., and Kageyama, R. (2011). The Role of Notch Signaling in Adult Neurogenesis. *Mol. Neurobiol.* 44 (1), 7–12. doi:10.1007/s12035-011-8186-0

Iribarne, M. (2021). Inflammation Induces Zebrafish Regeneration. *Neural Regen. Res.* 16 (9), 1693–1701. doi:10.4103/1673-5374.306059

Jaerve, A., and Müller, H. W. (2012). Chemokines in CNS Injury and Repair. *Cell Tissue Res* 349 (1), 229–248. doi:10.1007/s00441-012-1427-3

Jeansson, M., Gawlik, A., Anderson, G., Li, C., Kerjaschki, D., Henkelman, M., et al. (2011). Angiopoietin-1 Is Essential in Mouse Vasculature during Development and in Response to Injury. *J. Clin. Invest.* 121 (6), 2278–2289. doi:10.1172/JCI46322

Johansson, F. K., Göransson, H., and Westermark, B. (2005). Expression Analysis of Genes Involved in Brain Tumor Progression Driven by Retroviral Insertional Mutagenesis in Mice. *Oncogene* 24 (24), 3896–3905. doi:10.1038/sj.onc.1208553

Jones, C., Karajannis, M. A., Jones, D. T. W., Kieran, M. W., Monje, M., Baker, S. J., et al. (2017). Pediatric High-Grade Glioma: Biologically and Clinically in Need of New Thinking. *Neuro Oncol.* 19 (2), now101–161. doi:10.1093/neuonc/now101

Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A., and Belmonte, J. C. I. (2010). Zebrafish Heart Regeneration Occurs by Cardiomyocyte Dedifferentiation and Proliferation. *Nature* 464 (7288), 606–609. doi:10.1038/nature08899

Jurisch-Yaksi, N., Yaksi, E., and Kizil, C. (2020). Radial Glia in the Zebrafish Brain: Functional, Structural, and Physiological Comparison with the Mammalian Glia. *Glia* 68 (12), 2451–2470. doi:10.1002/glia.23849

Kanazawa, M., Hatakeyama, M., and Ninomiya, I. (2020). Angiogenesis and Neuronal Remodeling after Ischemic Stroke. *Neural Regen. Res.* 15 (1), 16–19. doi:10.4103/1673-5374.264442

Kanazawa, M., Miura, M., Toriyabe, M., Koyama, M., Hatakeyama, M., Ishikawa, M., et al. (2017). Microglia Preconditioned by Oxygen-Glucose Deprivation Promote Functional Recovery in Ischemic Rats. *Sci. Rep.* 7 (1), 42582. doi:10.1038/srep42582

Karayan-Tapon, L., Wager, M., Guilhot, J., Levillain, P., Marquant, C., Clarhaut, J., et al. (2008). Semaphorin, Neuropilin and VEGF Expression in Glial Tumours: SEMA3G, a Prognostic Marker? *Br. J. Cancer* 99 (7), 1153–1160. doi:10.1038/sj.bjc.6604641

Kaslin, J., Kroehne, V., Ganz, J., Hans, S., and Brand, M. (2017). Distinct Roles of Neuroepithelial-like and Radial Glia-like Progenitor Cells in Cerebellar Regeneration. *Development* 144 (8), 1462–1471. doi:10.1242/dev.144907

Katoh, M. (2002). Expression of Human SOX7 in normal Tissues and Tumors. *Int. J. Mol. Med.* 9 (4), 363–368. doi:10.3892/ijmm.9.4.363

Kha, C. X., Son, P. H., Lauper, J., and Tseng, K. A.-S. (2018). A Model for Investigating Developmental Eye Repair in *Xenopus laevis*. *Exp. Eye Res.* 169, 38–47. doi:10.1016/j.exer.2018.01.007

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a Fast Spliced Aligner with Low Memory Requirements. *Nat. Methods* 12 (4), 357–360. doi:10.1038/nmeth.3317

Kim, E., Kim, M., Woo, D.-H., Shin, Y., Shin, J., Chang, N., et al. (2013). Phosphorylation of EZH2 Activates STAT3 Signaling via STAT3 Methylation and Promotes Tumorigenicity of Glioblastoma Stem-like Cells. *Cancer Cell* 23 (6), 839–852. doi:10.1016/j.ccr.2013.04.008

Kim, I.-K., Kim, K., Lee, E., Oh, D. S., Park, C. S., Park, S., et al. (2018). Sox7 Promotes High-Grade Glioma by Increasing VEGFR2-Mediated Vascular Abnormality. *J. Exp. Med.* 215 (3), 963–983. doi:10.1084/jem.20170123

Kizil, C., Dudczig, S., Kyritsis, N., Machate, A., Blaesche, J., Kroehne, V., et al. (2012a). The Chemokine Receptor Cxcr5 Regulates the Regenerative Neurogenesis Response in the Adult Zebrafish Brain. *Neural Dev.* 7, 27. doi:10.1186/1749-8104-7-27

Kizil, C., Kaslin, J., Kroehne, V., and Brand, M. (2012b). Adult Neurogenesis and Brain Regeneration in Zebrafish. *Devel Neurobio* 72 (3), 429–461. doi:10.1002/dneu.20918

Kolde, R. (2012). Pheatmap: Pretty Heatmaps. *R. Package Version* 1 (2), 726.

Krafts, K. P. (2010). Tissue Repair. *Organogenesis* 6 (4), 225–233. doi:10.4161/org.6.4.12555

Kriegstein, A., and Alvarez-Buylla, A. (2009). The Glial Nature of Embryonic and Adult Neural Stem Cells. *Annu. Rev. Neurosci.* 32, 149–184. doi:10.1146/annurev.neuro.051508.135600

Kroehne, V., Freudenreich, D., Hans, S., Kaslin, J., and Brand, M. (2011). Regeneration of the Adult Zebrafish Brain from Neurogenic Radial Glia-type Progenitors. *Development* 138 (22), 4831–4841. doi:10.1242/dev.072587

Kyritsis, N., Kizil, C., Zocher, S., Kroehne, V., Kaslin, J., Freudenreich, D., et al. (2012). Acute Inflammation Initiates the Regenerative Response in the Adult Zebrafish Brain. *Science* 338 (6112), 1353–1356. doi:10.1126/science.1228773

Ladomersky, E., Scholtens, D. M., Kocherginsky, M., Hibler, E. A., Bartom, E. T., Otto-Meyer, S., et al. (2019). The Coincidence between Increasing Age, Immunosuppression, and the Incidence of Patients with Glioblastoma. *Front. Pharmacol.* 10, 200. doi:10.3389/fphar.2019.00200

Lange, C., Rost, F., Machate, A., Reinhardt, S., Lesche, M., Weber, A., et al. (2020). Single Cell Sequencing of Radial Glia Progeny Reveals Diversity of Newborn Neurons in the Adult Zebrafish Brain. *Development* 147 (1), 1855951. doi:10.1242/dev.185595

Langfelder, P., and Horvath, S. (2008). WGCNA: an R Package for Weighted Correlation Network Analysis. *BMC Bioinformatics* 9, 559. doi:10.1186/1471-2105-9-559

Law, J. W. S., and Lee, A. Y. W. (2012). The Role of Semaphorins and Their Receptors in Gliomas. *J. Signal Transduction* 2012, 1–14. doi:10.1155/2012/902854

Li, X., Nie, S., Lv, Z., Ma, L., Song, Y., Hu, Z., et al. (2021). Overexpression of Annexin A2 Promotes Proliferation by Forming a Glypican 1/c-Myc Positive Feedback Loop: Prognostic Significance in Human Glioma. *Cell Death Dis* 12 (3), 261. doi:10.1038/s41419-021-03547-5

Li, X., Wu, C., Chen, N., Gu, H., Yen, A., Cao, L., et al. (2016). PI3K/Akt/mTOR Signaling Pathway and Targeted Therapy for Glioblastoma. *Oncotarget* 7 (22), 33440–33450. doi:10.18632/oncotarget.7961

Lindsey, B. W., Douek, A. M., Loosli, F., and Kaslin, J. (2017). A Whole Brain Staining, Embedding, and Clearing Pipeline for Adult Zebrafish to Visualize Cell Proliferation and Morphology in 3-Dimensions. *Front. Neurosci.* 11, 750. doi:10.3389/fnins.2017.00750

Liu, H., Yan, Z.-Q., Li, B., Yin, S.-Y., Sun, Q., Kou, J.-J., et al. (2014). Reduced Expression of SOX7 in Ovarian Cancer: a Novel Tumor Suppressor through the Wnt/β-Catenin Signaling Pathway. *J. Ovarian Res.* 7, 87. doi:10.1186/s13048-014-0087-1

Louis, D. N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W. K., et al. (2016). The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a Summary. *Acta Neuropathol.* 131 (6), 803–820. doi:10.1007/s00401-016-1545-1

Louis, D. N., Perry, A., Wesseling, P., Brat, D. J., Cree, I. A., Figarella-Branger, D., et al. (2021). The 2021 WHO Classification of Tumors of the Central Nervous System: a Summary. *Neuro Oncol.* 23 (8), 1231–1251. doi:10.1093/neuonc/noab106

Love, M. I., Huber, W., and Anders, S. (2014). Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* 15 (12), 550. doi:10.1186/s13059-014-0550-8

Lu, H.-J., Yan, J., Jin, P.-Y., Zheng, G.-H., Zhang, H.-L., Bai, M., et al. (2018). Mechanism of MicroRNA-708 Targeting BAMBI in Cell Proliferation, Migration, and Apoptosis in Mice with Melanoma via the Wnt and TGF-β Signaling Pathways. *Technol. Cancer Res. Treat.* 17, 153303461875678. doi:10.1177/1533034618756784

Lymbouridou, R., Soufla, G., Chatzinikola, A. M., Vakis, A., and Spandidos, D. A. (2009). Down-regulation of K-Ras and H-Ras in Human Brain Gliomas. *Eur. J. Cancer* 45 (7), 1294–1303. doi:10.1016/j.ejca.2008.12.028

Magrassi, L., Conti, L., Lanterna, A., Zuccato, C., Marchionni, M., Cassini, P., et al. (2005). Shc3 Affects Human High-Grade Astrocytomas Survival. *Oncogene* 24 (33), 5198–5206. doi:10.1038/sj.onc.1208708

Mao, H., Lebrun, D. G., Yang, J., Zhu, V. F., and Li, M. (2012). Deregulated Signaling Pathways in Glioblastoma Multiforme: Molecular Mechanisms and Therapeutic Targets. *Cancer Invest.* 30 (1), 48–56. doi:10.3109/07357907.2011.630050

Marín-Juez, R., Marass, M., Gauvrit, S., Rossi, A., Lai, S.-L., Materna, S. C., et al. (2016). Fast Revascularization of the Injured Area Is Essential to Support Zebrafish Heart Regeneration. *Proc. Natl. Acad. Sci. USA* 113 (40), 11237–11242. doi:10.1073/pnas.1605431113

Marques, I. J., Lupi, E., and Mercader, N. (2019). Model Systems for Regeneration: Zebrafish. *Development* 146 (18). doi:10.1242/dev.167692

Méndez, O., Zavadil, J., Esencay, M., Lukyanov, Y., Santovasi, D., Wang, S.-C., et al. (2010). Knock Down of HIF-1α in Glioma Cells Reduces Migration *In Vitro* and Invasion *In Vivo* and Impairs Their Ability to Form Tumor Spheres. *Mol. Cancer* 9, 133. doi:10.1186/1476-4598-9-133

Merchant, T. E., Pollack, I. F., and Loeffler, J. S. (2010). Brain Tumors across the Age Spectrum: Biology, Therapy, and Late Effects. *Semin. Radiat. Oncol.* 20 (1), 58–66. doi:10.1016/j.semradonc.2009.09.005

Messali, A., Villacorta, R., and Hay, J. W. (2014). A Review of the Economic burden of Glioblastoma and the Cost Effectiveness of Pharmacologic Treatments. *Pharmacoeconomics* 32 (12), 1201–1212. doi:10.1007/s40273-014-0198-y

Morata, G., Shlevkov, E., and Pérez-Garijo, A. (2011). Mitogenic Signaling from Apoptotic Cells in Drosophila. *Dev. Growth Differ.* 53 (2), 168–176. doi:10.1111/j.1440-169X.2010.01225.x

Nasser, M. M., and Mehdipour, P. (2018). Exploration of Involved Key Genes and Signaling Diversity in Brain Tumors. *Cell Mol Neurobiol* 38 (2), 393–419. doi:10.1007/s10571-017-0498-9

National Cancer Institute (2020). *Genomic Data Commons Data Portal*. Bethesda, Maryland: National Cancer Institute.

Oh, K.-Y., Hong, K.-O., Huh, Y.-S., Lee, J.-I., and Hong, S.-D. (2017). Decreased Expression of SOX7 Induces Cell Proliferation and Invasion and Correlates with Poor Prognosis in Oral Squamous Cell Carcinoma. *J. Oral Pathol. Med.* 46 (9), 752–758. doi:10.1111/jop.12566

Ohgaki, H., and Kleihues, P. (2007). Genetic Pathways to Primary and Secondary Glioblastoma. *Am. J. Pathol.* 170 (5), 1445–1453. doi:10.2353/ajpath.2007.070011

Ohka, F., Natsume, A., and Wakabayashi, T. (2012). Current Trends in Targeted Therapies for Glioblastoma Multiforme. *Neurol. Res. Int.* 2012, 1–13. doi:10.1155/2012/878425

Ostrom, Q. T., Gittleman, H., Farah, P., Ondracek, A., Chen, Y., Wolinsky, Y., et al. (2013). CBTRUS Statistical Report: Primary Brain and central Nervous System Tumors Diagnosed in the United States in 2006-2010. *Neuro-Oncology* 15 (Suppl. 2), ii1–ii56. doi:10.1093/neuonc/not151

Oviedo, N. J., and Beane, W. S. (2009). Regeneration: The Origin of Cancer or a Possible Cure? *Semin. Cell Developmental Biol.* 20 (5), 557–564. doi:10.1016/j.semcdb.2009.04.005

Pellettieri, J., Fitzgerald, P., Watanabe, S., Mancuso, J., Green, D. R., and Sánchez Alvarado, A. (2010). Cell Death and Tissue Remodeling in Planarian Regeneration. *Developmental Biol.* 338 (1), 76–85. doi:10.1016/j.ydbio.2009.09.015

Peng, G., Wang, Y., Ge, P., Bailey, C., Zhang, P., Zhang, D., et al. (2021). The HIF1α-PDGFD-Pdgfrα axis Controls Glioblastoma Growth at Normoxia/mild-Hypoxia and Confers Sensitivity to Targeted Therapy by Echinomycin. *J. Exp. Clin. Cancer Res.* 40 (1), 278. doi:10.1186/s13046-021-02082-7

Pereira, J. D., Sansom, S. N., Smith, J., Dobenecker, M.-W., Tarakhovsky, A., and Livesey, F. J. (2010). Ezh2, the Histone Methyltransferase of PRC2, Regulates the Balance between Self-Renewal and Differentiation in the Cerebral Cortex. *Proc. Natl. Acad. Sci.* 107 (36), 15957–15962. doi:10.1073/pnas.1002530107

Pierce, G. F., Mustoe, T. A., Altrock, B. W., Deuel, T. F., and Thomason, A. (1991). Role of Platelet-Derived Growth Factor in Wound Healing. *J. Cell. Biochem.* 45 (4), 319–326. doi:10.1002/jcb.240450403

Prasad, G., and Haas-Kogan, D. A. (2009). Radiation-induced Gliomas. *Expert Rev. Neurotherapeutics* 9 (10), 1511–1517. doi:10.1002/pmic.20080080210.1586/ern.09.98

Rahaman, S. O., Harbor, P. C., Chernova, O., Barnett, G. H., Vogelbaum, M. A., and Haque, S. J. (2002). Inhibition of Constitutively Active Stat3 Suppresses Proliferation and Induces Apoptosis in Glioblastoma Multiforme Cells. *Oncogene* 21 (55), 8404–8413. doi:10.1038/sj.onc.1206047

Raphael, I., Nalawade, S., Eagar, T. N., and Forsthuber, T. G. (2015). T Cell Subsets and Their Signature Cytokines in Autoimmune and Inflammatory Diseases. *Cytokine* 74 (1), 5–17. doi:10.1016/j.cyto.2014.09.011

Ruan, L., Wang, B., ZhuGe, Q., and Jin, K. (2015). Coupling of Neurogenesis and Angiogenesis after Ischemic Stroke. *Brain Res.* 1623, 166–173. doi:10.1016/j.brainres.2015.02.042

Schäfer, M., and Werner, S. (2008). Cancer as an Overhealing Wound: an Old Hypothesis Revisited. *Nat. Rev. Mol. Cell Biol* 9 (8), 628–638. doi:10.1038/nrm2455

Schmidt, R., Beil, T., Strähle, U., and Rastegar, S. (2014). Stab Wound Injury of the Zebrafish Adult Telencephalon: a Method to Investigate Vertebrate Brain Neurogenesis and Regeneration. *JoVE* 90, e51753. doi:10.3791/51753

Schwartzbaum, J. A., Fisher, J. L., Aldape, K. D., and Wrensch, M. (2006). Epidemiology and Molecular Pathology of Glioma. *Nat. Rev. Neurol.* 2 (9), 494–503. quiz 491 p following 516. doi:10.1038/ncpneuro0289

Sherry, M. M., Reeves, A., Wu, J. K., and Cochran, B. H. (2009). STAT3 Is Required for Proliferation and Maintenance of Multipotency in Glioblastoma Stem Cells. *Stem Cells* 27 (10), 2383–2392. doi:10.1002/stem.185

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., et al. (2004). Identification of Human Brain Tumour Initiating Cells. *Nature* 432 (7015), 396–401. doi:10.1038/nature03128

Smedley, D., Haider, S., Ballester, B., Holland, R., London, D., Thorisson, G., et al. (2009). BioMart - Biological Queries Made Easy. *BMC Genomics* 10, 22. doi:10.1186/1471-2164-10-22

Soengas, M. S., Gerald, W. L., Cordon-Cardo, C., Lazebnik, Y., and Lowe, S. W. (2006). Apaf-1 Expression in Malignant Melanoma. *Cell Death Differ* 13 (2), 352–353. doi:10.1038/sj.cdd.4401755

Sonar, S., and Lal, G. (2015). Role of Tumor Necrosis Factor Superfamily in Neuroinflammation and Autoimmunity. *Front. Immunol.* 6, 364. doi:10.3389/fimmu.2015.00364

Speidel, D. (2015). The Role of DNA Damage Responses in P53 Biology. *Arch. Toxicol.* 89 (4), 501–517. doi:10.1007/s00204-015-1459-z

Stern, C. D. (2000). Conrad H. Waddington's Contributions to Avian and Mammalian Development, 1930-1940. *Int. J. Dev. Biol.* 44 (1), 15–22.

Stocum, D. L. (2019). Nerves and Proliferation of Progenitor Cells in Limb Regeneration. *Develop Neurobiol.* 79 (5), 468–478. doi:10.1002/dneu.22643

Stovall, D. B., Wan, M., Miller, L. D., Cao, P., Maglic, D., Zhang, Q., et al. (2013). The Regulation of SOX7 and its Tumor Suppressive Role in Breast Cancer. *Am. J. Pathol.* 183 (5), 1645–1653. doi:10.1016/j.ajpath.2013.07.025

Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J. B., et al. (2005). Radiotherapy Plus Concomitant and Adjuvant Temozolomide for Glioblastoma. *N. Engl. J. Med.* 352 (10), 987–996. doi:10.1056/NEJMoa043330

Suvà, M.-L., Riggi, N., Janiszewska, M., Radovanovic, I., Provero, P., Stehle, J.-C., et al. (2009). EZH2 Is Essential for Glioblastoma Cancer Stem Cell Maintenance. *Cancer Res.* 69 (24), 9211–9218. doi:10.1158/0008-5472.can-09-1622

Tamimi, A. F., and Juweid, M. (2017). "Epidemiology and Outcome of Glioblastoma," in *Glioblastoma*. Editor S. De Vleeschouwer (Brisbane, AU: Codon Publications).

Tan, C., Lu, N.-N., Wang, C.-K., Chen, D.-Y., Sun, N.-H., Lyu, H., et al. (2019). Endothelium-Derived Semaphorin 3G Regulates Hippocampal Synaptic Structure and Plasticity via Neuropilin-2/PlexinA4. *Neuron* 101 (5), 920–937. e913. doi:10.1016/j.neuron.2018.12.036

Tanase, C., Albulescu, R., Codrici, E., Calenic, B., Popescu, I. D., Mihai, S., et al. (2015). Decreased Expression of APAF-1 and Increased Expression of Cathepsin B in Invasive Pituitary Adenoma. *Ott* 8, 81–90. doi:10.2147/OTT.S70886

Tsarouchas, T. M., Wehner, D., Cavone, L., Munir, T., Keatinge, M., Lambertus, M., et al. (2018). Dynamic Control of Proinflammatory Cytokines Il-1β and Tnf-α by Macrophages in Zebrafish Spinal Cord Regeneration. *Nat. Commun.* 9 (1), 4670. doi:10.1038/s41467-018-07036-w

Tseng, A.-S., Adams, D. S., Qiu, D., Koustubhan, P., and Levin, M. (2007). Apoptosis Is Required during Early Stages of Tail Regeneration in *Xenopus laevis*. *Developmental Biol.* 301 (1), 62–69. doi:10.1016/j.ydbio.2006.10.048

Venkatesan, S., Lamfers, M. L., Dirven, C. M., and Leenstra, S. (2016). Genetic Biomarkers of Drug Response for Small-Molecule Therapeutics Targeting the

RTK/Ras/PI3K, P53 or Rb Pathway in Glioblastoma. *CNS Oncol.* 5 (2), 77–90. doi:10.2217/cns-2015-0005

Verkhratsky, A., and Butt, A. (2013). "General Pathophysiology of Neuroglia," in *Glial Physiology and Pathophysiology*. Editors A. Verkhratsky and A. Butt (Hoboken, NJ: Wiley), 431–450.

Viotti, J., Duplan, E., Caillava, C., Condat, J., Goiran, T., Giordano, C., et al. (2014). Glioma Tumor Grade Correlates with Parkin Depletion in Mutant P53-Linked Tumors and Results from Loss of Function of P53 Transcriptional Activity. *Oncogene* 33 (14), 1764–1775. doi:10.1038/onc.2013.124

Waddington, C. H. (1935). Cancer and the Theory of Organisers. *Nature* 135, 606–608. doi:10.1038/135606a0

Walter, W., Sánchez-Cabo, F., and Ricote, M. (2015). GOplot: an R Package for Visually Combining Expression Data with Functional Analysis: Fig. 1. *Bioinformatics* 31 (17), 2912–2914. doi:10.1093/bioinformatics/btv300

Wang, X., Pei, Z., Hossain, A., Bai, Y., and Chen, G. (2021). Transcription Factor-Based Gene Therapy to Treat Glioblastoma through Direct Neuronal Conversion. *Cancer Biol. Med.* 18, 860–874. doi:10.20892/j.issn.2095-3941.2020.0499

Wang, Y., Jin, K., Mao, X. O., Xie, L., Banwait, S., Marti, H. H., et al. (2007). VEGF-overexpressing Transgenic Mice Show Enhanced post-ischemic Neurogenesis and Neuromigration. *J. Neurosci. Res.* 85 (4), 740–747. doi:10.1002/jnr.21169

Watzlawik, J. O., Warrington, A. E., and Rodriguez, M. (2013). PDGF Is Required for Remyelination-Promoting IgM Stimulation of Oligodendrocyte Progenitor Cell Proliferation. *PLOS ONE* 8 (2), e55149. doi:10.1371/journal.pone.0055149

Webster, H. d. F. (1997). Growth Factors and Myelin Regeneration in Multiple Sclerosis. *Mult. Scler.* 3 (2), 113–120. doi:10.1177/135245859700300210

White, R. M., and Zon, L. I. (2008). Melanocytes in Development, Regeneration, and Cancer. *Cell Stem Cell* 3 (3), 242–252. doi:10.1016/j.stem.2008.08.005

Wickham, H. (2016). *Ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Wilson, S. E., Chaurasia, S. S., and Medeiros, F. W. (2007). Apoptosis in the Initiation, Modulation and Termination of the Corneal Wound Healing Response. *Exp. Eye Res.* 85 (3), 305–311. doi:10.1016/j.exer.2007.06.009

Wu, Y.-S., and Chen, S.-N. (2014). Apoptotic Cell: Linkage of Inflammation and Wound Healing. *Front. Pharmacol.* 5, 1. doi:10.3389/fphar.2014.00001

Xiong, D. D., Xu, W. Q., He, R. Q., Dang, Y. W., Chen, G., and Luo, D. Z. (2019). In$\blacksquare$silico Analysis Identified miRNA-based T-herapeutic A-gents against G-lioblastoma M-ultiforme. *Oncol. Rep.* 41 (4), 2194–2208. doi:10.3892/or.2019.7022

Xu, C., Hasan, S. S., Schmidt, I., Rocha, S. F., Pitulescu, M. E., Bussmann, J., et al. (2014). Arteries Are Formed by Vein-Derived Endothelial Tip Cells. *Nat. Commun.* 5, 5758. doi:10.1038/ncomms6758

Zacchetti, A., van Garderen, E., Teske, E., Nederbragt, H., Dierendonck, J. H., and Rutteman, G. R. (2003). Validation of the Use of Proliferation Markers in Canine Neoplastic and Non-neoplastic Tissues: Comparison of KI-67 and Proliferating Cell Nuclear Antigen (PCNA) Expression versus *In Vivo* Bromodeoxyuridine Labelling by Immunohistochemistry. *APMIS* 111 (3), 430–438. doi:10.1034/j.1600-0463.2003.t01-1-1110208.x

Zambusi, A., and Ninkovic, J. (2020). Regeneration of the central Nervous System-Principles from Brain Regeneration in Adult Zebrafish. *Wjsc* 12 (1), 8–24. doi:10.4252/wjsc.v12.i1.8

Zhang, J., Ji, F., Liu, Y., Lei, X., Li, H., Ji, G., et al. (2014). Ezh2 Regulates Adult Hippocampal Neurogenesis and Memory. *J. Neurosci.* 34 (15), 5184–5199. doi:10.1523/JNEUROSCI.4129-13.2014

Zhang, J., and Jiao, J. (2015). Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis. *Biomed. Res. Int.* 2015, 1–14. doi:10.1155/2015/727542

Zhang, Y., Dube, C., Gibert, M., Jr., Cruickshanks, N., Wang, B., Coughlan, M., et al. (2018). The P53 Pathway in Glioblastoma. *Cancers* 10 (9), 297. doi:10.3390/cancers10090297

Zhao, T., Yang, H., Tian, Y., Xie, Q., Lu, Y., Wang, Y., et al. (2016). SOX7 Is Associated with the Suppression of Human Glioma by HMG-Box Dependent Regulation of Wnt/β-Catenin Signaling. *Cancer Lett.* 375 (1), 100–107. doi:10.1016/j.canlet.2016.02.044

Zhou, B.-B. S., Zhang, H., Damelin, M., Geles, K. G., Grindley, J. C., and Dirks, P. B. (2009). Tumour-initiating Cells: Challenges and Opportunities for Anticancer Drug Discovery. *Nat. Rev. Drug Discov.* 8 (10), 806–823. doi:10.1038/nrd2137

Zoncu, R., Efeyan, A., and Sabatini, D. M. (2011). mTOR: from Growth Signal Integration to Cancer, Diabetes and Ageing. *Nat. Rev. Mol. Cell Biol* 12 (1), 21–35. doi:10.1038/nrm3025

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 14 February 2022
doi: 10.3389/fcell.2022.843775

The Onset of Whole-Body Regeneration in *Botryllus schlosseri*: Morphological and Molecular Characterization

Lorenzo Ricci[1,2], *Bastien Salmon*[1], *Caroline Olivier*[1], *Rita Andreoni-Pham*[1,2], *Ankita Chaurasia*[1], *Alexandre Alié*[1†] *and Stefano Tiozzo*[1*†]

[1]*Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), CNRS, Sorbonne University, Paris, France,* [2]*Institute for Research on Cancer and Aging in Nice (IRCAN), CNRS, INSERM, Université Côte d'Azur, Nice, France*

Colonial tunicates are the only chordates that regularly regenerate a fully functional whole body as part of their asexual life cycle, starting from specific epithelia and/or mesenchymal cells. In addition, in some species, whole-body regeneration (WBR) can also be triggered by extensive injuries, which deplete most of their tissues and organs and leave behind only small fragments of their body. In this manuscript, we characterized the onset of WBR in *Botryllus schlosseri,* one colonial tunicate long used as a laboratory model. We first analyzed the transcriptomic response to a WBR-triggering injury. Then, through morphological characterization, *in vivo* observations via time-lapse, vital dyes, and cell transplant assays, we started to reconstruct the dynamics of the cells triggering regeneration, highlighting an interplay between mesenchymal and epithelial cells. The dynamics described here suggest that WBR in *B. schlosseri* is initiated by extravascular tissue fragments derived from the injured individuals rather than particular populations of blood-borne cells, as has been described in closely related species. The morphological and molecular datasets here reported provide the background for future mechanistic studies of the WBR ontogenesis in *B. schlosseri* and allow to compare it with other regenerative processes occurring in other tunicate species and possibly independently evolved.

Keywords: tunicate, ascidian, evo devo, stem cell, wound healing, Styelidae, non embryonic development, dedifferentiation

OPEN ACCESS

Edited by:
Alessandro Minelli,
University of Padua, Italy

Reviewed by:
Sebastian Shimeld,
University of Oxford, United Kingdom
Jonathan P. Rast,
Emory University, United States

****Correspondence:***
Stefano Tiozzo
stefano.tiozzo@imev-mer.fr

†These authors share last authorship

Specialty section:
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Cell and Developmental Biology

Received: *26 December 2021*
Accepted: *19 January 2022*
Published: *14 February 2022*

Citation:
Ricci L, Salmon B, Olivier C, Andreoni-Pham R, Chaurasia A, Alié A and Tiozzo S (2022) The Onset of Whole-Body Regeneration in Botryllus schlosseri*: Morphological and Molecular Characterization. Front. Cell Dev. Biol. 10:843775. doi: 10.3389/fcell.2022.843775*

INTRODUCTION

Within the lifespan of a metazoan, sub-lethal damages or loss of body parts can occur frequently as a consequence of predation, competition, pathogens infections, or simply by accident. Animals cope with such traumatic events by developing a wide range of strategies, such as the synthesis of protective structure, scar formation, or various degrees of regeneration (Vorontsova and Liosner, 1961; Sinigaglia et al., 2022). The most extreme examples of regeneration occur when the entire functional body is restored from only minute fragments of the original organism, a *bona fide* ontogenesis generally referred to as whole-body regeneration (WBR). WBR has been described in many animal species belonging to different non-vertebrate taxa (Sinigaglia et al., 2022), and it is often correlated with the capacity of such organisms to reproduce asexually, i.e., a cyclical form of body

regeneration that suggests possible co-options of cellular and molecular mechanisms between the injury-triggered and the physiological WBRs (Martinez et al., 2005; Sánchez Alvarado and Yamanaka, 2014; Sinigaglia et al., 2022).

Colonial species of tunicates, the sister group of vertebrates, acquired the capacity to undergo WBR as part of their asexual life-cycle and also as a response to extreme injury, they are therefore promising models to compare these two forms of non-embryonic development, study their mechanisms and infer their evolution (Alié et al., 2020). During tunicate asexual reproduction, adult individuals called zooids regenerate cyclically the entire body through a non-embryonic developmental process generally called propagative budding (Nakauchi, 1982), ultimately leading to the formation of colonies of genetically identical individuals. The way the propagative budding processes unfold differs from one species to another, starting from different and often non-homologous cells and tissues, but often converging into a common stage of two concentric hollow vesicles, each of them formed of a monolayer epithelium, reviewed in Alié et al., 2020. From this phylotypic asexual stage of double-vesicle, the process of organogenesis begins, eventually leading to a *bauplan* that is shared by the whole subphylum (Alié et al., 2020). In many tunicates, the capacity of WBR is not only a characteristic of their life-cycle, but it can also be triggered in response to extensive injury (Tiozzo et al., 2008a), in which case the WBR process is referred to as survival budding (Nakauchi, 1982).

Both propagative and survival buddings have been studied mainly in the subfamily of *Botryllinae* (Brunetti, 2009) (**Supplementary Figure S1**), a widespread group of colonial tunicates composed of small zooids (<0.5 cm) embedded in a common soft extracellular matrix, the tunic, and connected by an extracorporeal network of the epidermal derived vessels (Manni et al., 2007; Tiozzo et al., 2008c). Throughout the vasculature, different types of mesenchymal cells, the hemocytes, circulate through the colony propelled by zooids' hearts and by the peristaltic movement of ampullae, the blind tips of the circulatory vessels (**Supplementary Movie S1**). Botryllids include several species of the genera *Botryllus* and *Botrylloides,* which undergo WBR via two modes of budding: peribranchial budding, a form of propagative budding that arises from a multipotent epithelium (Manni et al., 2014; Ricci et al., 2016b; Alié et al., 2020), and vascular budding (VB) that, depending on the species, can be propagative or triggered by injury (Oka and Watanabe, 1957; Oka and Watanabe, 1959; Milkman, 1967; Satoh, 1994) (**Supplementary Figure S1**). For instance, in *Botryllus primigenus* VB occurs routinely in a propagative fashion, while in other botryllids, such as *Botrylloides violaceus* (Brown et al., 2009) and *Botrylloides leachi* (Rinkevich et al., 2007), VB occurs upon the exogenous removal of the existing zooids. It has then been suggested that the source of cells forming both propagative and survival vascular buds is a population of hemocytes that aggregate in the vascular network. Recently, Kassmer and collaborators (Kassmer et al., 2020) identified a population of Integrin-alpha-6-positive (Ia6+) hemocytes as candidate stem cells responsible for induced VB in the species *Botrylloides diegensis*. Ia6+ hemocytes, which constantly divide in healthy colonies, also express genes associated with pluripotency. The latter findings strongly suggest that the presence of permanent population/s of circulating stem cells may be at the bases of WBR via vascular budding in botryllid tunicates.

The species *Botryllus schlosseri* has been widely used in the last several decades as a laboratory model for developmental biology, immunology, and regenerative biology (Manni et al., 2007; Kürn et al., 2011; Voskoboynik and Weissman, 2014; Gasparini et al., 2015; Munday et al., 2015; Kassmer et al., 2016; Manni et al., 2019). In *B. schlosseri*, VB occurs purely in response to injury, and it can be triggered in laboratory conditions by depleting the colony of the adult zooids and their peribranchial buds via microsurgery (Milkman, 1967; Sabbadin et al., 1975; **Supplementary Figures S2A–S2B**). While asexual propagation through peribranchial budding has been increasingly characterized these past years (Tiozzo et al., 2005; Manni et al., 2014; Di Maio et al., 2015; Ricci et al., 2016a; Ricci et al., 2016b; Pruenster et al., 2018; Prünster et al., 2019), only a few studies have addressed VB in this species (Milkman, 1967; Sabbadin et al., 1975; Voskoboynik et al., 2007; Ricci et al., 2016a; Nourizadeh et al., 2021). The cell populations and the tissues involved in the onset of *B. schlosseri* VB are still not well defined, and the morphogenetic events that lead to the regeneration of a functional adult zooid are poorly described. In this manuscript, we follow the dynamic of WBR upon injury in the laboratory model *Botryllus schlosseri*. We focus on the early stages of the process and characterize the transcriptome profile of the initial response of the whole colony to extensive injury; we describe the cytological and histological structures at the onset of the presumptive vascular bud and test the contribution of mesenchymal cells and vascular epithelia. The correlated observations suggest that WBR is initiated by extravascular tissue fragments derived from the injured zooids or buds, rather than a particular population of hemocytes as occurring in other closely related species.

MATERIALS AND METHODS

Animal Culturing and Surgical Procedure

Colonies of *Botryllus schlosseri* were raised on glass slides in a marine-culture system as described previously (Langenbacher et al., 2015). Colonies used for WBR induction experiments were transferred to an 18°C incubator in small containers (<1 L) in a closed system with filtered seawater (FSW) and bubblers, with a day/night cycle of 10 h/14 h and no feeding. The water was completely replaced every 2 days. Colonies of *B. schlosseri* at stage D (Lauzon et al., 2002) were dissected with microsurgery tools and syringe needles (30G, Terumo, SG2-3013) under a stereomicroscope. After the removal of all zooids and peribranchial buds, animals were cleaned and allowed to regenerate in FSW. Water was replaced every 2 days and vascular bud detection was performed by daily observations under a stereomicroscope allowing a 120X magnification. For fluorescent *in situ* hybridization (FISH) experiments, dissected colonies were fragmented into small pieces before fixation to facilitate the penetration of solutions (Prünster et al., 2019).

Video Acquisition and Processing

Regenerating colonies were placed in a room at 18°C in a petri dish filled with 150 ml of FSW. Photographs for time-lapse videos were taken every 5 min for up to 8 days post-injury using a Canon EOS 6D Mark II equipped with a 100 mm macro objective. Videos were assembled using Avidemux 2.7.8 (http://www/avidemux.org). The digital magnification of **Supplementary Movies S3–9** were focused on the area of the colony where WBRs occurred or were expected.

Immunohistochemistry

Whole *B. schlosseri* systems and regenerating colonies were anesthetized in natural seawater and MS222 0,3% (Sigma-Aldrich, #E10505-25G) and processed as previously described (Ricci et al., 2016a). Nuclei were counterstained by incubation at room temperature with 1 μg/ml Hoechst 33342 in PBS for 2h, then mounted in glycerol after quick washes in PBS. Confocal images were acquired with a Leica TCS SP5, SP8, or Stellaris microscope. Primary antibodies include: polyclonal, mouse anti-integrin-alpha 6 (DSHB, P2C62C4) diluted 1:10 in PBS; polyclonal, rabbit anti-phospho Histone H3 (Ser10), (Merk Millipore #06-570) diluted 1:1,000 in PBS; monoclonal, mouse anti acetylated tubulin (Sigma-Aldrich #T6793), diluted 1:1,000 in PBS; monoclonal, mouse anti tyrosinated tubulin, (Sigma-Aldrich #T90028), 1:1,000; monoclonal, mouse anti-gamma tubulin (Sigma-Aldrich, # T6557) 1:500; polyclonal rabbit anti-PKCξ C-20 (Santa-Cruz Biotechnology inc., #sc-216) 1:1,000; anti-phospho-tyrosine, 4G10® Platinum, (Merck Millipore, #05-1,050X) 1:500.

Transmission Electron Microscopy

Samples for ultra-thin sectioning were fixed with a 3% solution of glutaraldehyde in sodium cacodylate buffer (pH 7.3), post-fixed with osmium tetroxide (OsO4) 1% in cacodylate buffer, dehydrated using acetone, and embedded in epoxy resin. An UltracutE Reichert ultramicrotome was used for the ultra-thin sections (60–80 nm), which were contrasted with uranyl acetate and lead citrate and observed under a transmission electron microscope TEM JEM 1400 JEOL coupled with a MORADA SIS camera (Olympus).

Whole-Mount *in situ* Hybridization

Antisense mRNA probes were designed within the coding region of each gene (**Supplementary Figure S3**) FISH was carried out as previously described (Ricci et al., 2016a). DIG-probe detection was performed with bench-made FITC-Tyramide and TRITC-Tyramide by 3 h incubation.

In vivo Cells and Tissue Labeling and Imaging

Colonies were grown in Willco-dishes (Willco-Dish®, 50 × 7 × 0.17 mm). Once reached stage D (Lauzon et al., 2002) the colonies were injected with 1–2 μl per system of lipophilic dye FM® 4-64 Dye, (Life Technologies, #T-13320), diluted 1: 100 in PBS and with BSA Alexa Fluor® 488 conjugate, (Life Technologies, #A13100) at a concentration of 1 mg/ml, according to published parameters (Braden et al., 2014). Following injection, colonies were left to recover 3 hrs in FSW, then dissected to induce WBR, and left to regenerate in FSW. After vascular bud detection, the colony was observed with a confocal Leica TCS SP5 microscope vesicle.

Fusion-Chimera Assay and Genotyping Via Microsatellite

To trigger fusion, two isogenic and histocompatible colonies were selected from the clones present in the marine culture of LBDV (e.g., clone CB and DA) and sub-cloned side-by-side in the same glass slide. The colonies were allowed to grow until they fuse. Following fusion, hemocytes cells from both genotypes were immediately mixed in the plasma and circulated freely in the whole vascular system of the chimera. Around 48 h after fusion, the couple of colonies were separated, and, as soon as they reached stage D, WBR was induced as previously described (**Supplementary Figure S2C**). For micro-satellite sequencing, after fusion of allogeneic colonies, clear landmarks were established to delineate the vascular system of each colony by scratching the glass slide with a diamond pen and taking photographs of the colony prior and daily after fusion. Dissected colonies were left to regenerate until they produced a vascular bud that underwent organogenesis. Large vascular buds were dissected with microsurgery tools and syringe needles (30G, Terumo, SG2-3013) under a stereomicroscope. Stomach epithelium was isolated with thin forceps and repetitively washed in clean FSW to avoid blood cell contamination. Then, genomic DNA was extracted from the stomach tissue, using the NucleoSpin® Tissue XS kit for genomic DNA (Mascherey-Nagel, #740901.50) and eluted in 10 μl of elution buffer. Following elution, samples were stored at −20°C. Tissues from both fused colonies were collected separately before fusion and their genomic DNA was collected with the same procedure as used for vascular buds. Couples of forward and reverse primers complementary to a *Botryllus* non-coding genomic locus designed to amplify microsatellites sequences BS1 and PB49 were used (Stoner and Weissman, 1996; Ben-Shlomo et al., 2008). For each microsatellite locus, a 5′ tag made of a universal oligo was added to the forward primer. The sequence of this universal primer was used to design another forward primer, with a 6-FAM™ fluorescent tag at its 5′ end (Life Technologies). Three primers PCR amplification were performed using the Qiagen Multiplex PCR kit (206143) in a final volume of 20 μl, at the concentration of 0.01 μM of forward primer and 0.2 μM of each reverse and 6-FAM forward primer. 1 μl of gDNA was added to the reaction as a template. The cycling program was as follows: denaturation, 95°C, 15 min; amplification,(94°C, 30 s; 60°C, 90 s; 72°C, 60 s)x40; 60°C, 30 min for the BS1 locus. For the PB49 locus, the program was modified as follows: denaturation, 95°C, 15 min; amplification, (94°C, 30 s; 65°C, 1 min; 72°C, 1 min) x3 then (94°C, 30 s; 63°C, 1 min; 72°C, 1 min)x17 and (94°C, 30 s; 57°C, 1 min; 72°C, 1 min)x20; 60°C, 30 min. The success of the PCR was validated by electrophoresis on a 1.7%, agarose gel before genotyping. Genotyping was performed by the Plateforme Génome Transcriptome de Bordeaux, Site de Pierroton—INRA.

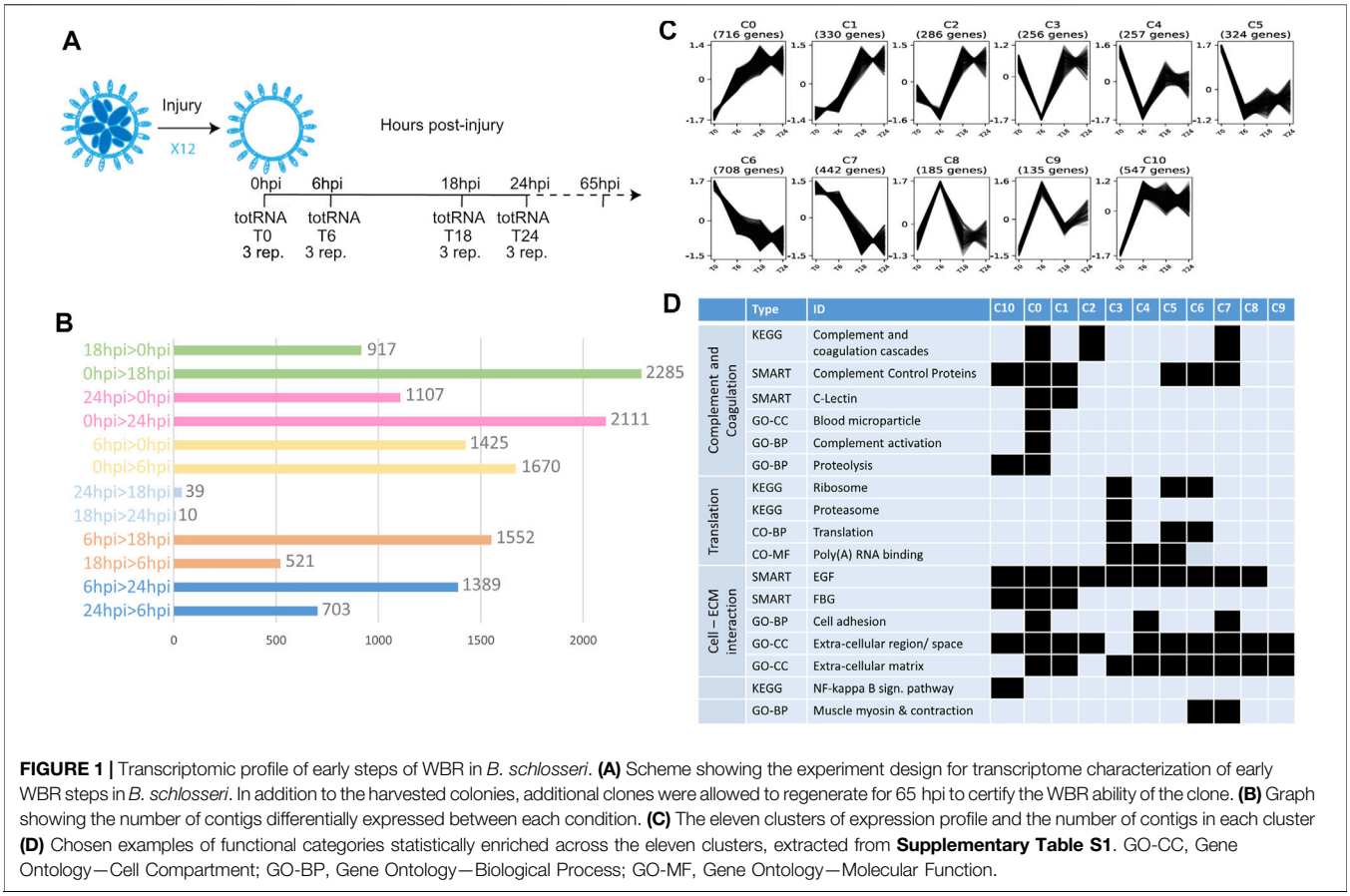


FIGURE 1 | Transcriptomic profile of early steps of WBR in *B. schlosseri*. **(A)** Scheme showing the experiment design for transcriptome characterization of early WBR steps in *B. schlosseri*. In addition to the harvested colonies, additional clones were allowed to regenerate for 65 hpi to certify the WBR ability of the clone. **(B)** Graph showing the number of contigs differentially expressed between each condition. **(C)** The eleven clusters of expression profile and the number of contigs in each cluster **(D)** Chosen examples of functional categories statistically enriched across the eleven clusters, extracted from **Supplementary Table S1**. GO-CC, Gene Ontology—Cell Compartment; GO-BP, Gene Ontology—Biological Process; GO-MF, Gene Ontology—Molecular Function.

Three primers PCR products were diluted in formamide to avoid excessive fluorescence, with a dilution factor of 50 or 100, according to the sample. They were subsequently analyzed with an ABI3730 analyzer, in parallel with LIZ-600 and LIZ-1200 size standards. Fragments sizes were then analyzed with the Peak Scanner™ Software v2.0 (Applied Biosystems).

RNA Extraction and Transcriptome Sequencing and Differential Expression Analyses

An isogenic strain of *Botryllus schlosseri* was tested for its ability to regenerate and produce vascular buds in an average time of 2–3 days. Twelve subclones of comparable size from this strain were separated with a razor blade and allowed to grow separately on individual glass slides. When colonies reached stage D (Lauzon et al., 2002), they were dissected with microsurgery tools and syringe needles (30G, Terumo, SG2-3013) under a stereomicroscope. After removal of all zooids and buds, animals were cleaned and either conditioned for further RNA extraction or allowed to regenerate in Filtered seawater (FSW) in small containers (<1 L) placed in an incubator at 19°C. When allowed to regenerate, colonies were left 6, 18, or 24 h post-injury (hpi) in FSW before preparing for RNA extraction. The regenerating colony was detached from the glass slide with a razor blade and then transferred to a tube and flash frozen before storage at −80°C and later RNA extraction. For each time point, three replicates were made, bringing the total number of samples to twelve (**Figure 1A**).

Extraction of total RNA was performed the same day in a single round, for the twelve samples, using the NucleoSpin® RNA XS Mascherey-Nagel kit (#740902.50). First, 500 µl of lysis buffer from the kit was added to the 1.5 ml tubes containing the samples. The latter was subsequently ground manually in the tube, using a plastic, RNAse free micropestle. All further steps were performed according to the user manual section for RNA extraction from animal tissue. For each sample, total RNA was eluted in 12 µm of nuclease-free water and stored at −80°C until sequencing.

Library preparation and sequencing were performed at the USC Epigenomic Center (Los Angeles, CA, United States) according to the Illumina HiSeq 2500 protocol. Approximately 70 M PE reads were sequenced for each of the twelve samples.

Transcriptome assembly and differential expression analysis were performed as follows. Step 1: removing of contaminating ribosomal RNA using SortmeRNA v2.1 (Kopylova et al., 2012); Step 2: cleaning, clipping, and filtering reads using Trimmomatic v0.30 (Bolger et al., 2014); Step 3: transcriptome assembly from the remaining reads, using Trinity v2.11.0 (Grabherr et al., 2011) with default parameters; Step 4: Recover the best ORF per contig using TransDecoder v5.5.0, using a minimum protein length of 90 amino-acids (-m parameter); Step 5: Reduce spurious redundancy by collapsing similar transcripts using cd-hit-est

v4.6 with default parameters (Fu et al., 2012); Step 6: Mapping the sequencing reads on the obtained transcriptome using Kallisto v0.43.1 using default parameters (Bray et al., 2016); Step 7: Identifying Differentially Expressed Genes using DeSeq2 (Love et al., 2014) through the iDEP v0.93 platform, following a between-sample normalization of expression values given by Kallisto to ensure a homogeneous distribution of expression data across samples (see **Supplementary Table S1**).

Genes being differentially expressed between at least two conditions (e-value < 0.05 and Fold-change >1) have been clustered by expression profile using Clust v1.12.0 (Abu-Jamous and Kelly, 2018) using raw ESTs (Kallisto output) as input data. Then contigs were named after their best tblastn hit against the human uniProt_proteome_UP000005640. Functional enrichment for each cluster was investigated using DAVID Bioinformatics Resources 6.8, using the whole transcriptome (from Step 5 above) as a reference dataset.

RESULTS

Wound Healing Response and Vascular Remodeling Precede Injury Activated Whole-Body Regeneration

To describe the transcriptomic response to extensive colony injury, colonies of *Botryllus schlosseri* were allowed to regenerate for 0, 6, 18 and 24 h post-injury (hpi) respectively (see Material and Methods, **Figure 1A**). Approximately 147 million reads were cleaned and assembled into 157,306 contigs (N50 = 707 nuc.) from which 32,561 open reading frames were retained for downstream analyses (**Supplementary Data S1**). Gene expression level across the four time-points was measured by mapping reads to the 32,561 contigs, leading to the identification of 6,007 contigs having a differential expression (adjusted p-value < 0.05 and Log2 Fold-change > 1) between at least two conditions (**Supplementary Table S1**; **Figure 1B**). Most of the variation in gene expression arises between 0, 6 and 18/24 hpi, while 18 hpi and 24 hpi have similar molecular profiles (**Figure 1B**). The 6,007 contigs were grouped into eleven clusters based on their expression profiles (**Figure 1C**). Clusters 0, 1 and 10 correspond to a general increase in expression upon surgery; clusters 5, 6, and 7 to a general decrease, while the other clusters show more complex profiles (**Figure 1C**). Taken together these results show a drastic transcriptomic response to injury in the first 18 h of WBR in *Botryllus*.

Functional enrichment of the retrieved clusters (**Supplementary Table S1**; **Figure 1D**) reflects the active role played by the circulatory system in injury response and WBR initiation, in line with the important vascular remodeling observed after zooid ablation (**Supplementary Movie S2**). Indeed, seven clusters are enriched in genes of the complement and coagulation cascade (**Figure 1D**), a mammalian proteolytic cascade in blood plasma acting as a defense mechanism against pathogens. More specifically, clusters 0, 1, 2 and 10 comprise orthologues of the complement components C3/C5—the core proteins of the complement cascade - as well as transcripts similar to MASP and Ficolins that activate the complement through the lectin pathway (**Supplementary Table S1**; **Supplementary Figure S4**, **Supplementary Data S2**). Clusters 5, 6 and 7 contain genes involved in coagulation (*e.g.*, orthologue or Coagulation factor XIII B chain) and platelet activation (*e.g.*, selectin-like genes). The enrichment of Fibrinogen (FBG) domain-containing genes (clusters 0, 1, 10) and of ECM components (all clusters) (**Figure 1D**) suggests a link between blood clotting and vascular remodeling by modulation of the physical interactions between vascular epithelium and extracellular matrix. Putative regulators of angiogenesis (the formation of new vessels from pre-existing ones) are numerous in clusters 0 and/or 1 (**Supplementary Table S1**), including transcripts similar to tenascins and angiopoietins, as well as orthologues of the Angiopoietin receptor (TIE1/2) and the transcription factors ETS1 and Sox7/17/18 (**Supplementary Data S2**). In mammals, ETS-1 controls endothelial cell migration and invasion (Iwasaka et al., 1996), while Sox17 promotes angiogenesis and endothelium regeneration (Liu et al., 2019). Finally, *Botryllus schlosseri* Gata-b, the orthologue of Gata1/2/3 that we previously found expressed in vascular buds (Ricci et al., 2016a), also belongs to cluster 0. In mammals, Gata-2 is central to maintaining endothelial cell identity (Kanki et al., 2011).

Functional enrichment analysis also revealed an expression increase of the NF-kappa B signaling pathway, involved in mammalian immunity and cell survival (Oeckinghaus et al., 2011), as well as a drop in the expression of translation-related genes, especially of ribosomal protein-coding genes (**Supplementary Table S1**). The biological significance of the latter is still unclear, but it may be linked to the translational response to stress (Advani and Ivanov, 2019).

Whole-Body Regeneration Origins From Extravascular Tissues That Migrate Into the Vasculature

To track the origin of WBR, we filmed with a high-resolution camera the entire colonies of *B. schlosseri* upon microsurgery (n = 9 colonies) and allowed them to regenerate until the morphogenesis of new zooids. The analyses of the digitally magnified areas of budding showed that, in the tunic near the dissection area, relatively small (50–70 μm) fragments of tissues start to move towards the vasculature, get surrounded by the latter, and eventually develop into a new zooid (white circles in **Supplementary Movies S3–5**). Such tissue fragments are not present in the tunic of undissected colonies suggesting that they may be debris of zooids or peribranchial buds, left behind after dissection. To better understand the dynamic of WBR, we followed *in vivo* the migrating tissues within the tunic until they got in contact with the vasculature. Then, we fixed and examined the details of the tissue interactions (**Figure 2**). From the observation of n = 11 putative WBR onsets from four different colonies, we detected the presence inside the tunic of double monolayered vesicles approaching (**Figures 2A–E"**; **Supplementary Movie S6**) and fusing to (**Figures 2F–Q"**; **Supplementary Movies S7–8**) the vasculature. We also

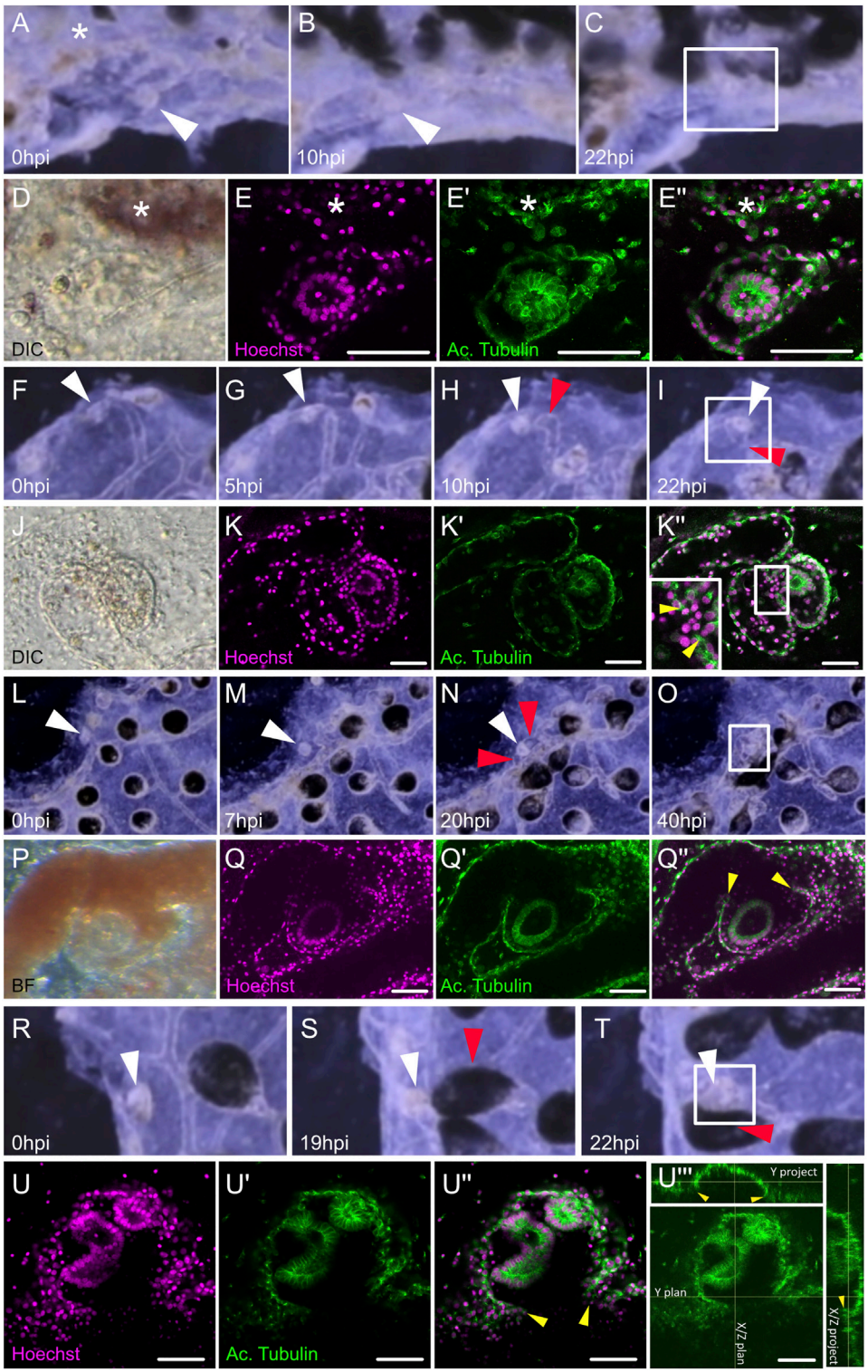


FIGURE 2 | Dynamic of the migration of extravascular tissues into the vascular network. **(A–C)** Screenshots from **Supplementary Movie S6** show tissue left-over getting in close contact to the vasculature from **(A)** 0 h post-injury (hpi) to **(B)** 10 hpi and **(C)** 22 hpi. **(D–E'')** Microscopic view of the areas squared in **(C)**. **(D)** Transmitted light with DIC filter, the double monolayer vesicle can be seen in the tunic. **(E)** Hoechst staining. **(E′)** Acetylated tubulin counter-staining. **(E'')** Composite. **(F–I)** Screenshots from **Supplementary Movie S7** showing tissue left-over fusing with the vasculature at the double vesicle stage, from **(F)** 0 hpi to **(G)** 5 hpi, **(H)** 10 hpi, and **(I)** 22 hpi. **(J–K'')** Microscopic view of the areas squared in **(I)**. **(J)** Transmitted light with DIC filter, the double monolayer vesicle can be seen in the tunic. **(K)** Hoechst staining. **(K-′)** Acetylated tubulin counter-staining. **(K'')** Composite, the insert is a magnification of the region of fusion. **(L–O)** Screenshots from **Supplementary Movie**

(*Continued*)

FIGURE 2 | S8 showing tissue left-over fusing with the vasculature at the double vesicle stage, from **(L)** 0 hpi to **(M)** 7 hpi, **(N)** 20 hpi, and **(O)** 40 hpi. **(J–K'')** Microscopic view of the areas squared in **(O)**. **(P)** Transmitted light, the double monolayer vesicle can be seen engulfed by the vasculature. **(Q)** Hoechst staining. **(Q')** Acetylated tubulin counter-staining. **(Q'')** Composite. **(R–T)** Screenshots from **Supplementary Movie S9** showing tissue left-over fusing with the vasculature from **(R)** 0 hpi to **(S)** 19 hpi and **(T)** 22 hpi. **(U–U'')** Microscopic view of the areas squared in **(T)**. **(U)** Hoechst staining. **(U')** Acetylated tubulin counter-staining. **(U''')** Orthogonal projections of the confocal stack show the histological continuity between the bud epithelium and the vascular wall. White arrowheads: tissue left-over, red arrowheads: ampullae fusing with the bud, yellow arrowheads: fusion between bud and vascular epithelium, asterisk: neighboring ampulla.

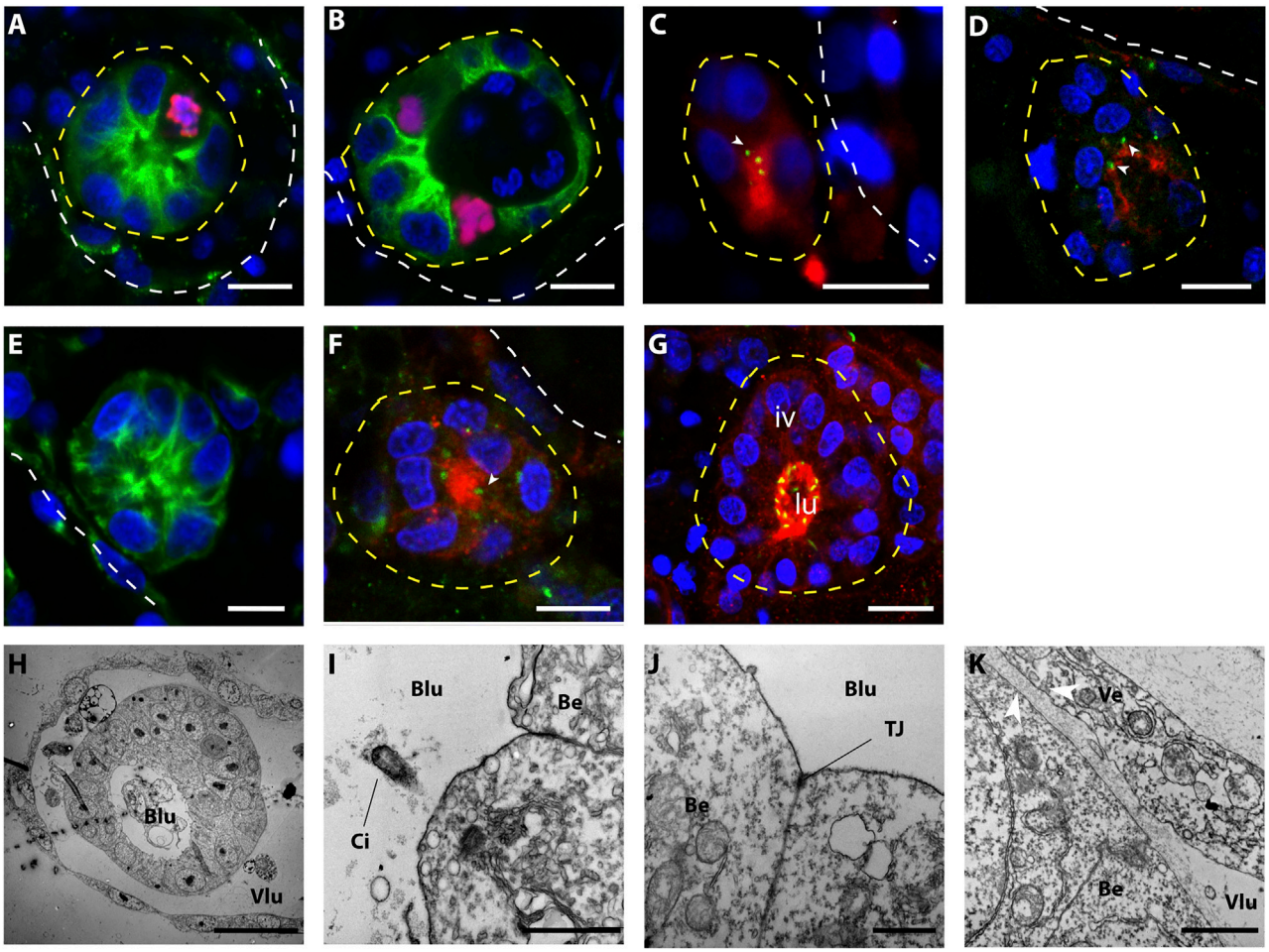


FIGURE 3 | Morphology of small (<40 μ) intravascular cell clusters. **(A–G)** Confocal images of intravascular cell clusters observed 72 h post-surgery. The white dotted line points out the epithelia of the vasculature, while the yellow dotted lines highlighted the intravascular cell clusters. Cell nuclei are stained with Hoescht (blue); in **(A,B,E)** anti-tyrosinated tubulin (green) outlines the cell bodies; in **(C,D,F,G)** anti-PKCξ (red) shows the apicobasal cell polarity and anti-gamma tubulin (green) suggest the presence of cilia. Iv, inner vesicle, lu, lumen. Scale bar 10 μ. **(H–K)** TEM imaging shows the ultra-structure of the vascular epithelium and the intravascular cell clusters. **(H)** Overview of an intravascular vesicle lining on the vessel wall; scale bar 20 μ. **(I)** detail of the epithelial cells of the vascular bud, showing monociliated cells (**I,** scale bar: 1 μ) and tight junction (**J**, scale bar: 1 μ) on the apical side (directed towards the bud lumen). **(K)** Detail of the intracellular cluster and the vessel epithelia. Note the thickening of the basal laminas between vessel epithelial cells and vascular bud cells (arrowheads), scale bar: 1 μ. Blu, bud lumen; Vlu, vessel lumen; Ci, cilia; Be, bud epithelium; TJ, tight-junction.

reported more complex epithelial structures already fused to the vasculature (n = 1) (**Figures 2R-U'''**, **Supplementary Movie S9**). The presence of such intravascular structures has never been observed in undissected colonies during their asexual growth (data not shown).

The localization of potential sites of vascular budding was also monitored *a posteriori*, i.e. by direct detection of clusters of cells in dissected colonies without the tracking via the corresponding movie. By screening different genotypes the first visible signs of putative WBR (n = 41 different colonies) were detected between 2 and 5 days after surgery (**Supplementary Table S2**). Also in these screening, we observed different scenarios: the WBR onset was often positioned on the side of the colony facing the surgery (internal side of the system), either in a protrusion of the peripheral vessel (**Supplementary Figure 5A**) or inside an ampullae (in 40 of the 41 colonies) (**Supplementary Figure**

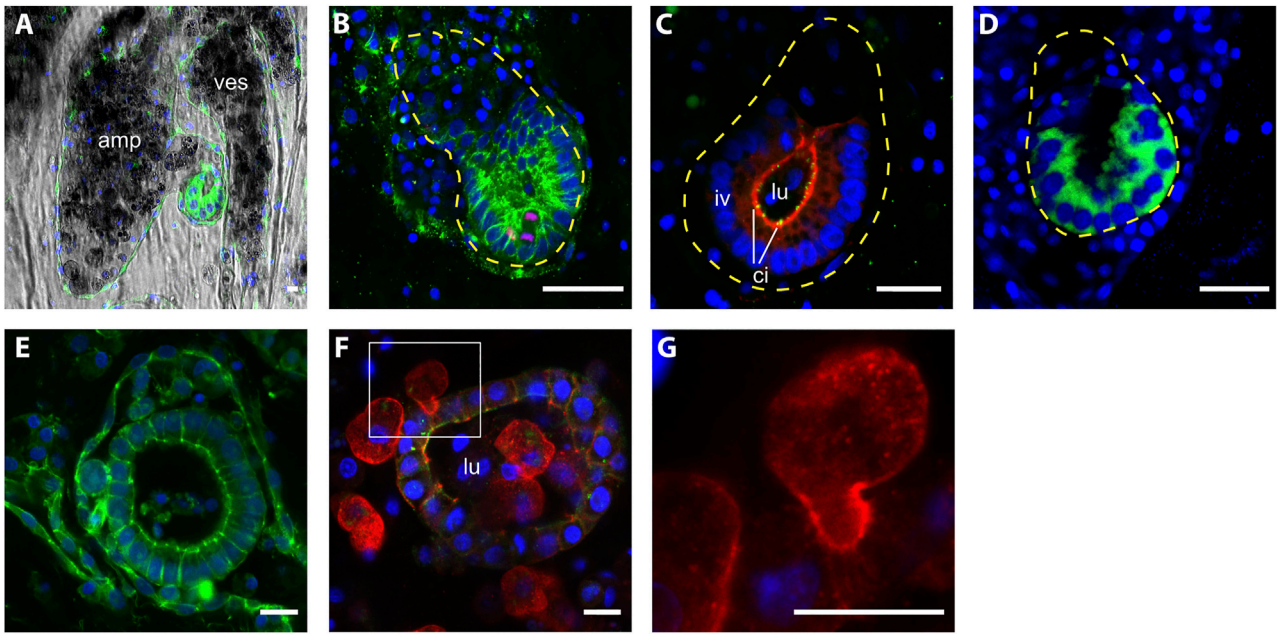


FIGURE 4 | Morphology of intravascular double vesicles.1 **(A)** The putative vascular bud grows in the protrusion of an ampulla and **(B)** it closely interacts with the vascular epithelia, the cells in contact with the vascular epithelia are thicker than the most distal cells. Cell shape is labeled with anti-tyrosinated tubulin (green) and proliferating cells are labeled with anti phospho HH3 (red). **(C)** Anti-PKCξ (red) shows the apicobasal cell polarity and anti-gamma tubulin (green) shows the presence of cilia. **(D)** The polarization of the intravascular vesicle is also highlighted by the transient localization of Wnt2 (green). **(E–F)** Bigger vesicle within the lumen mesenchymal cells, anti-tyrosinated tubulin (green), anti-pan-tyrosine kinase (red). **(G)** Details of a mesenchymal cell interacting with the epithelia of the vesicle. Cell nuclei are counterstained with Hoescht (blue). Amp, ampullae; ves, vessel; lu, lumen; iv, inner vesicle; ci, cilia. Scale bar, 10 μ.

S5B). In some cases, up to a dozen of hollow vesicles were observed in a single regenerating colony (**Supplementary Figure S5C**) with several of them being present in the same ampulla or vessel outgrowth (**Supplementary Figures S5D–E**). The presence of more complex epithelial structures was also detected (**Supplementary Figure 5F**).

Reconstruction of the Early Ontogenesis of the Intravascular Bud Onset

To better describe the morphology of the onset of WBR upon injury, as well as to infer the ontogeny of the process, we further described over a hundred ($n = 109$) proliferating intravascular cell clusters detected within the first 3 days after microsurgery. We coupled previously reported observations (Ricci et al., 2016a) with a higher number of observations and more accurate anatomical descriptions and attempted to assess the dynamics of the vascular bud development. The simplest intravascular structure detected upon microsurgery, and absent in undissected colonies, is a cluster of cells tightly associated with the vascular endothelium. These clusters of between 3 and 8 cells ($n = 9$, size ranging from 12 to 19 μm, average size = 16.1 +/−2.5 μm, **Figures 3A,B**) were found close to the vascular epithelium and proliferated (**Figure 3B**). Immunostaining revealed in the cells of such cluster a consistent localization of gamma-tubulin and PKCξ, suggesting that cells within the cluster have an apicobasal polarity (Parker et al., 2013) (**Figures 3C,D**). The size and the number of cells drew us to consider this intravascular structure a putative initial stage of WBR *via* vascular budding. The detection, very close to the vascular epithelia, of bigger spherical cell clusters (from 6 to 20 cells, $n = 28$; size ranging from 12 to 28 μm, average size = 16.8 +/− 4.3 μm) without a visible lumen, suggest a possible successive stage (**Figures 3E,F**). In larger vascular buds ($n = 16$; size ranging from 23 to 37 μm, average size = 29.6 +/− 4.3 μm), a lumen was detected in the center of the vesicle. These buds consisted of a spherical, hollow, monolayered epithelium. The apical localization of PKCξ, and the presence of cilia in the vascular bud cells, showed an epithelialization and cell polarization in the vascular buds (**Figures 3G,H**). The cells of the bud are monociliated, with their apical side facing the bud lumen and associated with tight junctions (**Figures 3I,J**), and the basal side facing the vessel lumen (**Figure 3K**). Mesenchymal cells, i.e., hemocytes, are detectable inside the vesicle.

Polarization of the whole vesicle could be observed in the majority of vascular buds of slightly bigger size ($n= 37$, size ranging from 23 to 55 μm, average size = 41.2 +/− 7.4 μm) (**Figures 4A–C**). In these buds, the side of the bud epithelium in close contact with the vascular endothelium (proximal side) exhibited big, cuboidal cells, with nuclei positioned on the side of the basal membrane. On the opposite side of the bud (distal side), facing the vessel lumen, cells appeared flattened, slightly bigger than their nuclei (**Figures 4A–C**). We also detected a polarized expression of Wnt2 (**Figure 4D**), which is also a marker of polarization in the peribranchial bud (Di Maio et al., 2015).

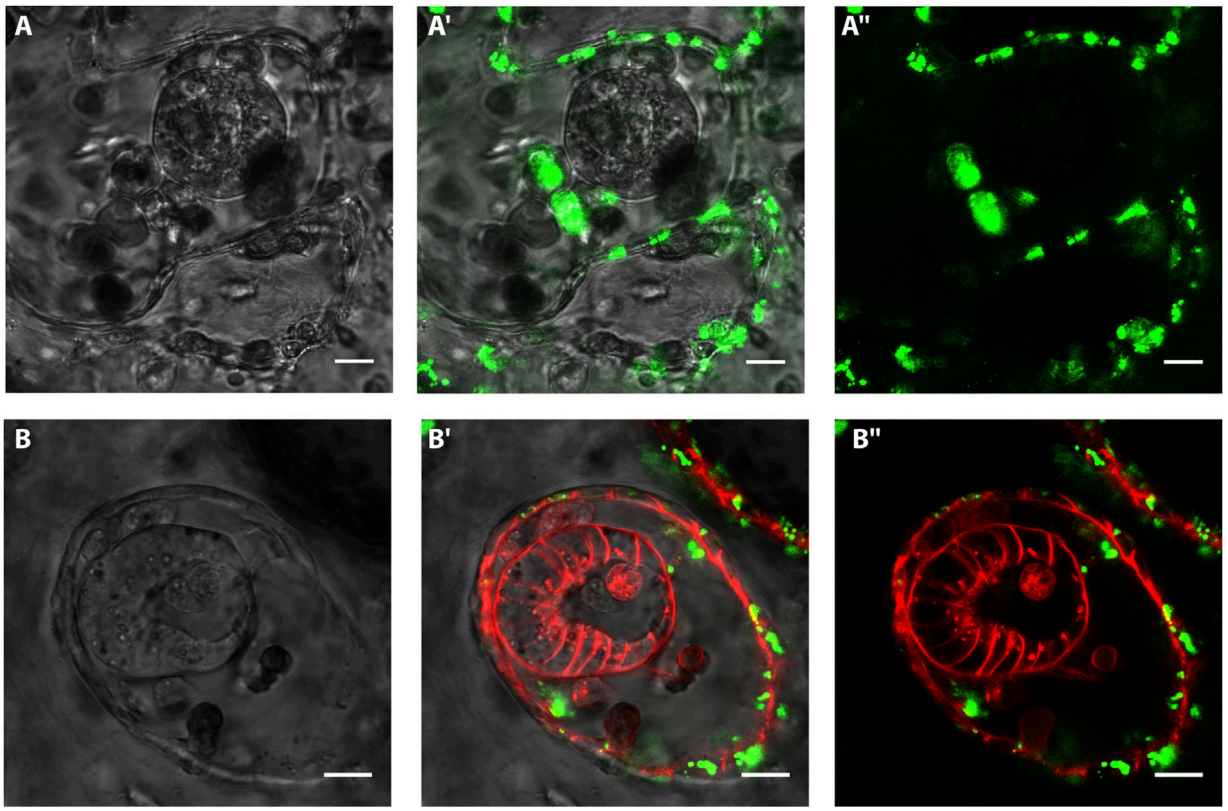


FIGURE 5 | *In vivo* labeling of vasculature epithelium and intravascular vesicles with BSA and FMR4-64 Confocal images of vascular buds after injection of BSA and FM4-64. Green: BSA Alexa Fluor 488 conjugate; red: FM4-64. **(A–A")** Confocal images of a spherical-shaped cluster of cells detected within 3 days after microsurgery. The vasculature is labeled with FITC-conjugated BSA (green). **(B–B ")** Confocal images of a polarized vesicle detected within 3 days after microsurgery. The vasculature is labeled with FITC-conjugated BSA (green), the cell membranes are labeled with FM4-64 (red). Scale bar= 10 μ.

Vesicles with even bigger size, yet without clear polarization were also found (n= 19; size ranging from 45 to 91 μm, average size = 62.6 +/− 13.5 μm) (**Figure 4E**). In these latter structures, mesenchymal cells are recurrently present inside the lumen. The position of some mesenchymal cells and their surface activity suggest a dynamic interaction with the vesicle (**Figures 4G,H**).

Inconstant Morphogenesis Following the Double-Vesicle Stage

After the recurrent scenarios described above, larger vascular buds detected over 3 days post dissections exhibited epithelial folds and compartmentalization of inner cavities, similarly to morphogenesis of peribranchial buds (Manni et al., 2014), although with a greater diversity of configurations of shapes, as suggested by Voskoboynik et al. (2007). While growing, the epithelium of the vascular buds takes the shape of the surrounding vessels and ampullae, resulting in buds distributed in different vascular compartments with completely aberrant forms when compared to blastogenic buds of the same size, including double-axis, *situs inversus,* or hyperplasias (**Supplementary Figure S6**).

Epithelia of the Vessels do not Contribute to the Vascular Budding Early Ontogenesis

To test the possible contribution of the vascular epithelia to the bud onset we took advantage of previous studies that showed the affinity of *B. schlosseri* vascular epithelia for BSA (Braden et al., 2014; Rodriguez et al., 2017; Rodriguez et al., 2018). First, to confirm the specificity of BSA to epithelial versus mesenchymal cells, uninjured colonies were injected with fluorescent-conjugated BSA and counterstained with Hoechst (nuclei) and FRM4-64 (cell walls). Within the first 48h, the presence of BSA is almost exclusively detected in vacuoles inside epithelial cells (98.62% ± 2.38, n= 10, (**Supplementary Figure S7**). After triggering WBR in 12 colonies, 40 VB onsets have been examined at different stages. In none of the vesicles the BSA signal has been detected (**Figure 5**). In 12 cases, BSA was detected in a cell that bridges the epithelial of the vessel with the inner vesicle (**Supplementary Figure S8**).

In Chimeric Colonies, Regenerating Zooids Preserve the Genotype of the Surrounding Tissues

To assess whether VB onset is originating from circulating mesenchymal cells, an approach based on allorecognition and

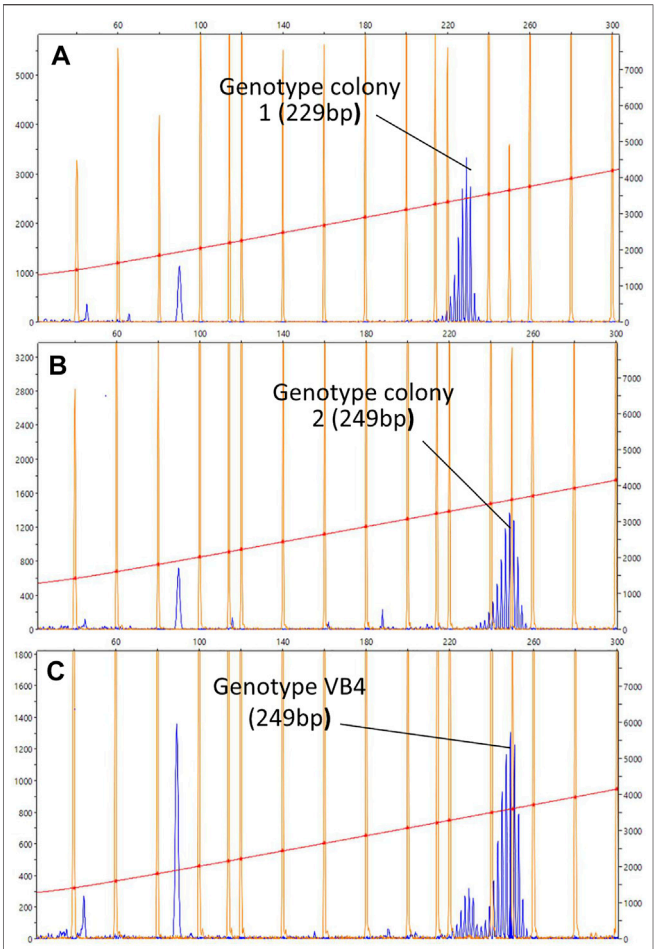


FIGURE 6 | Representative chromatogram for the microsatellites BS811. **(A–C)** Diagrams showing the size of the BS811 microsatellite locus amplified by PCR. The horizontal axis indicates the size in nucleotides and the vertical axis indicates the intensity of the fluorescence detected in the PCR product. The size is calculated with the default settings of the Peak Scanner software for the referenced standard size used in this experiment, LIZ600 (blue peaks = fluorescence of PCR products; orange peaks = standard size markers). Size of the fluorescence peak detected in the PCR carried out with the gDNA of colonies 1 **(A)** and 2 **(B)**, collected before the fusion, and **(C)** with the gDNA obtained from the stomach of a vascular bud, developed in the colony vascular system 2. The size is given in nucleotides. The peak at less than 100 bp could indicate other alleles for the same locus, but since the size of this microsatellite is normally between 200 and 300 bp, it is most likely a non-specific amplification product.

chimerism abilities of *Botryllus schlosseri* has been used (McKitrick and De Tomaso, 2010) (See Materials. and Methods). When two individual *B. schlosseri* colonies come into close contact, the ampullae reach out from each individual and come into contact. If the two colonies are histocompatible, the ampullae will fuse and form a single chimeric colony with a common vasculature. Yet, only hemocytes move from one original colony to the other, while the epithelia of vasculature remain separated (Braden et al., 2014; Taketa and De Tomaso, 2015).

After fusion, WBR was induced by depleting zooids and buds from the entire chimeric colony, and the regenerating zooids developed in separate regions of the vasculature. A total of 36 fusion experiments were performed and 7 of them produced vascular buds. Once they transformed into adult zooids, the gDNA was extracted from their stomachs. Then their genotype was assessed by analyzing four microsatellite loci and compared to the genotypes of the parental colonies (**Supplementary Figures S2C, S9, S10**). For the microsatellite BS811, we found alleles clearly different between the parental colonies (size 249 bp for colony A and 229 bp for colony B), and in six out of seven cases, the alleles amplified in the vascular buds corresponded to the genotype of the colony in which they originated (**Figure 6**). These results suggest a local vascular origin of WBR or a preferential association between hemocytes and cells of the vascular system of the same genotype. For the microsatellite PB41 and PB49, the results showed a presence of both genotypes. Yet, prevalent amplicon corresponded also to the genotype of the colony harboring the vascular buds.

Hemocytes and Hemoblasts Proliferating Activity is Stable Throughout the Vascular Budding Onset

To provide an overview of the dynamic of cell proliferation after the induction WBR, a time course of their mitotic activity was measured in the early phase of regeneration. While the distribution of mitotic cells appeared scattered through the whole colony all along the time course, since the VB has been detected within ampullae rather than along the vessels, the number of mitotic cells was counted within ampullae of identical volumes at 5 different time points upon injury. By analyzing 89 ampullae of approximately identical volume ($1{,}27*10^6 \pm 0{,}06*10^6$ microns^3) from 11 different colonies the mitotic activity was detected mainly among circulating hemocytes and it remains stable with a feeble increment at 72 h post-injury (**Figure 7** A-C).

In the closely related species *Botrylloides diegensis,* WBR has been reported to originate from a population of undifferentiated hemocytes, the hemoblasts, which behave like stem cells (Kassmer et al., 2020). Therefore, to explore the behavior of hemoblasts during the early stages of *B.schlosseri* WBR we used the putative hemoblast marker Integrin alpha 6 (Ia6) (Kassmer et al., 2020), assay the presence of Ia6+ and analyzed their dynamics via immunohistochemistry and *in situ* hybridization. With both techniques, we detected the presence of Ia6+. Yet, unlike what has been reported in *B.diegensis,* Ia6+ cells are rare and their number is stable throughout the onset of WBR, decreasing significatively only at 72 h post-injury (**Figures 7 C–F**, **Supplementary Figure S11**). Similar to *B. diegensis,* the majority of Ia6+ cells are proliferating (**Figure 7C**).

DISCUSSION

Among the different taxa that acquired WBR, the interest in tunicates regenerative abilities emerged due to their phylogenetic position as the sister group of vertebrates, and also because their regenerative capabilities are plastic within the sub-phylum,

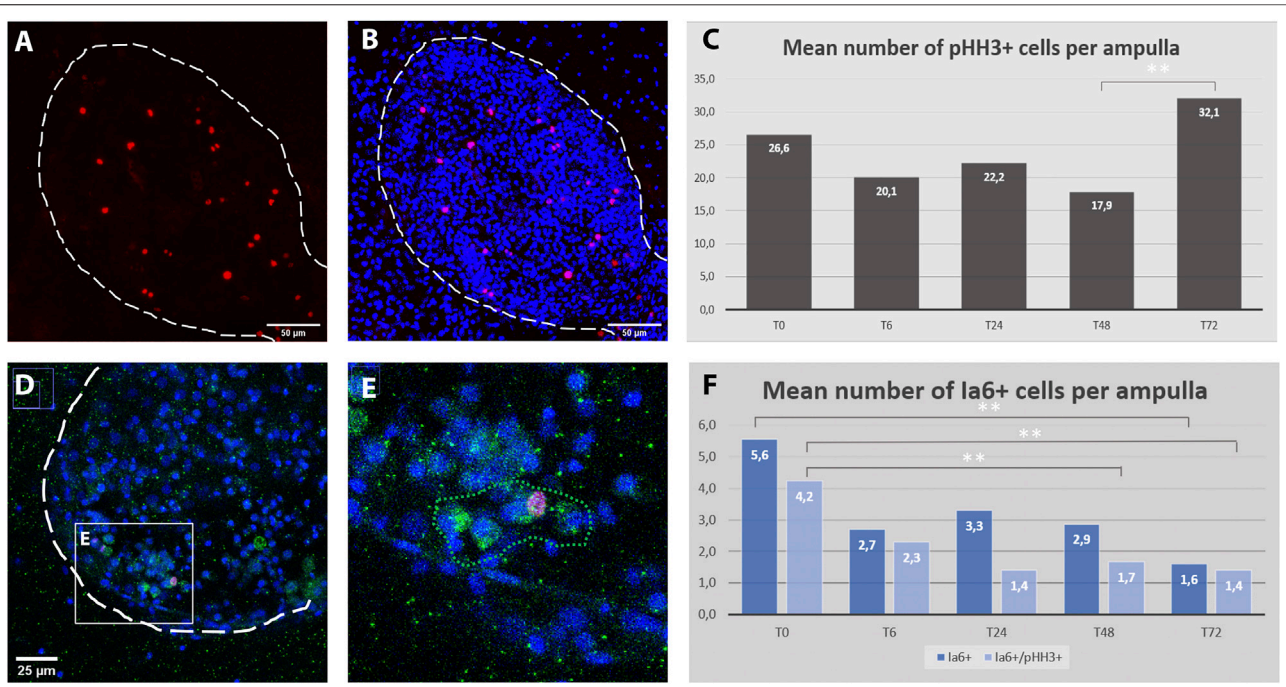


FIGURE 7 | Cell proliferation dynamics and Ia6 expression in ampullae during the first 3 days after microdissection. **(A–B)** Confocal z-stack showing the detail of an ampulla: proliferating cells are stained with anti-phospho-HH3 (red), cell nuclei are counter-stained with Hoechst (blue), ampulla shape is outlined with dotted lines. **(C)** Average number of pHH3+ cells per ampulla for five time-points within the first 3 days after microdissection. A significant difference has been detected only between 48 h (T48) and 72 (T72) hours post microdissection ($p = 0.025$). **(D–E)** ampulla containing a cluster of Ia6+ cells (green) including one co-labeled with anti-phospho-HH3 (red). **(F)** Histogram showing the proportion of proliferation Ia6+ cells and their dynamic within the first 3 days after microdissection ($p = 0.05$).

i.e., many species regenerate the whole body via asexual budding or upon extensive injury, others have more restrained regenerative potential (Alié et al., 2020; Nydam et al., 2021). Tunicates of the group of *Botryllinae* and in particular *Botryllus schlosseri* have been used for several decades as experimental laboratory models (Manni et al., 2019). The present study discloses previously undescribed dynamics of the phenomenon of injury triggered whole-body regeneration in *B. schlosseri*, and it adds anatomical and molecular elements that serve as a basis for further mechanistic studies in *B. schlosseri* as well as to compare regenerative processes among closely related chordate species.

Transcriptomic Response to Injury Suggests a Role of Angiogenesis and Complement Activation in Whole-Body Regeneration

Regardless of the extent and the nature of the lost part, regenerative response to an injury generally begins with a reparative event, such as wound-healing, followed by the activation of a developmental program that starts with the activation of precursors and eventually the unfolds of new morphogenesis (Carlson, 2007; Tiozzo and Copley, 2015). The overexpression of angiogenesis-related genes and ECM components, together with the extensive vascular remodeling observed during the first 24 h, point to an active role of the blood vessels in *Botryllus* WBR. In fact, similarities in the use of angiogenic factors between vertebrate endothelium and *Botryllus* vascular cells have already been identified (Gasparini et al., 2007; Tiozzo et al., 2008b; Braden et al., 2014; Gasparini et al., 2014; reviewed in Rodriguez et al., 2019). In the latter, VEGF and VEGFR regulate the active expansion of the vascular network by sprouting angiogenesis, which is key to the expansion of the colony and to maintain a proper connection between zooids (Gasparini et al., 2008; Gasparini et al., 2014). In addition, the plasticity of the vascular architecture is controlled by the epithelial cells' ability to synthesize the extracellular tunic (Gasparini et al., 2007) and to regulate its stiffness (Rodriguez et al., 2017). Finally, the ability of ampullae to actively migrate is central in the ability of *Botryllus* to regenerate its vasculature and is controlled by the expression of *BsVEGFR* in epithelial cells (Tiozzo et al., 2008c). Surprisingly, we could not find the *BsVEGFR* transcript in our RNAseq data. However, we found a dynamic expression of several angiogenic factors, of putative growth factors having EGF domains and of many components of the ECM, opening to further functional studies about the role of angiogenesis during WBR in *Botryllus schlosseri*.

Correct regeneration of lost organs in vertebrates necessitates a finely tuned interplay between inflammatory response, neovascularization and ECM remodeling to recruit stem/progenitor cells to the regenerative area and to organize the rebuilding tissues (reviewed in Mastellos et al., 2013). For instance, beyond its role as sentinels of immunity, C3 stimulate retina regeneration in chicken and mice (Haynes

et al., 2013; Peterson et al., 2021), while in mouse complement proteins regulate wound-healing and angiogenesis in a complex, not fully resolved manner (reviewed in Markiewski et al., 2020). In ascidians, C3 expression has been reported in various epithelial cells and hemocytes of several species (Pinto et al., 2003; Raftos et al., 2004; Giacomelli et al., 2012). In *Ciona intestinalis*, C3 and its putative receptor are expressed by phagocytic amoebocytes (Giacomelli et al., 2012) that show a chemotactic behavior toward sources of synthetic bioactive C3a, suggesting that amoebocytes may be recruited to inflammatory regions (Melillo et al., 2006). In *Botryllus schlosseri*, C3 and components of the lectin pathway are expressed by cytotoxic morula cells that promote phagocytosis of non-self particles (Franchi and Ballarin, 2014; Nicola and Loriano, 2017; Peronato et al., 2020). 2020). High level of expression of C3 orthologue and lectin pathway components (MASP, Ficolin) in the course of WBR in *Botryllus* suggests that the immune role of this pathway is important in the early steps of WBR. It also raises the intriguing possibility that C3 may be used to direct the migration of cells involved in regeneration, for instance, to orientate the vascular ampullae toward the tissue left-over. Finally, the high expression of coagulation-related genes immediately after injury suggests that the complement-coagulation interplay documented in vertebrates may also take place during *Botryllus* WBR to coordinate blood-clotting, defense against pathogen and tissue restoration.

Origin of WBR in *Botryllus schlosseri*

The cellular origin of WBR *via* vascular budding in *Botryllinae* has been attributed to undifferentiated hemocytes, referred to as hemoblasts, which home to areas of the vasculature and initiate to develop into the regenerating zooid (Rinkevich et al., 1995; Kassmer et al., 2020). The cluster of hemocytes proliferate and differentiate into a hollow monolayered vesicle, which grows in size and gets enclosed by the surrounding vascular epithelia (Brown et al., 2009; Kassmer et al., 2020). The resulting double vesicle is comparable to the one observed during other forms of budding across colonial tunicates, e.g. peribranchial budding in Stolidobranchs (Manni et al., 2014; Ricci et al., 2016a).

In our previous work, Ricci et al. (2016a) suggested that in *Botryllus schlosseri* the VB arises from a cluster of mesenchymal cells circulating in the vasculature that gives rise to vesicles eventually developing into a zooid. However, the study lacked longitudinal analyses to backtrack the origin of the clusters, and also missed detailed morphological descriptions to follow the ontogeny of the process (Ricci et al., 2016a). Discordantly, a recently published work by Nourizadeh et al. (2021) proposed that VB originates from, and occurs only, if parts of the blastogenic buds are left behind during the surgery, and therefore suggests an extra-vascular origin of the WBR. Nevertheless, this study also lacked to follow the *in vivo* dynamics of the process at the cellular level and therefore failed to detect any intravascular vesicle or cell cluster (Nourizadeh et al., 2021).

Indeed, our observations suggest that in *Botryllus schlosseri* vascular buds do not originate from mesenchymal cells resident inside the vasculature but tissues hailed from outside the vasculature and left behind during the injury. In our experimental setup, during the microdissection procedure, the whole blastogenic buds are removed, and so are the majority of the adult zooid tissues. Only small residues of the anterior part of the differentiated adult zooids (<50–70 μm) often remain attached to the tunic (**Supplementary Movies S3–S5**). According to the anatomy of *B. schlosseri,* these residues may contain parts of the epidermis, the epithelium of the endostyle, the branchial and peribranchial epithelia, and portions of the mantels with residues of muscle fibers and/or peripheral nerves (Tiozzo et al., 2008b; Manni et al., 2014). Starting from this scenario, we observed that within 72 h the heterogeneous tissue leftovers: a) migrate and fuse into the vascular network and b) re-shape into different types and numbers of monolayered vesicles (**Figure 8**). First, the migration dynamics potentially involve some form of chemotaxis, which allows the migration of the residual tissues through the tunic and towards the vascular network, as well as angiogenetic/vasculogenetic mechanisms that allow the active sprouting of the tip of the vessels towards the tissue leftover. Second, the reshaping of the tissues into vesicles that eventually gives rise to a complex body suggests the existence of an unforeseen level of tissue plasticity and cell potency. The possible presence in the leftover tissues of residues of endostyle, which has been suggested to be a somatic stem cell niche in *B. schlosseri* (Voskoboynik et al., 2008), may contribute to the initiation of the WBR via vascular budding. On the other hand, mechanisms of cell de- or transdifferentiation, reported in the WBR in other relatively close tunicate species (Kawamura and Fujiwara, 1995; Kawamura et al., 2018), cannot be ruled out. Without a high-resolution method to live-tracking the cells and tissues it was not possible to provide information concerning the exact nature of the left behind tissues and the mechanisms involved. Yet, the lack of a hemocyte proliferation burst following the injury, and the scarce presence of Ia6+ circulating cells, a marker of putative stem cells in the sister species *Botrylloides diegensis* (Kassmer et al., 2020) does not hint the presence of mesenchymal stem-cell-based mechanisms in *B. schlosseri.*

Morphological Convergence

The variability of both the site and the time of appearance of the first detectable intravascular structure supports the idea that these two variables are linked respectively to the location and the amount of the tissue left behind upon microdissection. These inconsistencies, which have also been recently reported by Nourizadeh et al. (2021), together with the lack of a proper live-tracking technique do not allow to detail the ontogenesis of the vascular bud once entered into the vasculature. Yet, in our experiments that originally were aimed to completely deplete all zooids and budding tissues, we consistently left behind clusters of 50–70 μ circa. In these conditions, the first intravascular structures were observed within a time window of 3 days (72 hpi). Except few cases which showed the presence of complex epithelial structures probably linked to clumsy microdissection (**Figures 2U–U"**), in most of the microsurgery experiments we detected a variety of intravascular monolayered vesicles. These vesicles were all made of polarized cells, with the apical side facing the lumen. They were all actively proliferating and, when they have been left to develop, they lead to the formation of growing vascular buds. Hence, the lack of contribution of the vascular epithelia and circulating hemoblasts (**Figures 5**, **6**), the absence of these structures in undissected colonies, and the dynamics seen in the movies strongly suggest that the cellular origin of the vesicle is the tissue leftover derived from the dissected adult.

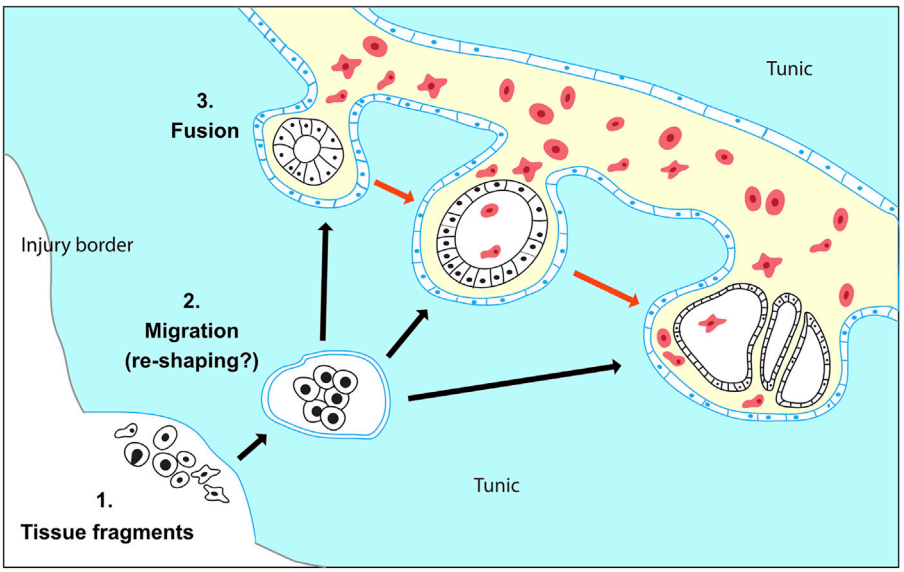


FIGURE 8 | Proposed model for WBR in *Botryllus schlosseri*. (1) WBR originates from heterogeneous tissue fragments of the adult zooid, which have been left behind during injury. (2) The tissue fragments migrate through the tunic (black arrows) and possibly re-shape into spherical vesicles enclosed by a monolayered epithelium (blue). (3) The double vesicles fuse with the vasculature and release the inner vesicle (black) in the vascular system. The vesicles proliferate and develop into the regenerating zooid (red arrows). The exact nature of the leftover cells and the dynamic of tissue reshaping during tissue migration are unknown.

The monolayered hollow vesicle, which becomes double-vesicle once enveloped by a layer of epithelial tissue, is a phylotypic stage common to many types of budding in tunicates (Alié et al., 2020). We previously documented that at this stage, the regionalized expression of germ-layers markers suggests a cell commitment (Ricci et al., 2016a). Therefore, the regular detection of this structure, its continuous proliferative activity, and the commitment of its cells suggest that a morphogenetic program is already in place. Unlike the vascular budding in other *Botryllinae*, the morphogenesis has been documented to unfold abnormally, regaining the normal developmental patterns only after a series of generations of blastogenic budding (Voskoboynik et al., 2007). While further observations are needed, the abnormalities detected seem to concern the patterning (axes and a/symmetries) rather than the cell differentiation, as the presence of differentiated muscles and the nervous system seems to suggest (**Supplementary Figure S6**).

Variation of Injury-Induced WBR Capacities Across Botryllinae

Among tunicates, both the diversity of the cellular onsets and the phylogenetic distribution suggests that the WBR capacity via propagative and survival budding is a plastic trait that evolved multiple times (Alié et al., 2018; Nydam, 2020). Mesenchymal stem cell-driven budding like vascular budding, has been suggested as a propagative and/or survival mode of WBR in several species of tunicates and it has been documented in *Botryllus schlosseri*'s closest related species such as *Botrylloides diegensis* (**Supplementary Figure S1**). In the latter, as well in other *Botryllinae* species, VB can be easily induced by isolating a small portion of the extracorporeal vasculature. On the other hand, in *B. schlosseri* a more structured vascular network and the presence of extravascular tissue seems to be necessary for the WBR to start (Sabbadin et al., 1975; Nourizadeh et al., 2021). Therefore, even if we cannot rule out a "leftover-free" initiation of VB, or multiple sources of budding, the data collected seems to suggest that *Botryllus schlosseri* does not undergo vascular budding as the other Botryllinae. Such phylogenetic proximity offers the opportunity to identify at the intra-generic level the genomic basis of developmental plasticity linked to whole-body regeneration.

DATA AVAILABILITY STATEMENT

The transcriptomic datasets generated in this study are deposited in the Gene Expression Omnibus repository, accession number GSE193805 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193805)

AUTHOR CONTRIBUTIONS

ST and LR designed the study; LR, BS, CO, AA, and ST performed the experiments; AA, RAP, and AC assembled the transcriptomes and analyzed the RNAseq dataset, ST and AA wrote and edited the manuscript. All authors read and commented on drafts, and approved the final version.

FUNDING

This work was supported by ANR (ANR-14-CE02-0019-01), INSB-DBM-2021, FRM (ING20140129231) and Canon

Foundation Fellowship 2020. LR was supported by UPMC-Emergence Grant an FRM Grant (FDT20140931163).

ACKNOWLEDGMENTS

We would like to thank the EMBRC-France and in particular Laurent Gilletta for setting up the aquaculture system, Mohamed Khamla for help with the video editing, Sonia Lotito for the technical support, Laurel Hiebert and Philippe Dru for help with the bioinformatics resources.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.843775/full#supplementary-material

REFERENCES

Abu-Jamous, B., and Kelly, S. (2018). Clust: Automatic Extraction of Optimal Co-expressed Gene Clusters from Gene Expression Data. *Genome Biol.* 19, 1–11. doi:10.1186/S13059-018-1536-8/FIGURES/5

Advani, V. M., and Ivanov, P. (2019). Translational Control under Stress: Reshaping the Translatome. *BioEssays* 41, 1900009. doi:10.1002/BIES.201900009

Alié, A., Hiebert, L. S., Scelzo, M., and Tiozzo, S. (2020). The Eventful History of Nonembryonic Development in Tunicates. *J. Exp. Zool (Mol Dev. Evol.* 336, 250–266. doi:10.1002/jez.b.22940

Alié, A., Hiebert, L. S., Simion, P., Scelzo, M., Prünster, M. M., Lotito, S., et al. (2018). Convergent Acquisition of Nonembryonic Development in Styelid Ascidians. *Mol. Biol. Evol.* 35, 1728–1743. doi:10.1093/molbev/msy068

Ben-Shlomo, R., Motro, U., Paz, G., and Rinkevich, B. (2007). Pattern of Settlement and Natural Chimerism in the Colonial Urochordate Botryllus Schlosseri. *Genetica* 132, 51–58. doi:10.1007/S10709-007-9148-3

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* 30, 2114–2120. doi:10.1093/BIOINFORMATICS/BTU170

Braden, B. P., Taketa, D. A., Pierce, J. D., Kassmer, S., Lewis, D. D., and De Tomaso, A. W. (2014). Vascular Regeneration in a Basal Chordate Is Due to the Presence of Immobile, Bi-functional Cells. *PLoS One* 9, e95460. doi:10.1371/journal.pone.0095460

Bray, N. L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal Probabilistic RNA-Seq Quantification. *Nat. Biotechnol.* 34, 525–527. doi:10.1038/nbt.3519

Brown, F. D., Keeling, E. L., Le, A. D., and Swalla, B. J. (2009). Whole Body Regeneration in a Colonial ascidian,Botrylloides Violaceus. *J. Exp. Zool.* 312B, 885–900. doi:10.1002/jez.b.21303

Brunetti, R. (2009). Botryllid Species (Tunicata, Ascidiacea) from the Mediterranean Coast of Israel, with Some Considerations on the Systematics of Botryllinae. *Zootaxa* 2289, 18–32. doi:10.11646/ZOOTAXA.2289.1.2

Carlson, B. M. (2007). *Principles of Regenerative Biology*. Amsterdam: Elsevier. doi:10.1016/B978-012369439-3/50006-4

Di Maio, A., Setar, L., Tiozzo, S., and De Tomaso, A. W. (2015). Wnt Affects Symmetry and Morphogenesis during post-embryonic Development in Colonial Chordates. *Evodevo* 6, 17. doi:10.1186/s13227-015-0009-3

Franchi, N., and Ballarin, L. (2014). Preliminary Characterization of Complement in a Colonial Tunicate: C3, Bf and Inhibition of C3 Opsonic Activity by Compstatin. *Dev. Comp. Immunol.* 46, 430–438. doi:10.1016/j.dci.2014.05.014

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: Accelerated for Clustering the Next-Generation Sequencing Data. *Bioinformatics* 28, 3150–3152. doi:10.1093/BIOINFORMATICS/BTS565

Gasparini, F., Burighel, P., Manni, L., and Zaniolo, G. (2008). Vascular Regeneration and Angiogenic-like Sprouting Mechanism in a Compound Ascidian Is Similar to Vertebrates. *Evol. Dev.* 10, 591–605. doi:10.1111/j.1525-142X.2008.00274.x

Gasparini, F., Caicci, F., Rigon, F., Zaniolo, G., and Manni, L. (2014). Testing an Unusual *In Vivo* Vessel Network Model: a Method to Study Angiogenesis in the Colonial Tunicate Botryllus Schlosseri. *Sci. Rep.* 4, 6460. doi:10.1038/srep06460

Gasparini, F., Longo, F., Manni, L., Burighel, P., and Zaniolo, G. (2007). Tubular Sprouting as a Mode of Vascular Formation in a Colonial Ascidian (Tunicata). *Dev. Dyn.* 236, 719–731. doi:10.1002/dvdy.21073

Gasparini, F., Manni, L., Cima, F., Zaniolo, G., Burighel, P., Caicci, F., et al. (2015). Sexual and Asexual Reproduction in the Colonial ascidian *Botryllus schlosseri*. *genesis* 53, 105–120. doi:10.1002/dvg.22802

Giacomelli, S., Melillo, D., Lambris, J. D., and Pinto, M. R. (2012). Immune Competence of the *Ciona intestinalis* Pharynx: Complement System-Mediated Activity. *Fish Shellfish Immunol.* 33, 946–952. doi:10.1016/J.FSI.2012.08.003

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length Transcriptome Assembly from RNA-Seq Data without a Reference Genome. *Nat. Biotechnol.* 29, 644–652. doi:10.1038/nbt.1883

Haynes, T., Luz-Madrigal, A., Reis, E. S., Echeverri Ruiz, N. P., Grajales-Esquivel, E., Tzekou, A., et al. (20132013). Complement Anaphylatoxin C3a Is a Potent Inducer of Embryonic Chick Retina Regeneration. *Nat. Commun.* 4 (4), 1–11. doi:10.1038/ncomms3312

Iwasaka, C., Tanaka, K., Abe, M., and Sato, Y. (1996). Ets-1 Regulates Angiogenesis by Inducing the Expression of Urokinase-type Plasminogen Activator and Matrix Metalloproteinase-1 and the Migration of Vascular Endothelial Cells. *J. Cell. Physiol.* 169, 522–531. doi:10.1002/(SICI)1097-4652(199612)169:3<522:AID-JCP12>3.0.CO;2-7

Kanki, Y., Kohro, T., Jiang, S., Tsutsumi, S., Mimura, I., Suehiro, J.-i., et al. (2011). Epigenetically Coordinated GATA2 Binding Is Necessary for Endothelium-Specificendomucinexpression. *EMBO J.* 30, 2582–2595. doi:10.1038/EMBOJ.2011.173

Kassmer, S. H., Langenbacher, A. D., and De Tomaso, A. W. (2020). Integrin-alpha-6+ Candidate Stem Cells Are Responsible for Whole Body Regeneration in the Invertebrate Chordate Botrylloides Diegensis. *Nat. Commun.* 11, 1–11. doi:10.1038/s41467-020-18288-w

Kassmer, S. H., Rodriguez, D., and De Tomaso, A. W. (2016). Colonial Ascidians as Model Organisms for the Study of Germ Cells, Fertility, Whole Body Regeneration, Vascular Biology and Aging. *Curr. Opin. Genet. Dev.* 39, 101–106. doi:10.1016/j.gde.2016.06.001

Kawamura, K., and Fujiwara, S. (1995). Cellular and Molecular Characterization of Transdifferentiation in the Process of Morphallaxis of Budding Tunicates. *Semin. Cell Biol.* 6, 117–126. doi:10.1006/scel.1995.0017

Kawamura, K., Yoshida, T., and Sekida, S. (2018). Autophagic Dedifferentiation Induced by Cooperation between TOR Inhibitor and Retinoic Acid Signals in Budding Tunicates. *Dev. Biol.* 433, 384–393. doi:10.1016/j.ydbio.2017.08.023

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: Fast and Accurate Filtering of Ribosomal RNAs in Metatranscriptomic Data. *Bioinformatics* 28, 3211–3217. doi:10.1093/BIOINFORMATICS/BTS611

Kürn, U., Rendulic, S., Tiozzo, S., and Lauzon, R. J. (2011). Asexual Propagation and Regeneration in Colonial Ascidians. *Biol. Bull.* 221, 43–61. doi:10.1086/bblv221n1p43

Langenbacher, A. D., Rodriguez, D., Di Maio, A., and De Tomaso, A. W. (2015). Whole-mount fluorescent in situ hybridization staining of the colonial tunicate *Botryllus schlosseri*. *Genesis* 53, 194–201. doi:10.1002/dvg.22820

Lauzon, R. J., Ishizuka, K. J., and Weissman, I. L. (2002). Cyclical Generation and Degeneration of Organs in a Colonial Urochordate Involves Crosstalk between Old and New: A Model for Development and Regeneration. *Dev. Biol.* 249, 333–348. doi:10.1006/dbio.2002.0772

Liu, M., Zhang, L., Marsboom, G., Jambusaria, A., Xiong, S., Toth, P. T., et al. (2019). Sox17 Is Required for Endothelial Regeneration Following Inflammation-Induced Vascular Injury. *Nat. Commun.* 10, 1–14. doi:10.1038/s41467-019-10134-y

Love, M. I., Huber, W., and Anders, S. (2014). Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* 15, 1–21. doi:10.1186/S13059-014-0550-8/FIGURES/9

Manni, L., Anselmi, C., Cima, F., Gasparini, F., Voskoboynik, A., Martini, M., et al. (2019). Sixty Years of Experimental Studies on the Blastogenesis of the Colonial Tunicate Botryllus Schlosseri. *Dev. Biol.* 448, 293–308. doi:10.1016/j.ydbio.2018.09.009

Manni, L., Gasparini, F., Hotta, K., Ishizuka, K. J., Ricci, L., Tiozzo, S., et al. (2014). Ontology for the Asexual Development and Anatomy of the Colonial Chordate Botryllus Schlosseri. *PLoS One* 9, e96434. doi:10.1371/journal.pone.0096434

Manni, L., Zaniolo, G., Cima, F., Burighel, P., and Ballarin, L. (2007). Botryllus Schlosseri: A Model Ascidian for the Study of Asexual Reproduction. *Dev. Dyn.* 236, 335–352. doi:10.1002/dvdy.21037

Markiewski, M. M., Daugherity, E., Reese, B., and Karbowniczek, M. (2020). The Role of Complement in Angiogenesis. *Antibodies* 9, 67. doi:10.3390/ANTIB9040067

Martinez, V. G., Menger, G. J., and Zoran, M. J. (2005). Regeneration and Asexual Reproduction Share Common Molecular Changes: Upregulation of a Neural Glycoepitope during Morphallaxis in Lumbriculus. *Mech. Dev.* 122, 721–732. doi:10.1016/j.mod.2004.12.003

Mastellos, D. C., DeAngelis, R. A., and Lambris, J. D. (2013). Complement-triggered Pathways Orchestrate Regenerative Responses throughout Phylogenesis. *Semin. Immunol.* 25, 29–38. doi:10.1016/J.SMIM.2013.04.002

McKitrick, T. R., and De Tomaso, A. W. (2010). Molecular Mechanisms of Allorecognition in a Basal Chordate. *Semin. Immunol.* 22, 34–38. doi:10.1016/j.smim.2009.12.001

Melillo, D., Sfyroera, G., De Santis, R., De, Graziano, R., Graziano, R., Lambris, J. D., et al. (2006). First Identification of a Chemotactic Receptor in an Invertebrate Species: Structural and Functional Characterization of *Ciona intestinalis* C3a Receptor. *J. Immunol.* 177, 4132–4140. doi:10.4049/JIMMUNOL.177.6.4132

Milkman, R. (1967). Genetic and Developmental Studies on Botryllus Schlosseri. *Biol. Bull.* 132, 229–243. doi:10.2307/1539891

Munday, R., Rodriguez, D., Di Maio, A., Kassmer, S., Braden, B., Taketa, D. A., et al. (2015). Aging in the Colonial chordate,Botryllus Schlosseri. *Invertebrate Reprod. Dev.* 59, 45–50. doi:10.1080/07924259.2014.938197

Nakauchi, M. (1982). Asexual Development of Ascidians: Its Biological Significance, Diversity, and Morphogenesis. *Am. Zool* 22, 753–763. doi:10.1093/icb/22.4.753

Nicola, F., and Loriano, B. (2017). Morula Cells as Key Hemocytes of the Lectin Pathway of Complement Activation in the Colonial Tunicate Botryllus Schlosseri. *Fish Shellfish Immunol.* 63, 157–164. doi:10.1016/J.FSI.2017.02.003

Nourizadeh, S., Kassmer, S., Rodriguez, D., Hiebert, L. S., and De Tomaso, A. W. (2021). Whole Body Regeneration and Developmental Competition in Two Botryllid Ascidians. *Evodevo* 12, 15. doi:10.1186/S13227-021-00185-Y

Nydam, M. L. (2020). Evolution of Allorecognition in the Tunicata. *Biology* 9, 1–13. doi:10.3390/biology9060129

Nydam, M. L., Lemmon, A. R., Cherry, J. R., Kortyna, M. L., Clancy, D. L., Hernandez, C., et al. (2021). Phylogenomic and Morphological Relationships Among the Botryllid Ascidians (Subphylum Tunicata, Class Ascidiacea, Family Styelidae). *Sci. Rep.* 11, 1–13. doi:10.1038/s41598-021-87255-2

Oeckinghaus, A., Hayden, M. S., and Ghosh, S. (2011). Crosstalk in NF-Kb Signaling Pathways. *Nat. Immunol.* 12 (12), 695–708. doi:10.1038/ni.2065

Oka, H., and Watanabe, H. (1959). Vascular Budding in Botrylloides. *Biol. Bull.* 117, 340–346. doi:10.2307/1538913

Oka, H., and Watanabe, H. (1957). Vascular Budding, a New Type of Budding in Botryllus ,. *Biol. Bull.* 112, 225–240. doi:10.2307/1539200

Parker, S. S., Mandell, E. K., Hapak, S. M., Maskaykina, I. Y., Kusne, Y., Kim, J.-Y., et al. (2013). Competing Molecular Interactions of aPKC Isoforms Regulate Neuronal Polarity. *Proc. Natl. Acad. Sci.* 110, 14450–14455. doi:10.1073/PNAS.1301588110/-/DCSUPPLEMENTAL

Peronato, A., Franchi, N., and Loriano, B. (2020). BsTLR1: A New Member of the TLR Family of Recognition Proteins from the Colonial Ascidian Botryllus Schlosseri. *Fish Shellfish Immunol.* 106, 967–974. doi:10.1016/J.FSI.2020.09.006

Peterson, S. L., Li, Y., Sun, C. J., Wong, K. A., Leung, K. S., de Lima, S., et al. (2021). Retinal Ganglion Cell Axon Regeneration Requires Complement and Myeloid Cell Activity within the Optic Nerve. *J. Neurosci.* 41, 8508–8531. doi:10.1523/JNEUROSCI.0555-21.2021

Pinto, M. R., Chinnici, C. M., Kimura, Y., Melillo, D., Marino, R., Spruce, L. A., et al. (2003). CiC3-1a-Mediated Chemotaxis in the Deuterostome InvertebrateCiona intestinalis(Urochordata). *J. Immunol.* 171, 5521–5528. doi:10.4049/JIMMUNOL.171.10.5521

Prünster, M. M., Ricci, L., Brown, F. D., and Tiozzo, S. (2019). De Novo neurogenesis in a Budding Chordate: Co-option of Larval Anteroposterior Patterning Genes in a Transitory Neurogenic Organ. *Dev. Biol.* 448, 342–352. doi:10.1016/j.ydbio.2018.10.009

Prünster, M. M., Ricci, L., Brown, F., and Tiozzo, S. (2018). Modular Co-option of Cardiopharyngeal Genes during Non-embryonic Myogenesis. *EvoDevo* 10, 3. doi:10.1186/s13227-019-0116-7

Raftos, D. A., Fabbro, M., and Nair, S. V. (2004). Exocytosis of a Complement Component C3-like Protein by Tunicate Hemocytes. *Dev. Comp. Immunol.* 28, 181–190. doi:10.1016/S0145-305X(03)00136-8

Ricci, L., Cabrera, F., Lotito, S., and Tiozzo, S. (2016a). Redeployment of Germ Layers Related TFs Shows Regionalized Expression during Two Non-embryonic Developments. *Dev. Biol.* 416, 235–248. doi:10.1016/j.ydbio.2016.05.016

Ricci, L., Chaurasia, A., Lapébie, P., Dru, P., Helm, R. R., Copley, R. R., et al. (2016b). Identification of Differentially Expressed Genes from Multipotent Epithelia at the Onset of an Asexual Development. *Sci. Rep.* 6, 27357. doi:10.1038/srep27357

Rinkevich, B., Shlemberg, Z., and Fishelson, L. (1995). Whole-body Protochordate Regeneration from Totipotent Blood Cells. *Proc. Natl. Acad. Sci.* 92, 7695–7699. doi:10.1073/pnas.92.17.7695

Rinkevich, Y., Douek, J., Haber, O., Rinkevich, B., and Reshef, R. (2007). Urochordate Whole Body Regeneration Inaugurates a Diverse Innate Immune Signaling Profile. *Dev. Biol.* 312, 131–146. doi:10.1016/j.ydbio.2007.09.005

Rodriguez, D., Braden, B. P., Boyer, S. W., Taketa, D. A., Setar, L., Calhoun, C., et al. (2017). *In Vivo* manipulation of the Extracellular Matrix Induces Vascular Regression in a Basal Chordate. *MBoC* 28, 1883–1893. doi:10.1091/mbc.e17-01-0009

Rodriguez, D., Nourizadeh, S., and De Tomaso, A. W. (2019). The Biology of the Extracorporeal Vasculature of Botryllus Schlosseri. *Dev. Biol.* 448, 309–319. doi:10.1016/j.ydbio.2018.10.013

Sabbadin, A., Zaniolo, G., and Majone, F. (1975). Determination of Polarity and Bilateral Asymmetry in Palleal and Vascular Buds of the Ascidian Botryllus Schlosseri. *Dev. Biol.* 46, 79–87. doi:10.1016/0012-1606(75)90088-3

Sánchez Alvarado, A., and Yamanaka, S. (2014). Rethinking Differentiation: Stem Cells, Regeneration, and Plasticity. *Cell* 157, 110–119. doi:10.1016/j.cell.2014.02.041

Satoh, N. (1994). in *Developmental Biology of Ascidians. Cambridge.* Editors P. W. Barlow, D. Bray, P. B. Green, and J. M. W. Slack (Cambridge: Cambridge University Press).

Sinigaglia, C., Alie, A., and Tiozzo, S. (1994). in *The Hazards of Regeneration: from Morgan's legacy to Evo-Devo*. Editors S. Blanchoud and B. Galliot. Whole-body Regeneration. in Methods Mol. Biol. (Berlin: Springer, USA).

Stoner, D. S., and Weissman, I. L. (1996). Somatic and Germ Cell Parasitism in a Colonial Ascidian: Possible Role for a Highly Polymorphic Allorecognition System. *Proc. Natl. Acad. Sci.* 93, 15254–15259. doi:10.1073/pnas.93.26.15254

Taketa, D. A., and De Tomaso, A. W. (2015). Botryllus Schlosseri Allorecognition: Tackling the enigma. *Dev. Comp. Immunol.* 48, 254–265. doi:10.1016/j.dci.2014.03.014

Tiozzo, S., Brown, F. D., and De Tomaso, A. W. (2008a). "Regeneration and Stem Cells in Ascidians," in *Stem Cells: From hydra to Man*. Editor T. C. Bosh (London: Springer), 95–112. doi:10.1007/978-1-4020-8274-0_6

Tiozzo, S., Christiaen, L., Deyts, C., Manni, L., Joly, J.-S. p., and Burighel, P. (2005). Embryonic versus Blastogenetic Development in the Compound ascidianBotryllus Schlosseri: Insights fromPitx Expression Patterns. *Dev. Dyn.* 232, 468–478. doi:10.1002/dvdy.20250

Tiozzo, S., and Copley, R. R. (2015). Reconsidering Regeneration in Metazoans: an Evo-Devo Approach. *Front. Ecol. Evol.* 3, 1–12. doi:10.3389/fevo.2015.00067

Tiozzo, S., Murray, M., Degnan, B. M., De Tomaso, A. W., and Croll, R. P. (2009b). Development of the Neuromuscular System during Asexual Propagation in an Invertebrate Chordate. *Dev. Dyn.* 238, 2081–2094. doi:10.1002/dvdy.22023

Tiozzo, S., Voskoboynik, A., Brown, F. D., and De Tomaso, A. W. (2008c). A Conserved Role of the VEGF Pathway in Angiogenesis of an Ectodermally-Derived Vasculature. *Dev. Biol.* 315, 243–255. doi:10.1016/j.ydbio.2007.12.035

Vorontsova, M. A., and Liosner, L. D. (1961). in *Asexual Propagation and Regeneration*. Editor U. C. Billet (London, New York: Pergamon Press).

Voskoboynik, A., Simon-Blecher, N., Soen, Y., Rinkevich, B., De Tomaso, A. W., Ishizuka, K. J., et al. (2007). Striving for Normality: Whole Body Regeneration through a Series of Abnormal Generations. *FASEB j.* 21, 1335–1344. doi:10.1096/fj.06-7337com

Voskoboynik, A., Soen, Y., Rinkevich, Y., Rosner, A., Ueno, H., Reshef, R., et al. (2008). Identification of the Endostyle as a Stem Cell Niche in a Colonial Chordate. *Cell Stem Cell* 3, 456–464. doi:10.1016/j.stem.2008.07.023

Voskoboynik, A., and Weissman, I. L. (2014). Botryllus Schlosseri, an Emerging Model for the Study of Aging, Stem Cells, and Mechanisms of Regeneration. *Invertebrate Reprod. Dev.* 59, 33–38. doi:10.1080/07924259.2014.944673

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 09 February 2023
DOI 10.3389/fevo.2022.979278

OPEN ACCESS

EDITED BY
Igor Schneider,
Federal University of Pará, Brazil

REVIEWED BY
Chiara Sinigaglia,
UMR7232 Biologie Intégrative des Organismes Marins (BIOM), France
Lucas Leclere,
UMR7009 Laboratoire de Biologie du Développement de Villefranche sur Mer, France

*CORRESPONDENCE
Marcin Adamski
✉ marcin.adamski@anu.edu.au
Maja Adamska
✉ maja.adamska@anu.edu.au

†PRESENT ADDRESSES
Oliver Mead,
CSIRO, Land and Water, Canberra, ACT, Australia
Aurelie Moya,
Limnological Institute, University of Konstanz, Konstanz, Germany

SPECIALTY SECTION
This article was submitted to Evolutionary Developmental Biology, a section of the journal Frontiers in Ecology and Evolution

RECEIVED 27 June 2022
ACCEPTED 21 December 2022
PUBLISHED 09 February 2023

CITATION
Xu J, Mead O, Moya A, Caglar C, Miller DJ, Adamski M and Adamska M (2023) Wound healing and regeneration in the reef building coral *Acropora millepora*. *Front. Ecol. Evol.* 10:979278. doi: 10.3389/fevo.2022.979278

Wound healing and regeneration in the reef building coral *Acropora millepora*

Jane Xu[1,2], Oliver Mead[1,2†], Aurelie Moya[3†], Cüneyt Caglar[1,2], David J. Miller[3,4], Marcin Adamski[1*] and Maja Adamska[1,2*]

[1]Research School of Biology, Australian National University, Canberra, ACT, Australia, [2]ARC Centre of Excellence for Coral Reef Studies, Australian National University, Canberra, ACT, Australia, [3]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD, Australia, [4]Comparative Genomics Centre and Department of Molecular and Cell Biology, James Cook University, Townsville, QLD, Australia

Branching scleractinian corals are niche-constructing organisms, providing continuously-growing, structural foundation for spectacularly biodiverse coral reef ecosystems. A large part of their success lies in the ability to quickly regenerate following mechanical damage. Even now, when the corals undergo great decline due to anthropogenic weather and storm extremes, it is surprising how little is known about molecular mechanisms governing regeneration in these iconic organisms. In this study, we used RNA-seq to identify genes involved in the regeneration of *Acropora millepora*, starting with the initial wound closure up to complete rebuilding of lost structures. Many of the differentially expressed genes we found in the wound healing steps are homologues of genes known to be involved in wound healing and regeneration of bilaterian and other cnidarian species, prominently including multiple components of FGF and Wnt signalling pathways. Comparison between genes involved in wound healing and continuous growth of the colony demonstrates both similarity and distinctiveness of the genetic programmes controlling these processes. A striking example is specific expression of c-Fos, a transcription factor with conserved role in early injury response, during the earliest stages of wound healing of *A. millepora*. By comparing results obtained in diverse experimental conditions including a closed-loop, recirculating aquarium and a flow-through system of marine station, we have demonstrated feasibility of using zooxanthellate scleractinian corals as experimental models in fundamental biology research, including studies of regeneration.

KEYWORDS

coral, regeneration, wound healing, FGF signalling pathway, Wnt signalling pathway, Fos

Introduction

The scleractinian coral holobionts (tight unions of animal hosts, their associated zooxanthellae and diverse prokaryotes) have been hugely successful in shallow tropical marine environments, creating complex habitats for multitude of other organisms.

However, this success is recently under threat due to anthropogenic climate change, resulting not only in increased water temperatures causing coral bleaching-related mortality, but also physical destruction of the reefs by increased intensity of storms in addition to direct damage by dredging and boating (e.g., Bak, 1978; Saphier and Hoffmann, 2005; Hughes et al., 2017). Maintenance and recovery of coral reefs greatly depends on the ability of individual corals to quickly heal the injuries, covering the exposed skeleton and then rebuilding lost structures. Indeed, zooxanthellate scleractinian corals have a high regenerative capacity, likely related to their continuous growth. For many branching corals, fragmentation is an important method of asexual reproduction, allowing significant dispersion and rapid recovery after physical disturbance (reviewed by Highsmith, 1982).

Ability to regenerate from fragments appears to also be an important recovery strategy after bleaching. For example, Diaz-Pulido et al. (2009) observed a bleached reef with coral cover reduced by 70–80% and the exposed coral skeletons overgrown by algae. Surprisingly, within 6 months of the event the reef had recovered to pre-bleaching coverage, likely as an effect of rapid growth from small fragments of coral tissue surviving at the bases of the colonies. Due to this ability, re-populating reefs with fragment-grown coral specimens is often considered as a strategy for reef restoration in face of the climate change (reviewed by Schmidt-Roach et al., 2020).

While several studies have addressed ecological aspects of coral regeneration (e.g., Sabine et al., 2015), the molecular mechanisms of regeneration in reef-building corals remain understudied. This is in contrast to extensive studies in many non-skeleton building cnidarians, which because of tremendous regeneration potential in this phylum and the ease of cultivation of several species have historically served as model systems for regenerative biology (Holstein et al., 2003). This is particularly true for the freshwater polyp Hydra, which even has the capacity to form new individuals from small clumps of cells (reviewed by Vogg et al., 2019). Regeneration ability is more limited – although still remarkable – in the starlet sea anemone *Nematostella vectensis*, which can re-grow complete polyp from a small, aboral section of body (Bossert and Thomsen, 2017). Intriguingly, cellular processes and genes involved in regeneration of these two model species vary to a large extent. Hydra regeneration appears to rely mainly on morphallactic processes (where new structures are formed by rearrangement of existing cells), while in *Nematostella* epimorphosis (where new structures are formed by cell proliferation) plays a larger role (Passamaneck and Martindale, 2012; DuBuc et al., 2014; Warner et al., 2018). As in many (if not all) animal regeneration processes, the Wnt pathway plays a major role in regeneration of both models; while the involvement of the TGF-beta pathway has been documented only in Hydra (e.g., Hobmayer et al., 2000; Reinhardt et al., 2004; Amiel et al., 2015; Petersen et al., 2015; Schaffer et al., 2016). A recent, candidate-gene driven study in *Tubastraea coccinea* demonstrated upregulation of several Wnt and FGF genes during regeneration of this azooxanthellate scleractinian (Luz et al., 2021).

Studies of molecular mechanisms of regeneration in the reef forming corals are an important endeavour from both fundamental biology and potential conservation perspectives, but are surprisingly limited, possibly due to practical and technical difficulties. In this study, we have sought to identify genes involved in regeneration of *Acropora millepora* using both laboratory and field systems. Our results reveal strong similarities between transcriptional signatures of the early wound healing phases and the leading edges of colonies spreading on the substrate, consistent with re-deployment of molecular machinery involved in normal growth of the colonies during regeneration. They also demonstrate involvement of the Wnt and FGF, but not TGF-beta pathways in coral regeneration, consistent with what was observed in previously studied anthozoans. Importantly, our results demonstrate feasibility of studying molecular and cellular aspects of reef building coral regeneration in inland laboratory conditions.

Materials and methods

Specimen collection, maintenance and wounding experiments

Inland laboratory experiments: Two colonies of *Acropora millepora* were obtained from Canberra Marine, collected by Cairns Marine (Cairns, Queensland) and placed in a closed-loop marine aquarium system in the Research School of Earth Sciences, Australian National University (ANU), Canberra. The main tank in the system measured 1.4 × 2.0 × 0.4 m, and was housing the corals, and a variety of cohabiting marine organisms associated with 'live rock' (natural aragonite framework serving as a habitat to a broad range or marine organisms in marine aquarium systems). The water movement was forced by circulation pumps (wave makers), and through a 1.2 × 0.6 × 0.6 m sump containing a protein skimmer, and physical and biological filters. Natural sea water in the tank was maintained at 27°C, 8.1–8.4 pH, ~35 mg/ml salinity, 440 mg/L calcium carbonate, 0.23–0.5 mg/L phosphate. The day/night cycling (12/12) used white and blue LED lights to illuminate the tank. The temperature fluctuated daily by approximately ±1°C (with day and night temperatures). Corals were fed 1/8th of a teaspoon of marine plankton (Reef Roids, PolypLab, United States) suspended in 50 ml of aquarium water twice a week. Approximately 2 ml suspension was target fed to each coral fragment with 20 ml plastic syringe (HSW Soft-Ject™, Germany) after wave makers were turned off. To obtain fragments for experiments, the two coral colonies were cut into fragments 4–8 cm in height, each containing two to three branches. They were cut using a diamond saw for the first colony and with a Dremel® 2001 with a 1–1/2″ EZ Lock Diamond Cut Wheel (Dremel®, Mexico) for the second colony. These were glued to plugs with Reef Glue™ (Seachem®, United States). Fragments were left to recover for a minimum of a week before starting

experiments. To create a lesion, one polyp and corallite were cut out from each branch using carbon steel Surgical Scalpel Blade No.15A (Swann-Morton®, England).

Marine station experiments: In April 2019, colonies of *Acropora millepora* were collected from locations near Orpheus Island, Australia (GBRMPA permit G17/39991.1). These colonies were transferred to flow-through unfiltered seawater tanks with oxygen stones and left to recover for 2 weeks before experiments were carried out.

In contrast to experiments carried in Canberra, the Orpheus Island colonies were not fragmented. Instead, to create lesions, one polyp and corallite per branch were cut out using carbon steel Surgical Scalpel Blade No.15A (Swann-Morton®, England).

Sampling

Tissues removed during initial wounding manipulation were discarded. To collect samples for RNA-Seq analysis, the area around the lesion, healed coenosarc or regenerating polyps, as well as matching controls were cut out following the timeline established during pilot observations (see Figure 1 in the Results section). To obtain enough tissue for extraction, three samples of the same regeneration stage and the same colony were pooled in 2 ml microcentrifuge tubes containing two 3 mm tungsten carbide beads (to aid subsequent sample disruption, Qiagen cat#69997) and snap frozen in liquid nitrogen.

RNA extraction and quality analysis

Samples were ground in the TissueLyser LT (Qiagen®, Netherlands) at 50 Hz with a TissueLyser adapter cooled to −20°C. The extraction was performed using TRIzol™ Reagent according to manufacturer's manual with the following changes: Samples in TRIzol™ and chloroform were transferred to a new 1.5 ml microcentrifuge tube which contained 100 µl autoclaved vacuum grease (Dow Corning®, United States). The RNA Nano Chip on Agilent Bioanalyzer® 2100 was used for RNA quality and quantification analyses.

cDNA library preparation

cDNA libraries were prepared following the TruSeq RNA Library Kit v2 manual (Illumina). Approximately 76–266 ng of total RNA per sample was used to prepare libraries, with the lower bound of this range determined by the amount of RNA available after quality and quantification analyses were carried in the initial experiments (see Supplementary Table 1). After library preparation, a SPRIselect (Beckmann Coulter) size selection was done according to the user guide on Both Side Size Selection with a few changes: Agencourt AMPure XP Beads (Beckman Coulter) with ratios 0.5× and 1.2× were used. Libraries were analysed on Aglient 2100 Bioanalyzer and pooled to obtain equal molarity. Samples were then sent to the

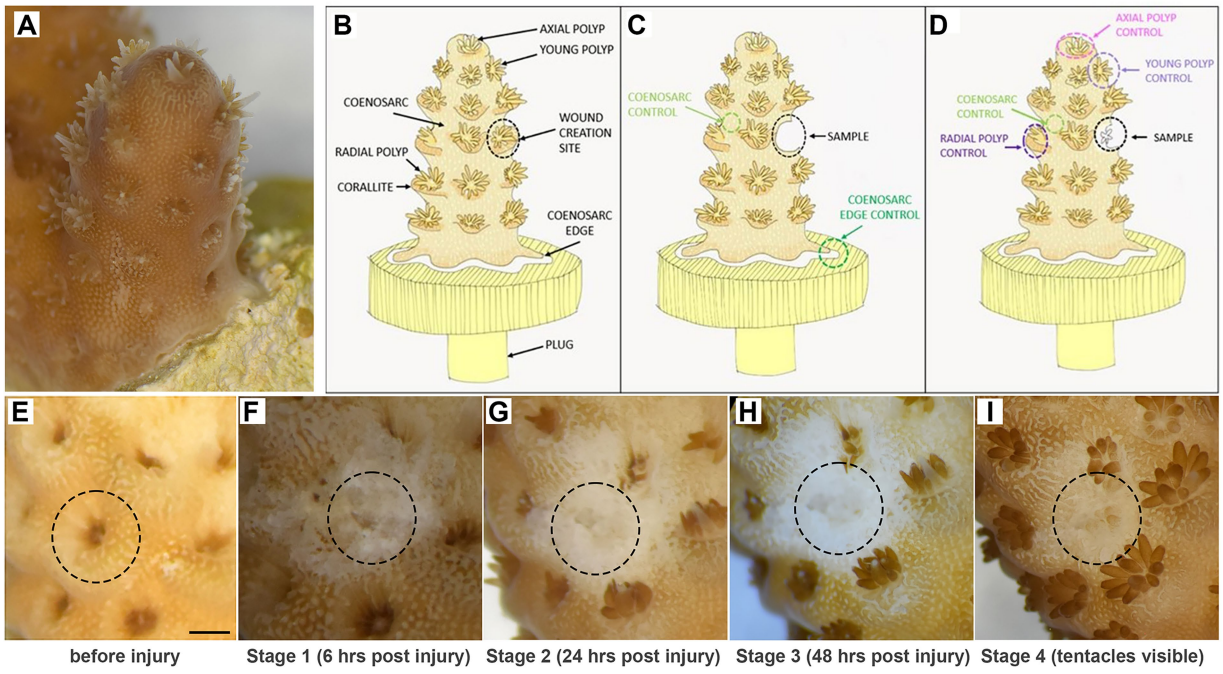


FIGURE 1
Experimental system. **(A,B)** Small branch of *Acropora millepora* glued to a plug, with features used for sampling indicated in the diagram. **(C,D)** Sampling strategy for regeneration stages before **(C)** and after **(D)** polyp formation. **(E,F)** Example images of a single polyp tracked before **(E)** and after **(F)** injury and until tentacles of the regenerated polyp became visible **(G–I)**. Scale bar represents 1mm.

Biomolecular Resource Facility (ANU) for single end 75 bp sequencing on high output flowcells using the NextSeq500 Illumina platform. Raw Illumina RNA-Seq reads generated in this study have been deposited in ENA Short Read Archive under study PRJEB55598 (samples ERS12852684–ERS12852781, runs ERR10123024–ERR10123121).

Differential gene expression analysis

Read mapping

RSEM v1.3.3 (Li and Dewey, 2011) with the Bowtie 2 v2.5.0 short-read aligner (Langmead and Salzberg, 2012) were used to map the RNA-Seq reads to the *A. millepora* protein-coding gene models (Ying et al., 2019, see Supplementary material 1 for the sequences of gene models used in this manuscript). The 'expected counts' from RSEM were used to perform the detection and analysis of the differentially expressed genes, focusing on protein coding genes.

Identification and visualisation of differentially expressed genes

The analysis was performed in R v4.2.2 with the edgeR v3.40.0 (Chen et al., 2016), and the Limma v3.54.0 (Ritchie et al., 2015) packages following the protocol from Law et al. (2016). The experimental incomplete block design was implemented with the type of the sample (the stages 1, 2, 3, 4 and Coenosarc) set as fixed effect directly in the model matrix, and the blocking factor (combined the replica and the colony) set as pairing-block with the duplicateCorrelation() function in Limma. The significance level for the detection of differentially expressed genes was set at 0.05 (the BH adjusted *p*-value was used), and the minimum $\log_2$ fold change was 1/−1 (details in the R code provided in the supplementary file amil_dge.R).

Transcript annotation

Translated *A. millepora* gene models were annotated with the gene name of their top blast-p hit among previously functionally annotated *A. digitifera* gene models (Shinzato et al., 2011). To increase specificity of the FGF and Wnt ligands annotation, the *A. millepora* sequences annotated as such were used to recover further *A. digitifera* and *N. vectensis* proteins based on their sequence similarity (with blast-p). The *H. sapiens* Wnt or FGF ligands recovered from UniProt were then added to the cnidarian sequences, and two sets of multiple sequence alignments were created in ClustalX 2.1 (Larkin et al., 2007). The alignments were then manually edited to remove divergent segments. Maximum Likelihood phylogenetic trees were computed in RAxML 8.2.11 using the PROTGAMMAAUTO model (allowing RAxML to choose the best available model; the LG model was chosen for both Wnt and FGF ligand alignments), and fast bootstrap of 100. The consensus tree annotated with the bootstrap values was displayed in Mega7 (Kumar et al., 2016).

Gene ontology enrichment

GO terms were linked to the gene models with InterProScan (Quevillon et al., 2005), and the enrichment analysis was performed with TopGO v2.50.0 (Alexa et al., 2006) using the 'weight01' algorithm with the Fisher statistic and applying the 0.05 *p*-value significance cut off (details in the R code provided in the supplementary file amil_topgo.R).

Clustering of gene expression profiles

Gene expression profiles were clustered with Clust 1.18.0. Clust was run on generated with edgeR normalized log-cpm values using default arguments except the tightness parameter-t set to 0.5 and with automatic choice for the normalisation method (z-score quantile normalisation was chosen, code 101 4; Abu-Jamous and Kelly, 2018).

Results

Staging of wound healing and polyp regeneration

Intact, healthy *Acropora millepora* fragments have brown colouration (due to presence of zooxanthellae) and clearly identifiable polyps: one large and symmetrical axial polyp at the tip of each branch, a few small (recently developed) radial polyps near the tip, and fully developed radial polyps uniformly distributed along the branches (Figures 1A,B). Polyp tentacles can either be extended or retracted, depending on time of the day or recent disturbance (Figures 1A–E). In fragments experimentally attached to plugs, the coenosarc forms a 'skirt' at the attachment area; the coenosarc edge grows to spread on the plug surface (Figure 1A,B). In long-term culture, polyps can also form on the coenosarc covering the plug, but never directly on the edge (not shown).

To choose potentially informative time points and appropriate controls for gene expression analysis during regeneration, we initially carried out low-magnification microscopic observations of experimentally wounded fragments. One radial polyp and its corallite, sited approximately 3–5 polyps down from the tip of the branch, were removed using a scalpel blade to create the lesion (Figures 1A,B). Immediately after wounding, and without any apparent changes within the first hours, the exposed calcium carbonate skeleton was clearly visible at the wounding site (Figure 1F). We informally referred to this stage as 'rough' reflecting its appearance and refer to this stage as Stage 1 of regeneration. By 24 h post-injury, the wound surface appeared smooth, with coenosarc apparently healed over the debris (Figure 1G); we refer to this as Stage 2. At Stage 3 (48 h), there was no obvious morphological change from Stage 2, with translucent (zooxanthellae-poor) tissue covering the wound (Figure 1H). The first morphological change – formation of tentacles of the regenerating polyp – could be observed between 3 and 10 days post-wounding (Figure 1I).

It is important to note that the initial translucent nature of regenerating coral tissue was problematic when determining the presence of tentacles, so that tentacles could only be identified when zooxanthellae became abundant enough in the tentacles to make them clearly visible. Therefore, the actual tentacle formation may have occurred earlier than noticed in some samples. While the first three regeneration stages are defined by time (6, 24, 48 h respectively), we only used polyps with visible tentacles to represent Stage 4 in our analysis. In some analyses we have additionally separated Stage 4 samples into an earlier stage, with only tentacles visible (4a) and a later one with completely regenerated polyps (4b).

Given potentially different cell types (and/or proportions of the same cell types) constituting the polyps and coenosarc, as well as the expected differences in gene expression between actively growing and non-growing tissues, we have taken different types of control samples to identify genes specifically changing expression during regeneration (see Supplementary Table 1 for details of the samples used). Therefore, for stages 1–3 (before regenerating polyp became visible), coenosarc samples from the areas between polyps (we call these samples 'coenosarc' for simplicity throughout the manuscript) and the actively growing coenosarc from the edge of the fragment ('coenosarc edge') were taken as controls (Figure 1C). For stage 4 (when regenerating polyp's tentacles were apparent) the following samples were taken as controls: an intact radial polyp from the same level as the regenerating polyp, a young polyp of similar size to the regenerating polyp, the axial polyp and coenosarc from between intact polyps (Figure 1D).

Gene expression changes during wound healing and polyp regeneration

We carried out wounding experiments on fragments derived from two colonies in a closed aquarium system in Canberra and two colonies in an open (flow-through) system on Orpheus Island (see section Material and Methods for detailed description of the two systems). Because of small sizes of the polyps and thus low tissue volumes surrounding the lesion, three tissue fragments from the same colony (and usually, the same branch) were pooled for each sample. Given that each of the colonies used was individually collected from the wild, we consider samples derived from different colonies to be biological replicates, in contrast to samples derived from different fragments of the same colony which we consider to be technical replicates. While we initially aimed to generate both technical and biological replicates for each regeneration stage and its matching controls, the need to pool samples resulted in a lower number of replicates. Overall, we generated at least two technical replicates for each sample type for colony one, and at least three biological replicates for each sample type (Supplementary Table 1 and Supplementary Figure 1).

As the first step to assess quality of the experiment we visualised the overall gene profiles as multi-dimensional scaling (MDS) plots. In the first and second dimension the samples clustered by colony of origin rather than sample type (Figure 2). In particular, the two Canberra samples formed two independent clusters, while the Orpheus colonies clustered together, perhaps reflecting closer genetic similarity (or even identity) of the Orpheus colonies. Intriguingly, regeneration stage 1 and coenosarc edge samples appeared to segregate from the remaining samples of colony 1 (for which we generated highest number of samples).

We have next generated an MDS plot for dimensions three and four to see whether in these dimensions sample-type expression signatures would drive clustering (Figure 3). Strikingly, regeneration Stage 1 and coenosarc edge samples appear to segregate from the remaining samples in dimension 4, indicating that these samples are distinct from the remaining ones, and perhaps similar to each other. The remaining samples are intermingled with each other, indicating lack of strong gene expression signatures distinguishing the sample types, although Stage 2 samples are all relatively close to Stage 1 and coenosarc edge samples.

To find out whether inter-colony differences are obscuring gene expression changes driven by response to wounding, we have also generated MDS plots for colony one and two separately, as well as Canberra and Orpheus Island colonies. In all of these plots, segregation or of Stage 1 and coenosarc edge samples from other sample types was apparent in the 1st and/or 2nd dimension separately (Supplementary Figure 1), and no further separation was detected in other dimensions (not shown). This result suggests that the earliest wound healing (Stage 1) and actively growing (coeanosarc edge) samples are the only sample types which are significantly distinct from other samples at the level of transcriptome.

Cluster analysis of gene expression profiles during regeneration

To gain insight into the molecular events during wound healing and polyp regeneration, we carried out cluster analysis of gene expression profiles, followed by Gene Ontology (GO) enrichment analysis. Aiming to generate a simple representation of a polyp regeneration time course, we excluded the coenosarc edge and axial polyp samples from this analysis. The time course starts with coenosarc samples, includes all the regeneration stages and culminates with the radial polyp samples. In line with the MDS visualisation which indicated that only the coenosarc edge and regeneration stage 1 samples are distinct from others, only two cluster profiles were identified, each showing dramatic gene expression change in the earliest stage of regeneration, and otherwise stable expression across all other samples (Figure 4). The first profile, C0, includes 2,818 genes which are strongly downregulated by 6 h post injury (regeneration stage 1) and return to the previous level of expression by 24–48 h post injury (regeneration stage 2–3). Gene Ontology (GO) terms enriched in

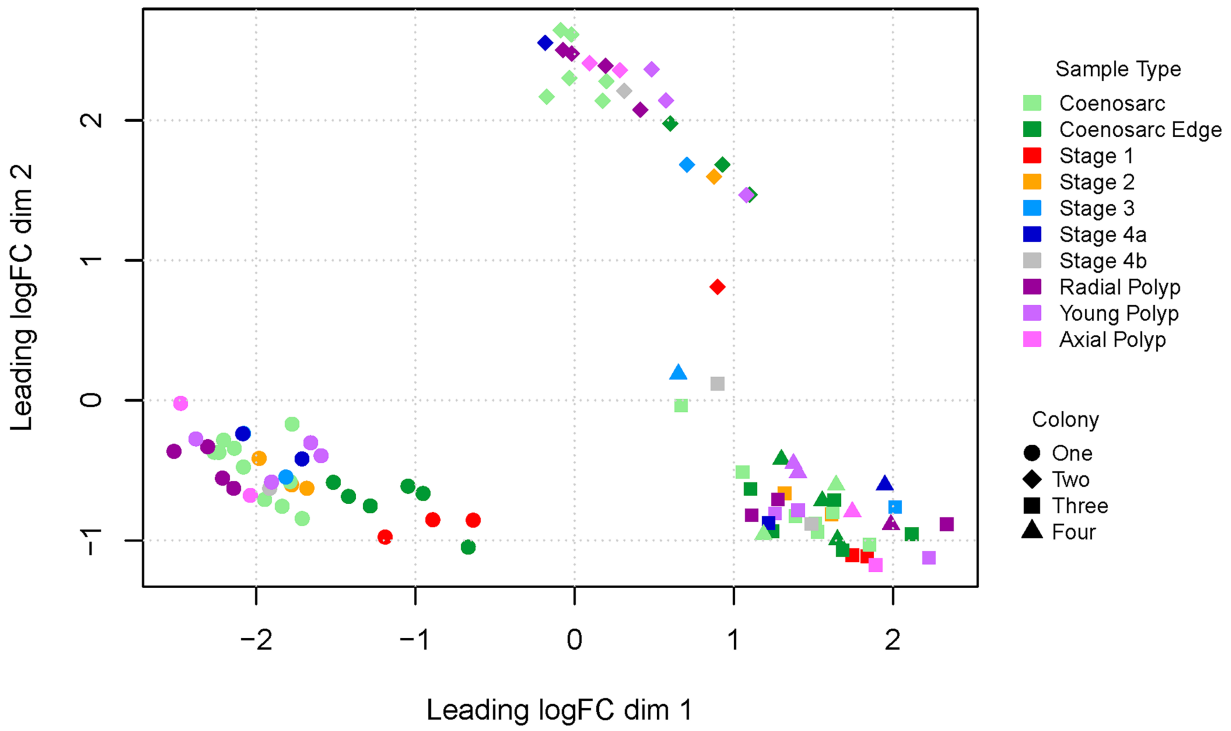


FIGURE 2
Multi-dimensional scaling plot (dimensions 1 and 2) of RNA-Seq samples representing regeneration of *A. millepora*. Colony one and two were housed in a closed system in Canberra, colony three and four were from open system in Orpheus Island.

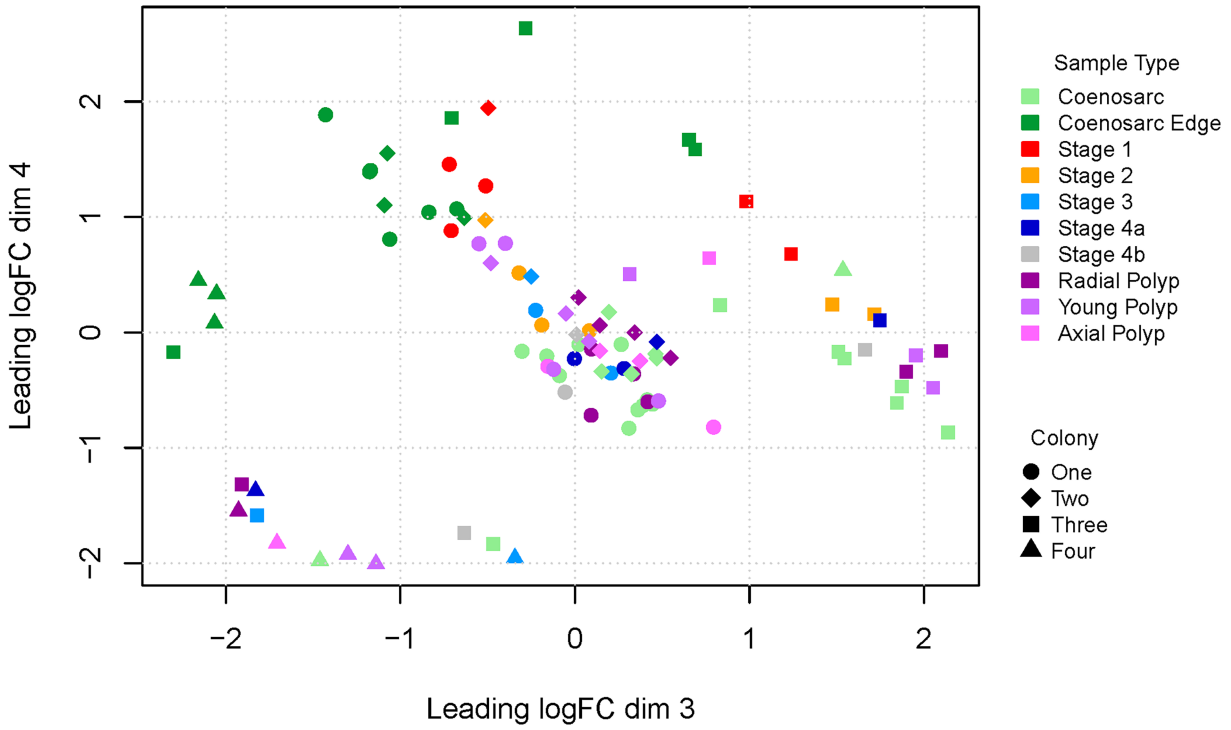


FIGURE 3
Multi-dimensional scaling plot (dimensions 3 and 4) of RNA-Seq samples representing regeneration of *A. millepora*. Colony one and two were housed in a closed system in Canberra, colony three and four were from open system in Orpheus Island. Note intermingled regeneration stage 1 and coenosarc edge samples segregating from other samples in dimension 4.

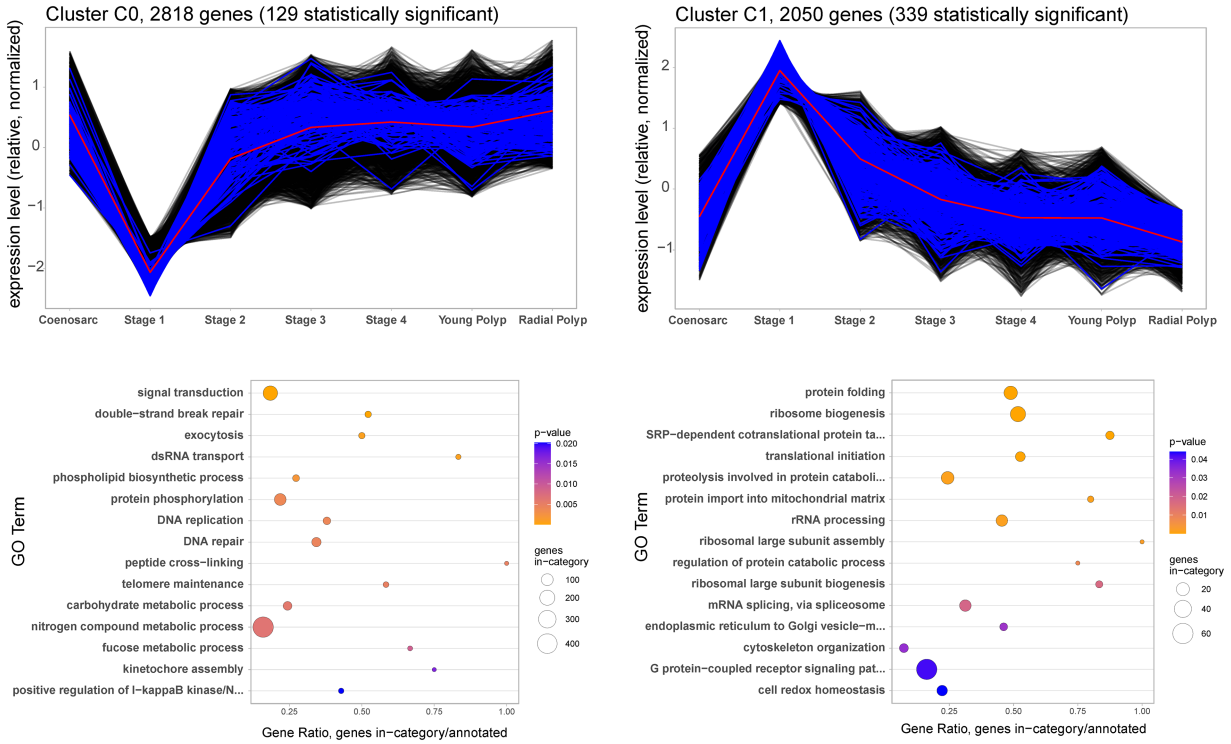


FIGURE 4
Gene expression clusters (top) and Biological Processes identified as Gene Ontology terms associated with them (bottom) during *A. millepora* wound healing and polyp regeneration. The black and blue lines show expression profiles of individual genes included in the cluster. The genes with expression statistically significantly different in Stage 1 (adj. value of $p \leq 0.05$) – lower for C0 and higher for C1 – are shown in blue, and those with not-significantly different expression in black. The red lines represent mean expression profile calculated from all the genes included in the cluster.

this cluster include signal transduction and exocytosis as well as terms connected with DNA repair and replication. These may be processes involved in the homeostasis/maintenance of cells which are downregulated during wound healing. The second cluster, C1, is composed of 2050 genes strongly upregulated 6 h after injury (stage 1) and returning to previous expression levels by 24–48 h post injury (stage 2–3). Enriched GO terms associated with this profile indicate increased ribosome biogenesis, splicing, translation and protein folding, as well as proteolysis and cytoskeleton reorganisation occurring soon after injury (Figure 4).

Differentially expressed genes

We next carried out direct pairwise gene expression level comparisons between regenerating samples and their matching controls (that is, Stage 1, Stage 2 and Stage 3 vs. coenosarc; Stage 4 vs. young and radial polyps) as well as between morphologically distinct parts of the colony (coenosarc edge and polyps vs. coenosarc, axial polyp vs. radial polyp). In line with both MDS plot visualisations and the cluster analysis described above, we have found hundreds of statistically significant differentially expressed genes between Stage 1 and coenosarc edge when compared to coenosarc, with less than 20 genes differentially expressed at each of the remaining stages of regeneration (Supplementary Table 2).

Given that regeneration Stage 1 and coenosarc edge samples appeared similar to each other in the MDS plot visualisation (Figure 3), we checked whether any of the identified differentially expressed genes are common between these samples. Indeed, 105 genes were found on both the Stage 1 vs. coenosarc (out of total 658) and coenosarc edge vs. coenosarc (out of total 404; Supplementary Table 2) gene lists. It is worth to note that as we only compare lists of genes with statistically significant difference in expression, it is possible that the non-overlapping genes share the same expression trends without reaching the significance threshold.

To gain insight into biological processes occurring during the earliest stage of regeneration, we carried Gene Ontology (GO) enrichments analysis. Genes upregulated during regeneration Stage 1 appear strongly associated with regulation of transcription, signal transduction and development in general. Moreover, the identified terms include three specific signalling systems, well known to be involved in wound healing and regeneration across the animal kingdom: the *Wnt* (e.g., Kawakami et al., 2006) and *FGF* signalling pathways (e.g., Maddaluno et al., 2017) and the JNK cascade (e.g., Rämet et al., 2002; Figure 5).

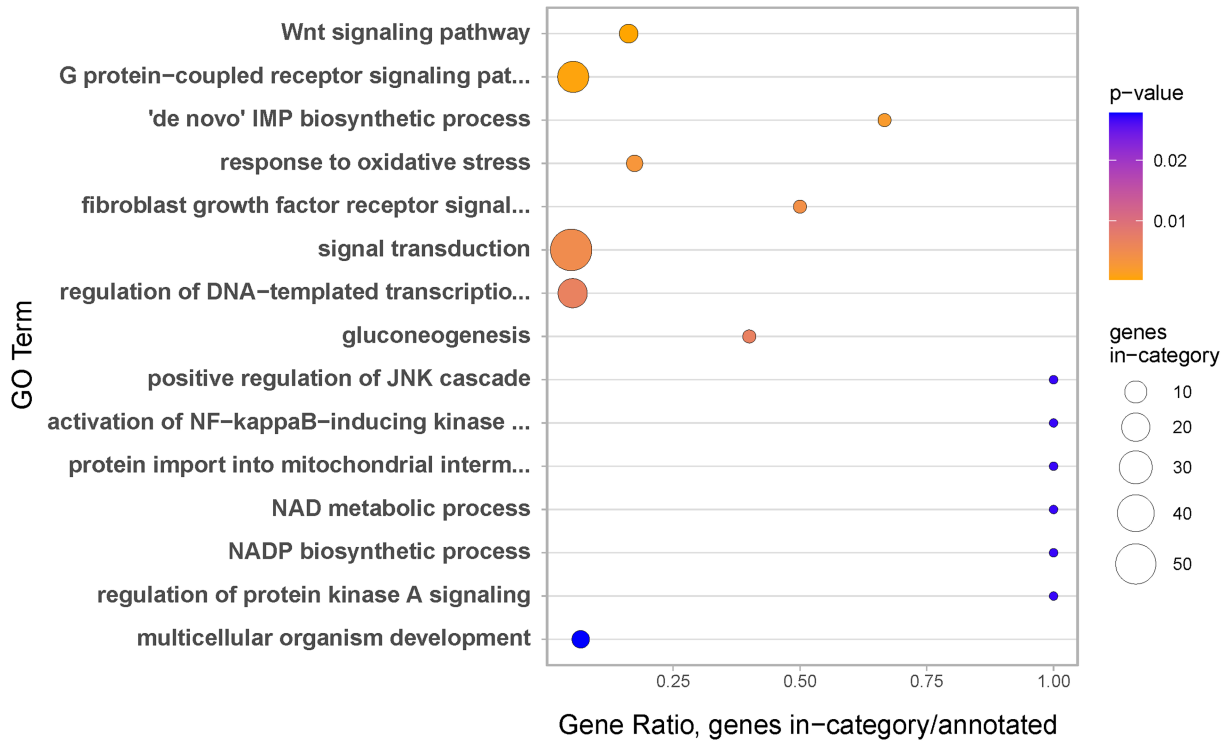


FIGURE 5
Biological Processes identified as gene ontology enriched terms in genes significantly upregulated at regeneration Stage 1 as compared to coenosarc.

We have next selected 39 Stage 1 upregulated genes which had meaningful annotations based on BLAST hit (including transcription factors, components of signalling pathways and genes implicated in coral skeleton formation, see Supplementary Table 2) to visualise their expression profiles across all samples.

In line with the enriched GO terms, these include four FGF ligands, one FGF antagonist (sprouty), one Wnt ligand and one Wnt pathway component (wntless), galaxin (component of the organic matrix of coral skeleton, e.g., Reyes-Bermudez et al., 2009), ADAMTS metalloproteases, belonging to a group of proteases found to be involved in ECM remodelling across phyla (e.g., Kuno and Matsushima, 1998) and previously shown to be involved in cnidarian regeneration (Schaffer et al., 2016; Stewart et al., 2017), and several transcription factors including c-Fos, known to be regulated by the JNK cascade and involved in injury response (e.g., Rämet et al., 2002). A heatmap representation of their expression demonstrates that in majority of cases the peak of expression coincides with the stage 1 of regeneration, although many are also upregulated in the coenosarc edge (Figure 6). Similar to the profiles identified by gene expression clustering analysis, while dramatically decreased as compared to Stage 1, expression of many of these genes is still elevated at Stage 2 or even 3.

As could be expected from the partly overlapping lists of genes differentially expressed in regeneration stage 1 and the coenosarc edge (both compared to coenosarc), GO enrichment analysis of the coenosarc edge regulated genes revealed both similar and strikingly contrasting terms. As in the case of regeneration, genes upregulated in the coenosarc edge were linked with regulation of transcription and cell signalling, likely reflecting the active growth at the edge (Figure 7). However, while for the coenosarc edge the top biological process indicated by GO enrichment analysis is calcium ion transmembrane transport (possibly linked with active formation of calcium-based skeleton of the coral), this term did not appear enriched during regeneration. This result is in line with the notion that transcriptional response to injury does not simply redeploy genes involved in growth.

The stringent analysis (limited to genes with statistically significant two-fold expression change) revealed multiple genes which are likely to be regulating the earliest stages of regeneration in scleractinian corals. However, we are aware that the small sample size and batch (colony) effects might be precluding detection of many other genes involved in the process if their expression or level of change are lower and/or more variable. We wondered whether including all samples in the analysis increases our power of detection of differentially expressed genes, or whether, conversely, the potential differences between the experimental conditions (and/or colony differences) are obscuring the expression changes in response to the injury. We have therefore generated and compare lists of DEGs obtained using only samples from the closed (colony 1 and 2) and open (colony 3 and 4)

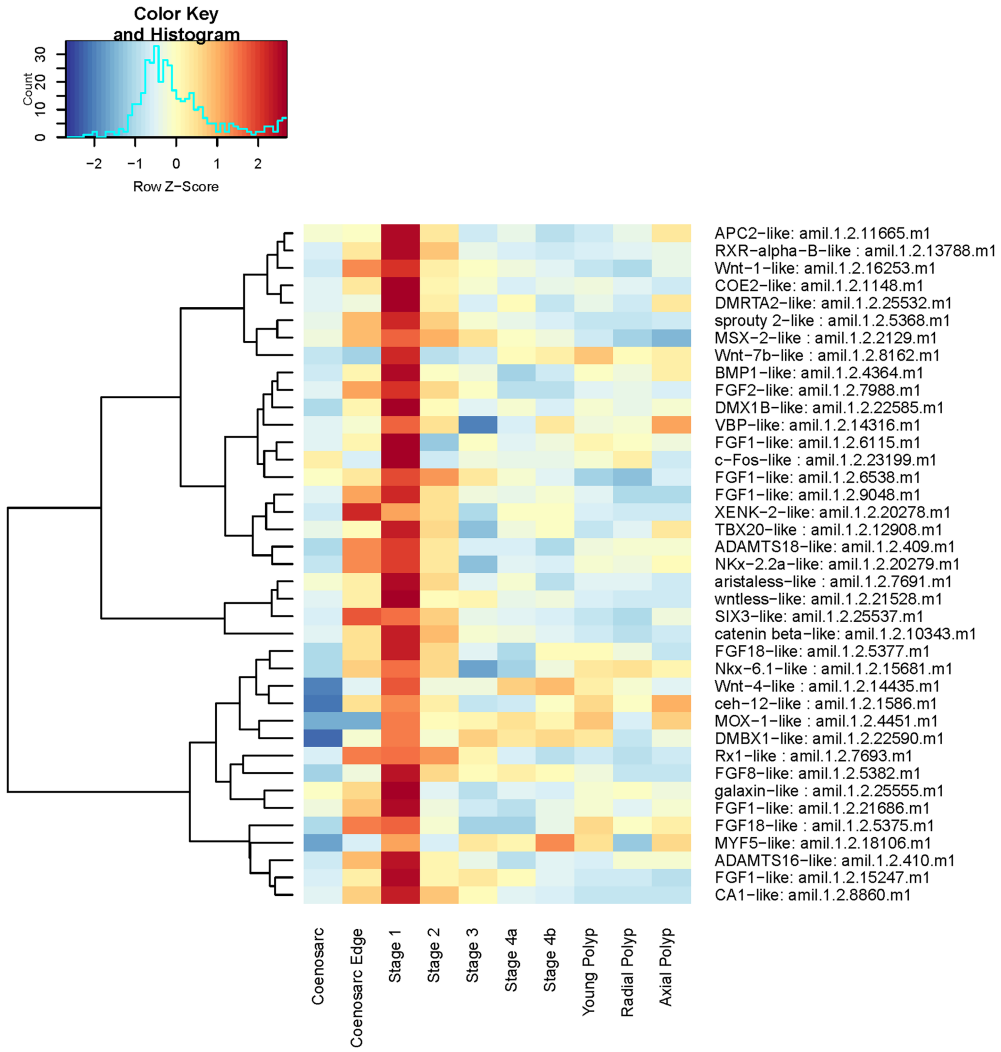


FIGURE 6
Expression heatmap of selected genes among those highly upregulated at regeneration Stage 1. Samples were pooled according to regeneration stage and colony part. Colour denotes an increase (red) or decrease (blue) in expression based on the z-score. The Histogram shows the distribution of the z-scores on the heatmap.

experimental systems (Supplementary Figure 2). The analyses using only subsets of data resulted in lower numbers of differentially expressed genes detected, especially when using only colonies 3 and 4 for which the lowest number of replicates was obtained. Importantly, over 68% of DEGs identified in the condition-limited analyses was also identified when using the entire dataset (Supplementary Figure 2), demonstrating that increasing number of replicates, even if these are biologically distinct and cultivated in different systems, increases the detection power of our analysis.

Notably, even the analyses limited to low numbers of replicates pointed to importance of FGF and Wnt pathways in the earliest step of *A. millepora* regeneration. Therefore, we decided to visualise expression of core components of these two signalling pathways across *A. millepora* regeneration timeline and in different parts of the colony. Based on *A. millepora* transcriptome annotation, we identified 16 ligands (Supplementary Figure 3), three receptors and two antagonists of the FGF pathway.

As can be seen on the expression heatmap, all 21 genes show dynamic expression across the regeneration and/or morphologically distinctive parts of the colony (Figure 8).

In line with the previous analyses, stage 1 of regeneration and coenosarc edge have strongest expression of FGF pathway components overall, with 16 of the components displaying peak of their expression in one (or both) of these two stages. Notably, expression of only one (FGFRb in Figure 8) of the identified three FGF receptors follows this trend, with two remaining ones conspicuously downregulated in early stages of regeneration and coenosarc edge (Figure 8). The peak of expression of both of the sprouty antagonists during early regeneration stages and in the coenosarc edge is consistent with peak of the FGF signalling activity in these stages, as in other experimental systems

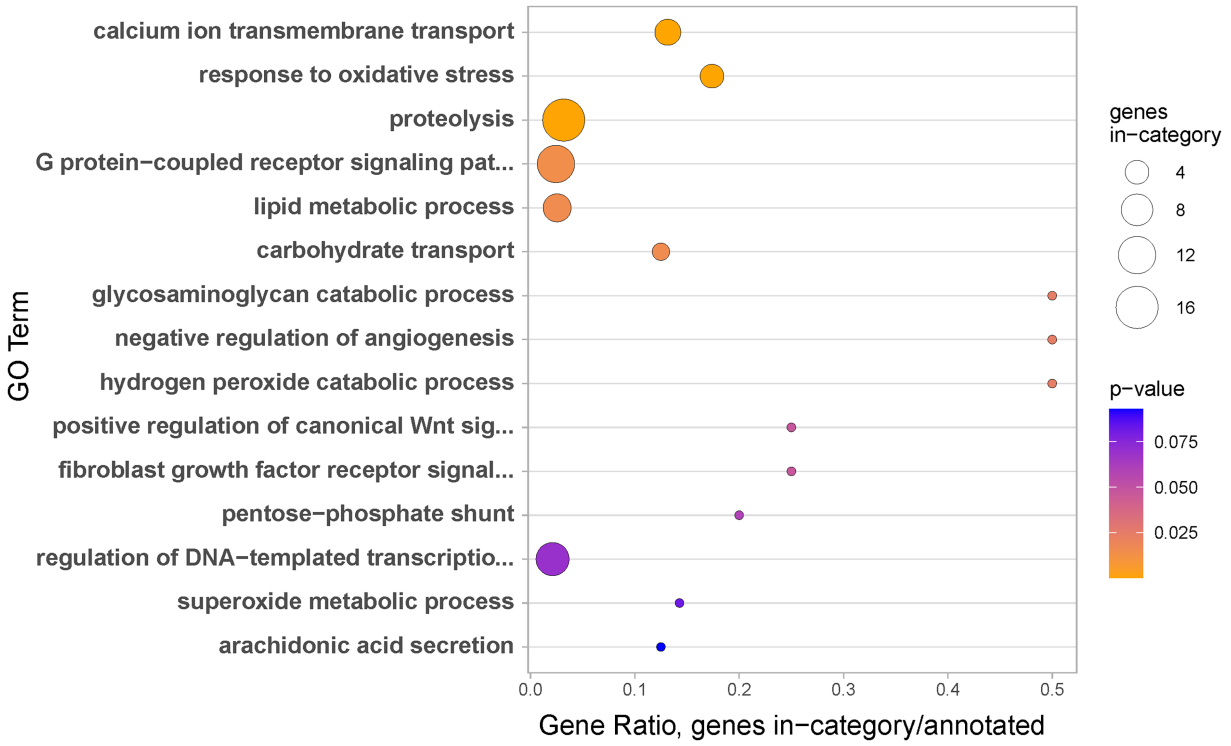


FIGURE 7
Biological processes identified as gene ontology enriched terms in significantly upregulated genes in coenosarc edge samples in comparison to coenosarc.

expression of sprouty has been documented to be positively regulated by the pathway activity (e.g., Minowada et al., 1999). Intriguingly, neither of the sprouties, but several of the FGF ligands and the two FGF receptors not upregulated during regeneration show differential expression between the three different polyp types we included in the analysis (young, radial and axial). This result must be interpreted with caution given our stringent analysis failed to identify significant expression differences between polyp types, and we have not seen these samples segregating in MDS plots.

Next, we visualised expression of all Wnt ligands (Supplementary Figure 4), along with Wntless (protein involved in Wnt secretion), beta-catenin and APC which we already found to be upregulated in regeneration stage 1 (Supplementary Table 2, Figure 6). In addition to the anticipated peak in stage 1 of regeneration (6 Wnt ligands, one of which was identified in the differential gene expression analysis), 4 Wnts have highest expression at Stage 3, the stage after wound healing is complete and just before polyp tentacles emerge, perhaps indicating a role in patterning (Figure 9). Interestingly, of the three Wnt transcripts upregulated at the coenosarc edge, two are also upregulated at the earliest stage of regeneration, but expression of Wnt10a-like appears downregulated. Given the critical role of Wntless in Wnt secretion (Bänziger et al., 2006), it is important to note that while its expression clearly peaks at the earliest stage of regeneration, it is also elevated at the coenosarc edge and throughout Stages 2 and 3 of regeneration, consistent with involvement of the Wnt pathway in growth, wound healing and regeneration (Figure 9).

We have next sought to identify genes involved in polyp morphogenesis and those responsible for morphological differences between radial and axial polyps. No genes were found as statistically significantly differentially expressed in the following comparisons: axial vs. radial polyps, axial polyp vs. coenosarc, stage 4 (when tentacles are first visible) vs. any polyps. Only 16 genes were found to be differentially expressed between stage 4 and coenosarc and 43 genes between young polyp and coenosarc (Supplementary Table 2). There is a strong overlap between these two lists, with nine genes upregulated in both types of samples, including one encoding neuropeptide RF-amide, which was also identified as the only gene with significantly different expression between the radial polyp and coenosarc (Supplementary Table 2). This is a notable finding, as RF-amide, as well as LW-amide upregulated in young polyps, are neuropeptides previously shown to be specifically expressed in the nervous system of *A. millepora*, concentrated around the oral region and in the tentacles (Attenborough et al., 2019). Genes encoding receptors for peptide hormones and neurotransmitters were also found to be upregulated in the young polyps, perhaps also associated with the nervous system, as were multiple transcription factors. As visible on heatmap representation of their expression (Figure 10), majority of these genes are also

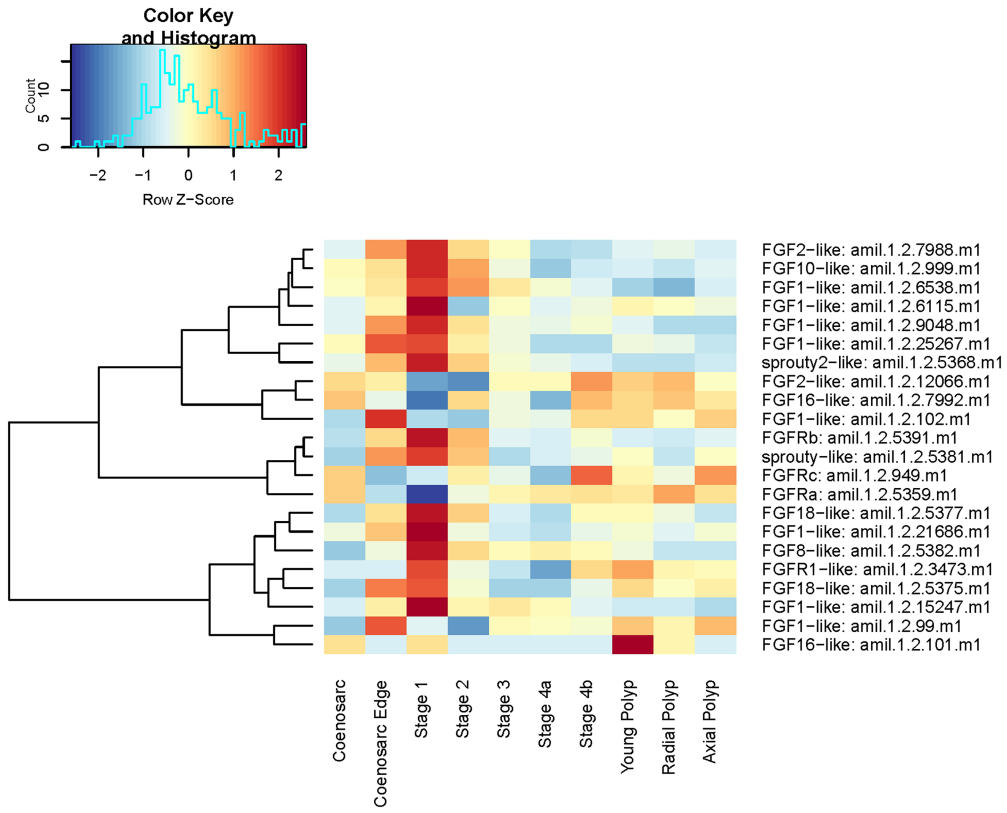


FIGURE 8
Expression heatmap of core components of the *A. millepora* FGF pathway. Samples are pooled according to regeneration stage and morphologically distinct colony part. Colour denotes an increase (red) or decrease (blue) in expression based on the z-score. The Histogram shows the distribution of the z-scores on the heatmap.

expressed higher than in coenosarc in regenerating and mature polyps, even though this upregulation is not statistically significant or high enough to be detected by our stringent analysis.

Discussion

Regeneration of scleractinian corals is important from ecological and environmental perspectives, especially given the increased intensity and frequency of reef framework damaging events. It is also fundamentally interesting from a developmental and regenerative biology perspectives, given the interlinked abilities of continuous growth and regeneration which are characteristic for this lineage. Here, by discovering genes involved in regeneration and comparing them to those involved in growth processes, we aimed to get insight into the molecular background of regeneration in *A. millepora*.

The first (and sometimes the only) step of regeneration is wound healing. In all animals capable of wound healing, it is a relatively fast process of tissue repairing/remodelling after injury, aimed at preventing infection and further loss of exposed tissues. In model system cnidarians, such as the starlet sea anemone *Nematostella vectensis*, wound healing and the subsequent regeneration have been shown to involve immune response, apoptosis and cell proliferation, as previously documented across diverse bilaterian species (e.g., DuBuc et al., 2014). For cnidarians with a calcium carbonate skeleton such as scleractinian corals, trauma-induced exposure of skeleton causes additional vulnerability to aragonite dissolving in the surrounding sea water (Frear and Johnston, 1929). Thus, to prevent loss of the skeleton as well as reduce the risk of infection, the damaged coenosarc must quickly extend over the entire wound surface. Importantly, one of key elements of normal coral growth is the extension of coenosarc around the edge of the substrate attachment zone. This growth is observed in young colonies derived from metamorphosed larvae, as well as fragments of established colonies. The fast growth rate of the coenosarc edge gives corals the ability to grow over substrates and extend their habitat (Forsman et al., 2015).

Here we asked whether wound healing in *A. millepora* utilises the same gene toolkit which is used during coenosarc edge growth, or whether an independent network of wound-healing specific genes is deployed upon injury. The issue of distinctiveness of regeneration vs. normal development/growth programmes

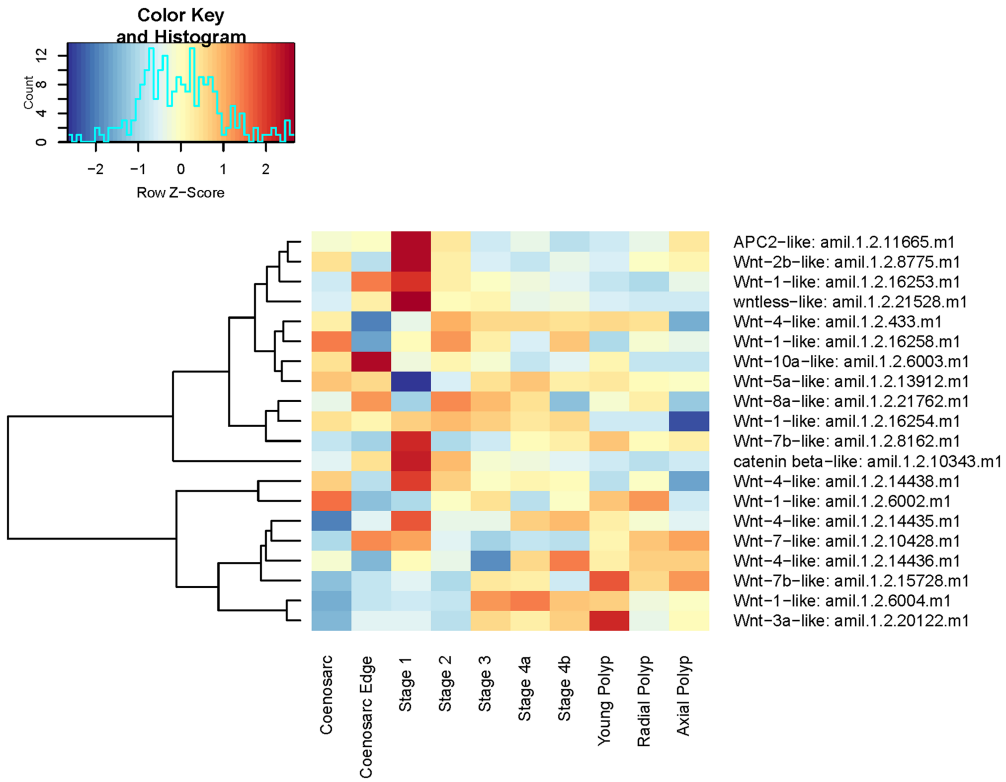


FIGURE 9
Expression heatmap of *A. millepora* Wnt genes and core components of the Wnt pathway. Samples are pooled according to regeneration stage and morphologically distinct colony part. Colour denotes an increase (red) or decrease (blue) in expression based on the z-score. The Histogram shows the distribution of the z-scores on the heatmap.

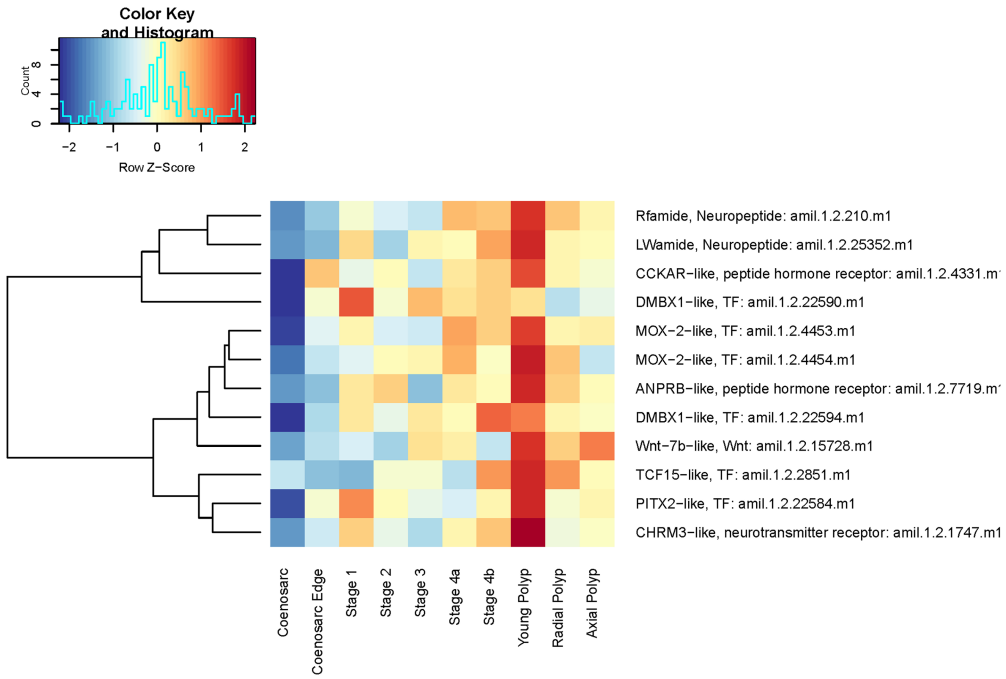


FIGURE 10
Expression heatmap of selected genes identified as upregulated in recently formed (young) polyps of *A. millepora*. Samples are pooled according to regeneration stage and morphologically distinct colony part. Colour denotes an increase (red) or decrease (blue) in expression based on the z-score. The Histogram shows the distribution of the z-scores on the heatmap.

continues to be a fascinating challenge for over a century (posed by Morgan, 1901; reviewed by Vervoort, 2011, recently addressed, among others, by Soubigou et al., 2020; Johnston et al., 2021; Sinigaglia et al., 2022).

As could be expected if a similar set of genes was used during both processes, the multi-dimensional scaling (MDS) plot showed that coenosarc edge (continues growth) and earliest wound healing stages are more similar to each other then they are to the remaining samples (Figure 3). Moreover, consistent with their previously described roles in other experimental systems, including cnidarians, (e.g., DuBuc et al., 2014; Stewart et al., 2017), components of both Wnt and FGF pathways are strongly upregulated at both the coenosarc edge and at regeneration stage 1 (Figures 8, 9, Supplementary Table 2).

We have hypothesised that because *A. millepora* specimens continuously grow in favourable conditions, redeployment of the 'growth genes' is a likely scenario, perhaps supplemented by additional genes involved in clearing and remodelling of the damaged tissues upon injury. Surprisingly, only 16% of genes we found to be regulated as response to injury were also found to be differentially expressed between the actively growing coenosarc edge vs. more 'static' (not expanding) coenosarc between mature radial polyps. Conversely, 26% of genes differentially expressed between the coenosarc edge and the between-polyps coenosarc were also detected as involved in the wound healing.

It is formally possible that these apparent differences are not due to real differences in gene expression, but our ability to identify differentially expressed genes with sufficient statistical significance. However, a candidate-centred approach, where we visualised expression of Wnt and FGF pathway components as well as particularly interesting genes identified as upregulated at the earliest stage of regeneration suggests otherwise (Figures 6, 8, 9). In fact, while some FGF ligands, transcription factors (e.g., NK2) and the metalloprotease ADAMTS18-like are upregulated in both the coenosarc edge and the earliest stages of regeneration, it is clear that regulation of other genes is independent in these two processes. Therefore, we conclude that while similarities in regulatory gene usage exists between growth and regeneration, these processes are clearly distinct.

One of the most striking examples of genes used uniquely during the earliest stages of regeneration is transcription factor c-Fos (Supplementary Table 2 and Figure 6). c-Fos has been implicated in injury and stress response in multiple model systems, including *N. vectensis* (Kovács, 2008; DuBuc et al., 2014). In addition to direct identification of c-Fos as specifically upregulated during the earliest stages of regeneration, we have also found JNK cascade as implicated in this process through Gene Ontology (GO) enrichment analysis (Figure 5). This is meaningful, as the JNK cascade is known to regulate c-Fos across bilaterians (e.g., Rämet et al., 2002). While involvement of c-Fos in cnidarian wound healing is not a new finding, we believe that our ability to discover this in an unbiased (not candidate-driven) analysis in a reef building coral demonstrates that *A. millepora* is a valid model for regenerative biology research even in land-locked laboratories such as ours. Moreover, results obtained in laboratory conditions could be combined with those from a more natural, flow-through marine station system, underscoring experimental reproducibility across different genetics and experimental conditions. We hope that *A. millepora* and other scleractinian corals will provide useful models to address further questions in developmental and regenerative biology, such as genetic (and/or epigenetic) mechanisms governing identity of colony units (e.g., axial vs. radial polyps), interactions between diverse members of the coral holobiont in the changing environmental conditions and roles of specific cell types in growth and regeneration.

Data availability statement

Raw Illumina RNA-Seq reads generated in this study have been deposited in ENA Short Read Archive under study PRJEB55598 (samples ERS12852684–ERS12852781, runs ERR10123024–ERR10123121).

Author contributions

MAdamska, JX, OM, MAdamski, and DM: project design. JX: laboratory experiments. JX and AM: field work. JX, MAdamska, MAdamski, OM, and CC: data analysis and interpretation. JX, MAdamska, MAdamski, CC, and OM: manuscript writing. All authors contributed to the article and approved the submitted version.

Funding

The work was supported by the ARC through Centre of Excellence for Coral Reef Studies CE140100020.

Acknowledgments

We appreciate support of S. Eggins, M. Ellwood, and O. Branson who shared with us their saltwater aquarium system at the Research School of Earth Sciences, ANU, as well as the excellent support of the technical staff of Orpheus Island Research Station. We thank members of J.X. Honours committee: E. Ball, A. Fahrer and G. van Dooren for stimulating discussions throughout the duration of the project and Hua Ying for sharing *A. millepora* gene models prior to publication. Sequencing of all samples have been carried at Biomolecular Research Facility, ANU, which is a service node of Bioplatforms Australia. The project has been funded by ARC through Centre of Excellence for Coral Reef Studies CE140100020.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.979278/full#supplementary-material

References

Abu-Jamous, B., and Kelly, S. (2018). Clust: automatic extraction of optimal co-expressed gene clusters from gene expression data. *Genome Biol.* 19:172. doi: 10.1186/s13059-018-1536-8

Alexa, A., Rahnenführer, J., and Lengauer, T. (2006). Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics 22*, 1600–1607. doi: 10.1093/bioinformatics/btl140

Amiel, A. R., Johnston, H. T., Nedoncelle, K., Warner, J. F., Ferreira, S., and Röttinger, E. (2015). Characterization of morphological and cellular events underlying oral regeneration in the sea anemone, nematostella vectensis. *Int. J. Mol. Sci.* 16, 28449–28471. doi: 10.3390/ijms161226100

Attenborough, R. M. F., Hayward, D. C., Wiedemann, U., Forêt, S., Miller, D. J., and Ball, E. E. (2019). Expression of the neuropeptides RFamide and LWamide during development of the coral *Acropora millepora* in relation to settlement and metamorphosis. *Dev. Biol.* 446, 56–67. doi: 10.1016/j.ydbio.2018.11.022

Bak, R. P. M. (1978). Lethal and sublethal effects of dredging on reef corals. *Mar. Pollut. Bull.* 9, 14–16. doi: 10.1016/0025-326X(78)90275-8

Bänziger, C., Soldini, D., Schütt, C., Zipperlen, P., Hausmann, G., and Basler, K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cells* 125, 509–522. doi: 10.1016/j.cell.2006.02.049

Bossert, P., and Thomsen, G. H. (2017). Inducing complete polyp regeneration from the Aboral Physa of the Starlet Sea anemone *Nematostella vectensis*. *J. Vis. Exp.* 2017:54626. doi: 10.3791/54626

Chen, Y., Lun, A. T. L., and Smyth, G. K. (2016). From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Res.* 5:1438. doi: 10.12688/f1000research.8987.2

Diaz-Pulido, G., McCook, L. J., Dove, S., Berkelmans, R., Roff, G., Kline, D. I., et al. (2009). Doom and boom on a resilient reef: climate change, algal overgrowth and coral recovery. *PLoS One* 4:e5239. doi: 10.1371/journal.pone.0005239

DuBuc, T. Q., Traylor-Knowles, N., and Martindale, M. Q. (2014). Initiating a regenerative response; cellular and molecular features of wound healing in the cnidarian *Nematostella vectensis*. *BMC Biol.* 12:24. doi: 10.1186/1741-7007-12-24

Forsman, Z. H., Page, C. A., Toonen, R. J., and Vaughan, D. (2015). Growing coral larger and faster: micro-colony-fusion as a strategy for accelerating coral cover. *PeerJ* 3:e1313. doi: 10.7717/peerj.1313

Frear, G. L., and Johnston, J. (1929). The solubility of calcium carbonate (calcite) in certain aqueous solutions at 25°. *J. Am. Chem. Soc.* 51, 2082–2093. doi: 10.1021/ja01382a014

Highsmith, R. C. (1982). Reproduction by fragmentation in corals. *Mar. Ecol. Prog. Ser.* 7, 207–226. doi: 10.3354/meps007207

Hobmayer, B., Rentzsch, F., Kuhn, K., Happel, C. M., von Laue, C. C., Snyder, P., et al. (2000). WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra. *Nature* 407, 186–189. doi: 10.1038/35025063

Holstein, T. W., Hobmayer, E., and Technau, U. (2003). Cnidarians: an evolutionarily conserved model system for regeneration? *Dev. Dyn.* 226, 257–267. doi: 10.1002/dvdy.10227

Hughes, T., Barnes, M., Bellwood, D., Cinner, J. E., Cumming, G. S., JBC, J., et al. (2017). Coral reefs in the Anthropocene. *Nature* 546, 82–90. doi: 10.1038/nature22901

Johnston, H., Warner, J. F., Amiel, A. R., Nedoncelle, K., Carvalho, J. E., and Röttinger, E. (2021). Whole body regeneration deploys a rewired embryonic gene regulatory network logic *bioRxiv* 658930. doi: 10.1101/658930. [PREPRINT]

Kawakami, Y., Esteban, C. R., Raya, M., Kawakami, H., Martí, M., Dubova, I., et al. (2006). Wnt/β-catenin signaling regulates vertebrate limb regeneration. *Genes Dev.* 20, 3232–3237. doi: 10.1101/gad.1475106

Kovács, K. J. (2008). Measurement of immediate-early gene activation-c-*fos* and beyond. *J. Neuroendocrinol.* 20, 665–672. doi: 10.1111/j.1365-2826.2008.01734.x

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Kuno, K., and Matsushima, K. (1998). ADAMTS-1 protein anchors at the extracellular matrix through the thrombospondin type I motifs and its spacing region. *J. Biol. Chem.* 273, 13912–13917. doi: 10.1074/jbc.273.22.13912

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Law, C. W., Alhamdoosh, M., Su, S., Dong, X., Tian, L., Smyth, G. K., et al. (2016). RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. *F1000 Fac. Rev.* 5:141. doi: 10.12688/f1000research.9005.1

Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323

Luz, B. L. P., Miller, D. J., and Kitahara, M. V. (2021). High regenerative capacity is a general feature within colonial dendrophylliid corals (Anthozoa, Scleractinia). *J. Exp. Zool. B. Mol. Dev. Evol.* 336, 281–292. doi: 10.1002/jez.b.23021

Maddaluno, L., Urwyler, C., and Werner, S. (2017). Fibroblast growth factors: key players in regeneration and tissue repair. *Development* 144, 4047–4060. doi: 10.1242/dev.152587

Minowada, G., Jarvis, L. A., Chi, C. L., Neubüser, A., Sun, X., Hacohen, N., et al. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* 126, 4465–4475. doi: 10.1242/dev.126.20.4465

Morgan, T. H. (1901). *Regeneration*. New York: Macmillan.

Passamaneck, Y. J., and Martindale, M. Q. (2012). Cell proliferation is necessary for the regeneration of oral structures in the anthozoan cnidarian *Nematostella vectensis*. *BMC Dev. Biol.* 12:34. doi: 10.1186/1471-213X-12-34

Petersen, H. O., Höger, S. K., Looso, M., Lengfeld, T., Kuhn, A., Warnken, A., et al. (2015). A comprehensive transcriptomic and proteomic analysis of hydra head regeneration. *Mol. Biol. Evol.* 32, 1928–1947. doi: 10.1093/molbev/msv079

Quevillon, E., Silventoinen, V., Pillai, S., Harte, N., Mulder, N., Apweiler, R., et al. (2005). InterProScan: protein domains identifier. *Nucleic Acids Res.* 33, W116–W120. doi: 10.1093/nar/gki442

Rämet, M., Lanot, R., Zachary, D., and Manfruelli, P. (2002). JNK signaling pathway is required for efficient wound healing in *Drosophila*. *Dev. Biol.* 241, 145–156. doi: 10.1006/dbio.2001.0502

Reinhardt, B., Broun, M., Blitz, I. L., and Bode, H. R. (2004). HyBMP5-8b, a BMP5-8 orthologue, acts during axial patterning and tentacle formation in hydra. *Dev. Biol.* 267, 43–59. doi: 10.1016/j.ydbio.2003.10.031

Reyes-Bermudez, A., Lin, Z., Hayward, D. C., Miller, D. J., and Ball, E. E. (2009). Differential expression of three galaxin-related genes during settlement and metamorphosis in the scleractinian coral *Acropora millepora*. *BMC Evol. Biol.* 9:178. doi: 10.1186/1471-2148-9-178

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., et al. (2015). Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43:e47. doi: 10.1093/nar/gkv007

Sabine, A. M., Smith, T. B., Williams, D. E., and Brandt, M. E. (2015). Environmental conditions influence tissue regeneration rates in scleractinian corals. *Mar. Pollut. Bull.* 95, 253–264. doi: 10.1016/j.marpolbul.2015.04.006

Saphier, A. D., and Hoffmann, T. C. (2005). Forecasting models to quantify three anthropogenic stresses on coral reefs from marine recreation: anchor damage, diver contact and copper emission from antifouling paint. *Mar. Pollut. Bull.* 51, 590–598. doi: 10.1016/j.marpolbul.2005.02.033

Schaffer, A. A., Bazarsky, M., Levy, K., Chalifa-Caspi, V., and Gat, U. (2016). A transcriptional time-course analysis of oral vs. aboral whole-body regeneration in the sea anemone *Nematostella vectensis*. *BMC Genomics* 17:718. doi: 10.1186/s12864-016-3027-1

Schmidt-Roach, S., Duarte, C. M., Hauser, C. A. E., and Aranda, M. (2020). Beyond reef restoration: next-generation techniques for coral gardening, landscaping, and outreach. *Front. Mar. Sci.* 7:672. doi: 10.3389/fmars.2020.00672

Shinzato, C., Shoguchi, E., Kawashima, T., Hamada, M., Hisata, K., Tanaka, M., et al. (2011). Using the *Acropora digitifera* genome to understand coral responses to environmental change. *Nature* 476, 320–323. doi: 10.1038/nature10249

Sinigaglia, C., Almazán, A., Lebel, M., Sémon, M., Gillet, B., Hughes, S., et al. (2022). Distinct gene expression dynamics in developing and regenerating crustacean limbs. *Proc. Natl. Acad. Sci. U. S. A.* 119:e2119297119. doi: 10.1073/pnas.2119297119

Soubigou, A., Ross, E. G., Touhami, Y., Chrismas, N., and Modepalli, V. (2020). Regeneration in the sponge *Sycon ciliatum* partly mimics postlarval development. *Development* 147:dev193714. doi: 10.1242/dev.193714

Stewart, Z. K., Pavasovic, A., Hock, D. H., and Prentis, P. J. (2017). Transcriptomic investigation of wound healing and regeneration in the cnidarian Calliactis polypus. *Sci. Rep.* 7:41458. doi: 10.1038/srep41458

Vervoort, M. (2011). Regeneration and development in animals. *Biol. Theory* 6, 25–35. doi: 10.1007/s13752-011-0005-3

Vogg, M. C., Galliot, B., and Tsiairis, C. D. (2019). Model systems for regeneration: Hydra. *Development* 146. doi: 10.1242/dev.177212

Warner, J. F., Guerlais, V., Amiel, A. R., Johnston, H., Nedoncelle, K., and Röttinger, E. (2018). NvERTx: a gene expression database to compare embryogenesis and regeneration in the sea anemone *Nematostella vectensis*. *Development* 145:dev162867. doi: 10.1242/dev.162867

Ying, H., Hayward, D. C., Cooke, I., Wang, W., Moya, A., Siemering, K. R., et al. (2019). The whole-genome sequence of the coral *Acropora millepora*. *Genome Biol. Evol.* 11, 1374–1379. doi: 10.1093/gbe/evz077

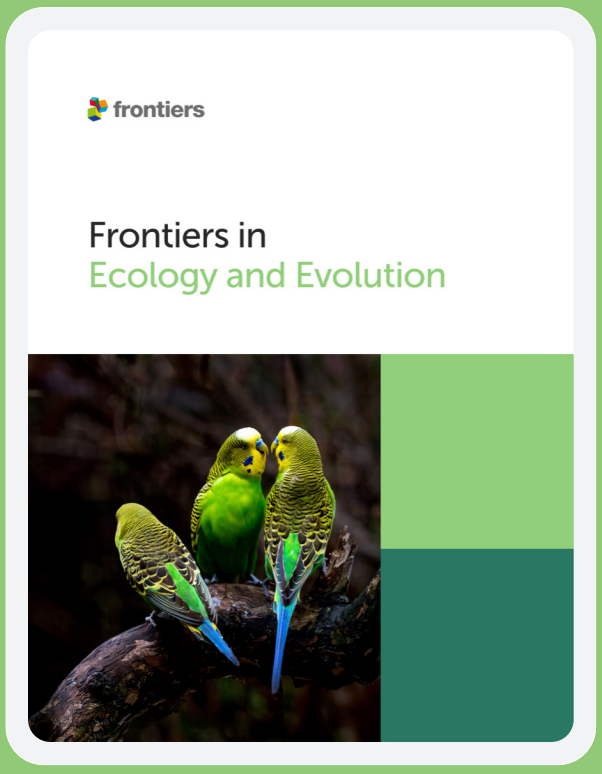
Frontiers in
Ecology and Evolution

Ecological and evolutionary research into our natural and anthropogenic world

This multidisciplinary journal covers the spectrum of ecological and evolutionary inquiry. It provides insights into our natural and anthropogenic world, and how it can best be managed.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact