CANCER STEM CELLS IN ENDOCRINE TUMORS

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-256-4
DOI 10.3389/978-2-88971-256-4

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CANCER STEM CELLS IN ENDOCRINE TUMORS

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Citation: Stassi, G., Di Franco, S., Luconi, M., Pellegata, N. S., eds. (2021). Cancer Stem Cells in Endocrine Tumors. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-256-4

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frontiers in Endocrinology

EDITORIAL
published: 28 June 2021
doi: 10.3389/fendo.2021.722790

Editorial: Stem Cells in Endocrine Tumors

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Keywords: endocrine tumor, cancer stem cells (CSCs), biomarker, EMT, tumor metabolism

Editorial on the Research Topic

Cancer Stem Cells in Endocrine Tumors

Endocrine tumors (ETs) include both benign and malignant diseases, originated from the boosted proliferation capacity of the hormone-producing glands of the endocrine system, and are characterized by a high degree of complexity and cellular heterogeneity. In this context, a crucial role in ET initiation and progression seems to be played by cancer cells endowed with stem cell properties, called cancer stem cells (CSCs), only recently identified in ETs, which presence is often associated with chemoresistance and metastatic potential. The aim of this Research Topic is to summarize all the findings regarding the role of CSCs in ETs, the peculiar properties of cancer versus healthy stem cells in different endocrine organs, highlighting the progress made in this research field, as well as the current contradictions. Recent findings have demonstrated that CSCs could arise from normal stem cells, upon acquisition of (epi)genetic hits, or by de-differentiation of progenitor/differentiated cells. For this reason, a better study of CSCs' healthy counterpart could lead to a better understanding of cancer cell biology and treatment.

In this context, Mariniello et al. study the role of healthy stem cells in the development and self-renewal of endocrine organs (pituitary, adrenal gland, thyroids, parathyroids, gonads, pancreas), as well as their functions in regenerative medicine, highlighting how alterations in the mechanisms acting in stem cells may lead to development of endocrine cancer.

An example of the biological mechanisms underlying transformation of normal to CSCs is reported in the review by Veschi, Verona, Lo Iacono et al. Indeed, despite several thyroid tumorigenesis models have been recently proposed, the thyroid CSC genetic mutation model has been reported to elucidate the molecular mechanisms underlying the distinct thyroid cancer (TC) histopathology and clinical behavior (1). In this review the authors examined the principal pathways and epigenetic alterations sustaining TCSC survival and the complex crosstalk between TCSCs and the tumor microenvironment cell populations, to define more effective therapeutic strategies, as immunotherapy and epigenetic drugs.

In line with the model describing CSCs as derived from healthy stem cells, the review article "Cancer Stem Cells and Neuroblastoma: Characteristics and Therapeutic Targeting Options"

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *09 June 2021*
Accepted: *15 June 2021*
Published: *28 June 2021*

Citation:
Di Franco S, Pellegata NS, Luconi M and Stassi G (2021) Editorial: Cancer Stem Cells in Endocrine Tumors. Front. Endocrinol. 12:722790. doi: 10.3389/fendo.2021.722790

Veschi et al. dissected what is already known about the origin and the isolation of CSCs in neuroblastoma (NB), the most common extracranial neoplasm in children. Targeting CSC subset may overcome NB recurrence and resistance to therapy. Of note, the authors discussed the mesenchymal (S-type) and adrenergic (N-type) phenotypes of NB-CSCs with tumor-initiating properties and their epigenetic traits, and explored the most recent therapeutic applications and strategies for CSCs targeting.

A tumor type that shares the same cell of origin as NB is pheochromocytoma, which originates from the chromaffin cells located in the adrenal medulla (pheochromocytoma-PCC) or in parasympathetic paraganglia (paraganglioma-PGL). In their review, Scriba et al. summarize where we stand with the identification of normal chromaffin stem cells (SCs), and of putative CSCs in PCC/PGL. Although the precise role of these cells, characterized by the expression of nestin, Oct4, Nanog, and Sox2, is still unclear, their involvement in the maintenance of stem cell niche, as well as in tumorigenesis is discussed, with particular focus on the development of targeted therapies for the treatment of metastatic tumors.

The pioneering work of Hugo Vankelecom set the stage for the identification of SCs in the pituitary gland, having roles in embryogenesis and in adult tissues, as well as in pituitary tumors (2, 3). Pituitary SCs and somatotroph cells express the proto-oncogene RET, which plays a role in cell survival. The study by Pradilla Dieste et al. systematically tackled the expression of the RET co-receptors GFRα 1,2,3, and 4 across the lobes and individual cell types of both rat and human pituitaries. Following extensive stainings, they could demonstrate that GFRα co-receptors are expressed in pituitary SCs at the niche in both species. The differential distribution of GFRαs is novel and provocative, indicating that some co-receptors' expression is retained in differentiated cells. This study suggests a potential involvement of GFRα co-receptors in maintaining stemness of the pituitary SC niche, and in directing proper lineage differentiation of adenopituitary cells, further supporting a role for RET/GFRα signaling in pituitary plasticity.

Although the isolation and characterization of cancer cells endowed with stemness properties has been achieved in several malignancies, the identification of tumor stem cells is not always child's play. In their review Mantovani et al. summarize the current knowledge about the tumor stem cells (TSCs) from pituitary tumors, a relatively recent research line, which is born following the identification of normal stem cells in the adult pituitary gland (4), with the first study describing a population of cells in pituitary tumors presenting CSC properties published by Xu et al. in 2009 (5). However, contrasting and not fully convincing data have been reported about the *in vivo* tumorigenic capacity of TSCs in mouse models, and the lack of reproducible data on long-term and serial *in vivo* growth. Another important feature of TSCs is the therapy resistance, as observed in many other tumor models. Even in this context, different studies have highlighted that pituitary TSCs are sensitive to drugs currently used in clinical setting, thus questioning role of these cells in driving the pharmacological resistance observed in pituitary tumor patients (6, 7). On the same page regarding the clinical implication of pituitary adenoma stem cells, and the controversial findings obtained in their isolation and *in vitro*/*in vivo* experimentation is the group of Würth et al., who analyzed in depth the experimental settings used to isolate and characterize the pituitary adenoma stem cell population, with a focus on their specific activated signaling pathways associated with the regulation of stemness and EMT (*i.e.* Notch, Shh, Wnt and Hippo). These studies emphasize that, although consistent data are available regarding the existence of pituitary adenoma stem cells, there is still an urgent need to address some unsolved questions with future studies, to design effective therapeutic protocols for the treatment of chemoresistant/invasive pituitary tumors. Cancer stemness has been often associated with the acquisition of mesenchymal traits, driven by a process known as epithelial-to-mesenchymal transition (EMT) that sustains cancer cell progression, which has been observed in few endocrine cancers including pancreatic, prostate, thyroid, and pituitary cancers (8). In their review, Bhattacharya and Scimè analyze in detail the main metabolic processes involved in EMT, such as the shift in glucose utilization towards aerobic glycolysis and the changes in lipid metabolism, typical stem cell-like feature that characterize these transitions in pancreatic, prostate, thyroid, and pituitary cancers. Interestingly, acquisition of stem-like properties is not restricted to cancer cell only, but this process has also been described for fibroblast (cancer-associated fibroblasts) and adipose cells (cancer-associated adipose cells) in the context of tumor microenvironment (9). These findings highlight the metabolic plasticity in endocrine tumors of the cancer cells and of the tumor microenvironment components which can reciprocally induce re-acquisition of stem-like properties supporting cancer progression.

All these findings highlight the controversial origin and the key role of CSCs in different steps of ETs' progression, thus underlining the clinical importance of their study for the development of innovative target therapies, to improve the quality of life of ETs' patients. Nonetheless, further studies are urgently needed to solve the existing controversial findings, and to develop novel and reliable *in vitro*/*in vivo* models to increase the scientific soundness and reproducibility of the obtained results.

AUTHOR CONTRIBUTIONS

SF, NP, ML, and GS participated in the design of the manuscript, analyzed the bibliographic data, collected them, and drafted the manuscript. ML and GS critically revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funding from German Research Foundation (DFG) project # 391523415-SFB824 (B8) and project # 314061271-TRR 205 (B11), German Cancer Aid (# 70112383), and Wilhelm Sander Stiftung foundation (# 2017.012.1) to NP; AIRC-CRF Multi-user Equipment Program ID 19515, and AIRC Investigator Grant IG2015 ID17691 to ML; AIRC IG (21445), and PRIN 830 2017WNKSLR to GS.

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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 Endocrinology

MINI REVIEW
published: 25 October 2019
doi: 10.3389/fendo.2019.00745

Stem Cells in Pituitary Tumors: Experimental Evidence Supporting Their Existence and Their Role in Tumor Clinical Behavior

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Although generally benign, pituitary tumors frequently show local invasiveness and resistance to pharmacological therapy. After the demonstration of the existence of pituitary gland stem cells, over the past decade, the presence of a stem cell subpopulation in pituitary tumors has been investigated, analogous to the cancer stem cell model developed for malignant tumors. This review recapitulates the experimental evidence supporting the existence of a population of stem-like cells in pituitary tumors, focusing on their potential role in tumor initiation, progression, recurrence and resistance to pharmacological therapy.

Keywords: pituitary tumors, stem cells, tumourigenesis, resistance, invasiveness, recurrence

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *30 July 2019*
Accepted: *14 October 2019*
Published: *25 October 2019*

Citation:
Mantovani G, Giardino E, Treppiedi D, Catalano R, Mangili F, Spada A, Arosio M and Peverelli E (2019) Stem Cells in Pituitary Tumors: Experimental Evidence Supporting Their Existence and Their Role in Tumor Clinical Behavior. Front. Endocrinol. 10:745. doi: 10.3389/fendo.2019.00745

INTRODUCTION

Pituitary tumors represent 10–25% of intracranial neoplasms. They can be classified based on their secretory activity into non-functioning pituitary tumors (NFPTs) or hormone-secreting tumors, including prolactin (PRL)-, growth hormone (GH)-, and adenocorticotropic hormone (ACTH)-secreting tumors. Pituitary tumors may cause visual field deficits and neurologic manifestations from mass effects and/or endocrine syndromes with specific signs and symptoms.

The pathogenetic mechanisms of pituitary tumourigenesis may be genetic or epigenetic and include cell cycle dysregulation, activation of oncoproteins, alterations in growth factors signaling, changes in the intrapituitary microenvironment, and germline or somatic mutations [reviewed in (1)]. In rare cases, germline pathogenetic mutations predispose individuals to pituitary tumourigenesis, often in the setting of familial genetic syndromes, whereas in the majority of sporadic cases, the exact molecular pathogenetic mechanism remains unknown.

Neurosurgery by the transsphenoidal approach is the treatment of choice in the majority of cases, except for PRL-secreting tumors, for which dopamine receptor type 2 (DRD2) agonists (DAs) represent the first-line therapy. Pharmacological treatment of GH- and ACTH-secreting tumors is based on the use of somatostatin (SS) analogs (SSAs). However, a subset of patients (approximately 10% of PRL-, 30% of GH-, and 50–70% of ACTH-secreting tumors) is resistant to these drugs (2–5).

Although classified as benign, pituitary tumors are frequently highly invasive into surrounding tissues (6) and can exhibit clinically aggressive behavior. Invasive tumors are associated with incomplete tumor resection and a high rate of recurrence (7, 8). Pituitary carcinomas are extremely rare, accounting for only 0.1–0.2% of pituitary tumors, but the prognosis is very poor, and 66% of patients die within the first year (9).

The discovery of a population of stem-like cells in pituitary tumors has raised the question about the role these cells in tumourigenesis, growth, invasiveness, recurrence and resistance to pharmacological treatment, similar to the cancer stem cell (CSC) model theorized for malignant tumors.

CANCER STEM CELLS: CHARACTERISTICS, MARKERS, AND THERAPIES

The cancer stem cell model states that tumor initiation, progression, growth and recurrence are promoted by a subpopulation of tumor stem cells (10). CSCs were first identified and studied in human leukemia (11, 12) and then in different types of solid tumors, including breast (13), brain (14) and liver (15) cancers. Although most studies have demonstrated that CSC are relatively rare in a number of tumor types, recent works show that tumorigenic potential is a common attribute of melanoma cells (16, 17).

CSCs have indefinite self-renewal capacity, an ability shared with normal tissue stem cells. However, unlike normal stem cells, CSCs also have tumor-generating potential (10). Moreover, CSCs have multilineage differentiation potential and are able to give rise to progenitors that can differentiate into all cell types that compose the bulk of the tumor mass. The multipotency of CSCs provides an explanation for intra-tumor heterogeneity (17). However, committed cells and even completely differentiated cells retain the ability to dedifferentiate, suggesting a high level of plasticity instead of a hierarchical one-direction differentiation (17). In this scenario, a CSC is considered a cell state that can switch from stem cell to differentiated cell, and vice-versa.

Based on the CSC model, while CSCs trigger tumor recurrence and metastatic spreading, the non-CSC population of the tumor does not contribute to the long-term growth of the tumor, as it can undergo only transient proliferation. In addition, CSCs are resistant to chemotherapy and radiotherapy (18) due to their specific features, e.g., slower rate of proliferation or quiescence state, small size, high expression of drug efflux pumps, alterations in apoptosis pathways and expression of telomerase.

The origin of CSCs is still uncertain. It has been proposed that they derive from the oncogenic transformation of normal tissue-specific stem cells (19, 20), progenitors or differentiated cells after reprogramming and dedifferentiation (21).

Signaling pathways that regulate the balance between self-renewal and differentiation of both normal and cancer stem cells are classically associated with oncogenesis, such as Notch, Sonic hedgehog, octamer-binding transcription factor 4 (OCT4) and Wnt. For example, overactivation of the Wnt/β-catenin pathway leads to the transformation of intestinal crypt stem cells into CSCs (19).

Both normal stem cells and CSCs reside in specific microenvironments called niches that are mainly composed of fibroblasts and endothelial, mesenchymal and immune cells, are crucial for the regulation of their self-renewal, activation and differentiation (22) and contribute to drug resistance of the tumor (23).

CSC surface markers have not been clearly established, mainly because of significant inter- and intra-tumor heterogeneity. CSCs isolated from different cancers share markers typical of physiological stem cells, such as CD24, CD44, CD90, CD123, CD133, OCT4, SOX2, Nanog, Nestin, c-kit, ABCG2, and ALDH1 (17, 24). In many cases CSCs can only be identified by a combination of different markers, and most of the CSCs markers are also expressed by normal stem cells (17, 25).

CSCs have the ability to form multicellular three-dimensional floating spheres *in vitro* when grown in non-adherent serum-free conditions (26, 27). This *in vitro* assay allows the growth and propagation of CSCs, and their subsequent molecular characterization.

The gold standard assay for identifying CSCs is serial tumor transplantation at limited dilutions in immunodeficient mice, which can assess both self-renewal and multipotency of a putative CSC subpopulation (26). Caveats reside in the choice of suitable CSC surface markers for the isolation of CSCs for transplantation and technical issues such as the dissociation of the tumor mass, which inevitably results in the disruption of cell-to-cell contacts, attachment to the extracellular matrix, and signals from the microenvironment, with possible consequences on tumor-initiating potential.

Recently developed experiments of genetic lineage tracing and cell ablation have overcome some of these limitations and confirmed *in situ* that many solid tumors contain stem cells in dedicated niches (20, 28–30).

According to the CSC model, complete tumor eradication requires a combination of conventional treatment directed toward bulk tumor cells with CSC-targeted drugs to prevent recurrence, resistance and metastasis, all of which are sustained by the CSC population. The first idea of anti-CSC therapy was based on early observation that leukaemic cells were blocked in an undifferentiated state. Drugs able to induce terminal differentiation of CSCs were thus proposed (differentiation therapy), with successful applications in patients with leukemia (31). However, limited evidence of differentiation therapy efficacy is available in solid tumors [revised in (32)]. A recent study in osteosarcoma stem cells demonstrated that the ROCK inhibitor fasudil significantly suppressed cell growth *in vitro* and tumourigenicity *in vivo* by inducing cell differentiation (33). In cultured CSCs of non-small cell lung cancer, an inhibitor of GSK3β exhibited a strong antiproliferative effect (34). In glioblastoma and neuroblastoma the inhibition of AKT/mTOR pathway selectively targeted the CSC population (35).

Another strategy is based on antibodies targeting CSCs, but the main limitation of this approach resides in the identification of reliable CSC-associated antigens and in possible damage to normal stem cells. The use of markers differentially expressed on normal stem cells and CSCs has allowed the specific targeting of leukemia stem cells in acute myeloid leukemia (36). An attractive alternative to directly targeting CSCs is represented by targeting their niche, e.g., by blocking stem cell niche signals (10).

TABLE 1 | Published studies describing isolation of TSCs from human pituitary tumors.

Study	Ability to grow as cell spheres	Expression of stem cells markers	Expression of pituitary specific markers	Ability to self-renew	Differentiation in pituitary hormone-producing cells	Resistance to chemotherapeutics	Resistance to DAs/SSAs	High efflux capacity	*In vivo* tumourigenicity	
									Mouse	Zebrafish
Xu et al. (38)	Yes	Yes	Yes	Yes	Yes	Yes	ND	ND	Yes	ND
Chen et al. (39)	Yes	Yes	ND	Yes	ND	ND	ND	ND	Yes	ND
Mertens et al. (40)	Yes	Yes	ND	Yes	Yes	ND	ND	Yes	NO	ND
Manoranjan et al. (41)	Yes	Yes	ND	Yes	ND	ND	ND	ND	Yes	ND
Würth et al. (42)	Yes	Yes	ND	Yes	Yes	ND	NO	ND	NO	Yes
Peverelli et al. (43)	Yes	Yes	Yes	Yes	ND	ND	NO	ND	ND	Yes

The assessment of the main features typical of CSCs is reported. ND, Not Determined.

TABLE 2 | Stem cell markers expressed in TSCs spheres isolated from human pituitary tumors.

	Xu et al. (38)	Chen et al. (39)	Mertens et al. (40)	Manoranjan et al. (41)	Würth et al. (42)	Peverelli et al. (43)
CD133	X	X			X	
CD15				X		
CD90	X					X
CXCR4					X	
DLL1	X					
JAG2	X					
KLF4						X
MSI	X					
NANOG					X	
Nestin	X	X	X		X	X
NOTCH1					X	
NOTCH4	X					
OCT4	X				X	X
SOX2			X		X	X

TUMOR STEM CELLS (TSCs) IN PITUITARY TUMORS

The CSC theory was initially developed for malignant tumors in which CSCs were originally isolated and characterized. However, the identification of normal stem cells in the adult pituitary gland [revised in (37)] has prompted the investigation of the presence of a CSC subpopulation in benign pituitary tumors. In the last decade, experimental evidence has accumulated demonstrating that it is possible to isolate cells from human pituitary tumors that fulfill some or all the features typical of CSCs, namely, clonogenic ability *in vitro*, expression of stem cell markers, ability to grow as rounded cell spheres, multipotency, resistance to chemotherapeutic agents, high efflux capacity, and propagation of tumor tissue following xenotransplantation (**Tables 1**, **2**). Since pituitary tumors are mostly benign, these cells are more correctly referred to as 'tumor stem cells' (TSCs) (44).

Isolation of TSCs From Pituitary Tumors

The first study describing a population of cells in pituitary tumors presenting CSC properties was published in 2009 (38). These cells, isolated from one NFPT and one GH-secreting tumor, were able to form floating spheres when grown in stem cell-permissive medium (DMEM/F12, B27 supplement, bFGF, and EGF) (**Figure 1**), as described for normal pituitary stem cells (45); expressed stem cell markers (OCT4, CD133 NOTCH4 and Nestin) (**Table 2**) and pituitary-specific markers (PIT1 and <GSU); and could be subcloned in culture (self-renewal assays) and differentiated into pituitary hormone-producing cells. In addition, these TSCs displayed resistance to chemotherapeutics (carboplatin and etoposide), that might be due to the upregulation of anti-apoptotic genes (38) or to an upregulation of multidrug transporters ABCB1 and ABCG2 (40).

These TSCs were able, when transplanted into the forebrains of immunodeficient NOD/SCID mice, to give rise to tumors that recapitulated the phenotypes of human primary tumors, although convincing evidence was not provided. This study presents some inconsistencies regarding hormone production by TSCs derived from NFPT that can produce LH and by TSCs derived from GH-secreting tumors that can secrete PRL and TSH after stimulation. These observations suggest that spheres may include differentiated, hormone-producing cells derived

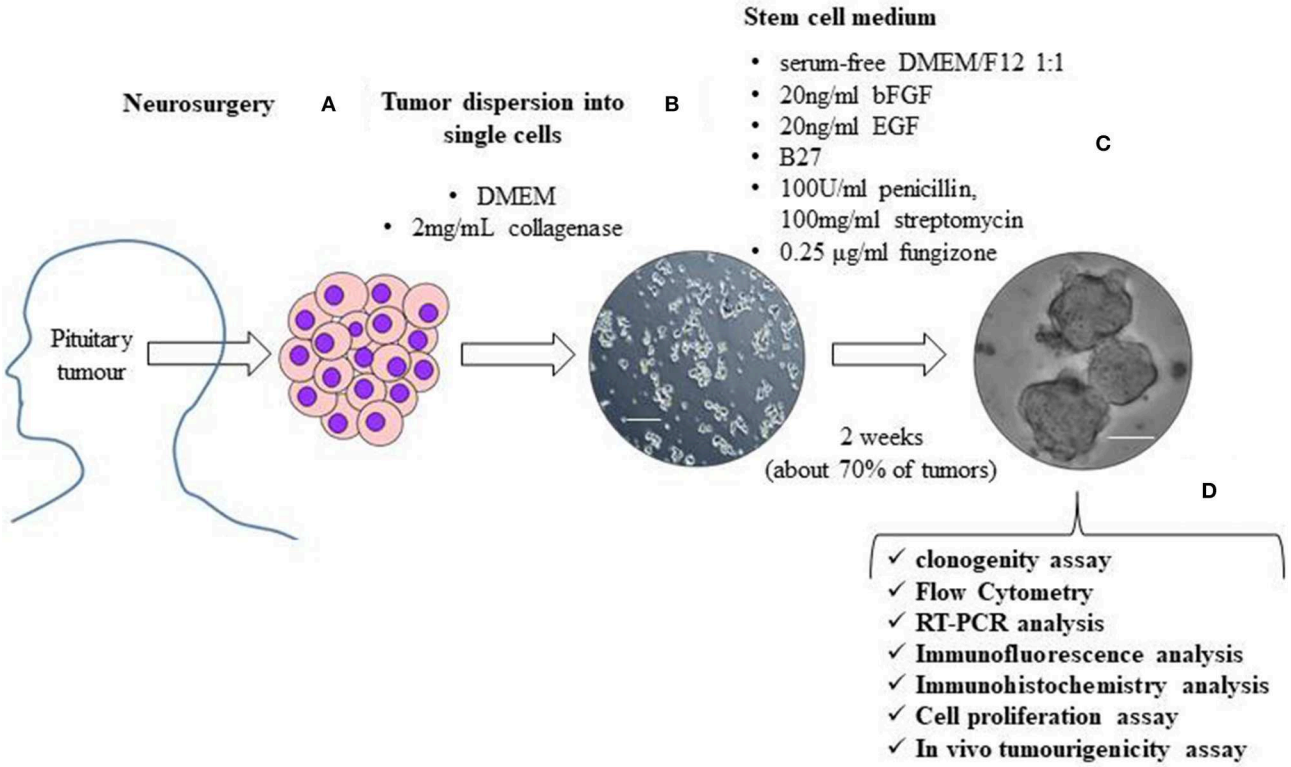

FIGURE 1 | The figure shows a schematic representation of the isolation and characterization of TSCs from surgically removed pituitary tumors. Human pituitary tumors tissues, obtained by the transsphenoidal route, are mechanically and enzymatically (with 2 mg/mL collagenase in DMEM) dissociated **(A)**. To obtain TSCs spheres, tumor cells are resuspended in stem cell permissive medium (serum-free DMEM/F12 1:1 medium) in the presence of 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), B27, 100 units/ml penicillin, 100 mg/ml streptomycin and 0.25 µg/ml fungizone **(B)**. About 2 weeks after cell seeding in stem cell permissive medium, non-adherent round cell spheres appear in a subset (about 70%) of cultured tumor cells **(C)**. Spheres are then phenotypically characterized for self-renewal ability, stem-cell markers expression, biological behavior and *in vivo* tumourigenicity **(D)**. Scale bar 50µm.

from the differentiation of stem cells, even if cultured in stem cell-permissive medium.

The hypothesis of the existence of pituitary TSCs was further supported 5 years later by a study by Chen et al. (39) that isolated pituitary tumor cells that were grown as floating spheres *in vitro*, expressed the neural stem/progenitor cell markers CD133 and Nestin, and were able to generate daughter cells that could differentiate into three neural lineages (**Tables 1**, **2**). These cells generated slow-growing, synaptophysin-positive tumors after subcutaneous xenotransplantation into SCID mice. The limitations of this study reside in the choice of markers, whose specificity is controversial, and in the lack of demonstration of production of pituitary hormones.

One year later, Mertens and colleagues found a side population (SP) composed of cells with high efflux capacity (40) in pituitary tumors, as previously described for normal pituitary stem cells (46). The SP, when purified from endothelial and immune cells, expressed a panel of CSC markers, including CD44, CXCR4, KIT, KLF4, SOX2, and Nestin, overexpressed genes related to epithelial-to-mesenchymal transition (EMT) and angiogenesis, formed floating spheres in culture, had clonogenic potential and differentiated into pituitary hormone-producing cells. However, in contrast with previous studies, these cells were not able to originate tumors following xenotransplantation into NOD-SCID mice (40) (**Tables 1**, **2**). Tumor-forming potential in mice was instead exhibited by an SP with high efflux capacity that was identified in the mouse pituitary corticotrope tumor cell line AtT20 (40) and by a subpopulation of murine stem-like cells that was isolated from pituitary tumors spontaneously occurring in Rb± mice (47).

A subsequent study (41) on differential gene expression profiles and flow cytometric characterization identified CD15, a CSC marker in other brain tumors, as a marker for pituitary TSCs. The $CD15^{+}$ cell subpopulation displayed high sphere-forming capacity, elevated SOX2 gene expression and tumourigenic potential in mice.

In contrast, the lack of tumor-generating ability of human pituitary TSCs in mice was reported in another study, in which stem-like cells were successfully isolated from 38 of 56 pituitary tumors of different types (GH-, GH/PRL-, and ACTH-secreting tumors and NFPTs) (42).

Since contrasting data have been reported about the *in vivo* tumourigenicity in mice (**Table 1**), it can be hypothesized that the failure of this assay is due to the typical clinical behavior of pituitary tumors, which is characterized by slow growth, as well as to methodological caveats related to the use of *in vitro* cultures

of stem cells prior to transplantation or to the isolation of stem cell populations with different proliferative rates. Moreover, we can speculate that benign tumors may depend more on their niche than malignant tumors, and thus the lack of a proper microenvironment in mice can explain the failure of tumor formation after xenograft (48). In addition, the *in vivo* results of the available studies remain questionable and not entirely convincing. The major issues reside in the excessive number of transplanted cells, lack of data on long-term growth and serial *in vivo* transplantation.

On the other hand, the pro-angiogenic and invasive potential of pituitary TSCs was successfully demonstrated in zebrafish embryos (42, 43) (**Table 1**), an alternative model that allows the detection of *in vivo* biological behaviors of tumor cells in a shorter time than that in mice, which is more compatible with the slow growth rate of pituitary tumors.

Würth et al. (42) and Peverelli et al. (43) isolated TSCs growing as floating spheres from a high number of NFPTs ($n = 32$) with a success rate of sphere formation nearly identical to that which was previously reported. In both of these studies, the sphere-forming cells were able to self-renew in culture and expressed several markers of stemness, including pluripotent embryonic stem cell markers (SOX2, OCT4, KLF4, and Nestin) (**Table 2**). In addition, NFPT-derived TSCs expressed embryonic pituitary-specific transcription factors involved in gonadotroph differentiation (DAX1, SF1, and EGR1), which is consistent with the gonadotroph origin of most NFPTs (43), and mature hormones (38, 40). Interestingly, the expression of pituitary tumor-specific receptors, SS receptors type 2 (SSTR2) and 5 (SSTR5) or DRD2 has been detected in cells in the spheres (42, 43). These observations suggest the presence of committed precursors in spheres cultured *in vitro* in stem cell-permissive medium.

Pituitary TSCs demonstrate long-term proliferation ability of up to 1–2 months in culture (42, 43), in striking contrast with differentiated cells from the same tumors, which actively proliferate only for 1 week (43), and with pituitary cell primary cultures, which usually survive *in vitro* for only a few days.

The analysis of stem cell marker expression in NFPT tissues revealed the expression of SOX2, OCT4, KLF4, and EGR1 (43). Interestingly, SOX2-positive cells, sparsely distributed in the tissue sections and representing approximately 4% of pituitary cells of NFPT tissues, were negative for pituitary hormones, and only 1% of $SOX2^+$ cells co-expressed LH (43). Similarly, in GH-secreting tumor tissues, no co-expression of GH with SOX2, OCT4, or Nestin was observed (42). Overall, these data indicate the presence of a hormone-negative cell subpopulation expressing stem cell markers in pituitary tumors.

Role of Pituitary TSCs in Pituitary Tumor Resistance to Medical Therapy

The molecular basis of pituitary tumor resistance to pharmacological therapy with DAs and SSAs is still poorly understood. The possible involvement of a drug-resistant TSC population has been independently investigated by two different groups with similar findings (**Table 3**).

TABLE 3 | The table summarizes the results of the published studies supporting or not a possible role of pituitary TSCs in human pituitary tumors clinical behavior.

	Role of TSCs	
	Yes	**No**
Resistance to medical therapy	(38) (carboplatin, etoposide)	(43) (BIM53097, BIM23120); (42) (BIM-23A760)
Invasiveness	(39, 40, 42, 43)	–
Recurrence	(41)	(49)

Peverelli et al. (43) tested the antiproliferative effects of the specific DRD2 agonist BIM53097 and SSTR2 agonist BIM23120 on cultured cells derived from NFPT tissues immediately after dispersion prior to sphere formation and on TSC spheres. Cell proliferation and cell cycle progression were evaluated by measuring BrdU incorporation, cyclin D3 and cyclin-dependent kinase inhibitor p27 levels. They found that in the subset of NFPTs in which DRD2 or SSTR2 agonists inhibited bulk tumor cell proliferation, the antiproliferative effect was maintained in the corresponding spheres.

In agreement, Würth et al. (42) observed a reduction in TSC survival measured by MTT assay upon somatostatin/dopamine chimera BIM-23A760 incubation.

Overall, these results suggest that pituitary TSCs are not characterized by resistance to the drugs currently used in the treatment of pituitary tumors, ruling out the hypothesis that these cells are responsible for the pharmacological resistance observed in a subset of patients, as also supported by the lack of difference in the frequency of TSC sphere formation between NFPTs that were resistant or sensitive to these drugs *in vitro* (43).

On the other hand, based on these data, pituitary TSCs can be considered a good target for pharmacological approaches using DAs and SSAs, supporting the use of these agents in adjuvant medical therapy. In agreement, it has been reported that DA therapy in patients with NFPTs was associated with a decreased prevalence of residual tumor regrowth after surgical resection (50).

Interestingly, TSC spheres derived from resistant NFPTs were significantly larger than those obtained from sensitive ones (43), suggesting an increased proliferative activity of TSCs in resistant tumors, which is consistent with the notion that resistance is associated with increased pituitary tumor aggressiveness.

Role of Pituitary TSCs in Pituitary Tumor Invasiveness

Since only approximately 70% of pituitary tumors gave rise to TSC spheres in culture (42, 43), a possible correlation between this ability and clinical tumor behavior has been hypothesized (**Table 3**). Comparison of clinicopathological and radiological features revealed that the formation of spheres was positively associated with cavernous sinus invasion, whereas no correlation with sex, age, tumor size, Ki67 and extrasellar extension was found (43). Accordingly, the number of $CD133^+$ cells was significantly increased in invasive tumors compared that of non-invasive pituitary tumors, as determined by both

immunocytochemistry and flow cytometry assays (39). In this respect, it is of interest to note that the SP isolated from pituitary tumors by Mertens et al. (40) overexpressed gene clusters involved in cell motility and migration. In addition, pituitary TSCs xenografted into zebrafish embryos migrated from the site of injection and showed strong invasive behavior (42, 43).

Together, these data suggest that pituitary TSCs may contribute to the local invasiveness of pituitary tumors.

Further studies are required to evaluate the differential cell migration and invasion ability of TSCs in comparison to that of differentiated bulk tumor cells. Moreover, since it has been shown that DAs and SSAs exert inhibitory effects on the migration and invasion of pituitary tumor cells (51, 52), it would be of great interest to test whether these effects are maintained in TSCs, possibly supporting the use of these agents in the control and prevention of tumor local invasiveness.

Role of Pituitary TSCs in Pituitary Tumor Recurrence

There is little data available about a possible contribution of pituitary TSCs to tumor recurrence, according to the CSC model (**Table 3**).

Manoranjan and colleagues found an enrichment of CD15 expression in recurrent tumors compared to that of their matched primary tumors (41), suggesting that $CD15^+$ TSCs may promote tumor relapse.

Yunoue et al. (49) reported no correlation between the expression of CD133 in pituitary tumors and the postoperative recurrence rate. Although CD133 is considered a common marker of CSCs in different tumors and its presence has been detected in pituitary tumors (39, 53), it is worth noting that contrasting data have been reported on CD133 expression in TSC spheres derived from pituitary tumors, ranging from positive to negative or low expression (38, 39, 41–43, 54), highlighting the need for the identification of more precise markers for pituitary TSCs.

Role of Pituitary TSCs in Tumourigenesis and Tumor Progression

Long-term proliferation ability and tumourigenic potential in animal models exhibited by pituitary TSCs suggest that TSCs may play a major role in the initiation process of pituitary tumourigenesis, as well as in pituitary tumor growth, but this hypothesis has yet to be proven.

Recently, non-secreting and aggressive pituitary tumors have been found in mice bearing deletion of the LATS gene, a kinase belonging to the Hippo pathway (LATS/YAP/TAZ signaling), which is crucial in the maintenance of active pituitary stem cell state and in the inhibition of differentiation (55). These murine tumors were composed predominantly of $SOX2^+$ cells, suggesting that loss of LATS, obtained by genetic manipulation, drives deregulation of $SOX2^+$ pituitary stem cells, leading to tumor formation. Interestingly, overexpression of Hippo pathway components has been reported in the SP of pituitary tumors (40).

In another pituitary tumor type, adamantinomatous craniopharyngioma (ACP), strong evidence supports a paracrine role of TSCs in tumor development and growth by stimulation of the proliferation of adjacent cells. Genetic linage tracing experiments in a mouse model of ACP showed that $SOX2^+$ cells targeted with mutant β-catenin did not autonomously give rise to the tumor mass but instead generated the tumor from neighboring cells in a paracrine manner (44, 56, 57).

CONCLUSIONS

In the past decade, the existence of a population of stem cells in pituitary tumors has been established, but the role of these pituitary TSCs in tumor initiation, progression, recurrence and resistance to pharmacological therapy, analogous to the CSC model, needs to be further elucidated. Although pituitary TSCs are not responsible for resistance to DAs and SSAs, their presence seems to be associated with tumor invasiveness and possibly recurrence.

The presence of stem cells in benign pituitary tumors may suggest that CSCs are not associated with malignancy. However, studies on CSCs in pituitary carcinomas or atypical pituitary adenomas would be required to clarify their role in pituitary tumor aggressiveness.

Admittedly, most of the published studies investigated TSCs in NFPTs, whereas little information is available for the other types of pituitary tumors.

Further studies aimed to clarify the relationship between tumor-initiating cells and pituitary TSCs are needed. Once the pituitary TSCs and the mechanisms involved in their regulation have been fully characterized, new therapeutic strategies targeting TSCs and new biomarkers predicting pituitary tumor clinical behavior could be developed.

AUTHOR CONTRIBUTIONS

GM, EG, DT, RC, FM, and EP wrote the manuscript. EP, AS, and MA contributed to manuscript revision. All authors read and approved the submitted version.

FUNDING

This work was supported by AIRC (Associazione Italiana Ricerca Cancro) grant to GM (IG 2017-20594) and by Italian Ministry of Health grant to GM (PE-2016-02361797).

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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 Endocrinology

REVIEW
published: 08 November 2019
doi: 10.3389/fendo.2019.00772

Stem Cells, Self-Renewal, and Lineage Commitment in the Endocrine System

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The endocrine system coordinates a wide array of body functions mainly through secretion of hormones and their actions on target tissues. Over the last decades, a collective effort between developmental biologists, geneticists, and stem cell biologists has generated a wealth of knowledge related to the contribution of stem/progenitor cells to both organogenesis and self-renewal of endocrine organs. This review provides an up-to-date and comprehensive overview of the role of tissue stem cells in the development and self-renewal of endocrine organs. Pathways governing crucial steps in both development and stemness maintenance, and that are known to be frequently altered in a wide array of endocrine disorders, including cancer, are also described. Crucially, this plethora of information is being channeled into the development of potential new cell-based treatment modalities for endocrine-related illnesses, some of which have made it through clinical trials.

Keywords: stem cells, development, self-renewal, regenerative medicine, plasticity

OPEN ACCESS

Edited by:
Michaela Luconi, University of Florence, Italy

Reviewed by:
Gail S. Prins, University of Illinois at Chicago, United States
Massimo Mannelli, University of Florence, Italy
Matteo A. Russo, San Raffaele Pisana (IRCCS), Italy

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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *30 August 2019*
Accepted: *23 October 2019*
Published: *08 November 2019*

Citation:
Mariniello K, Ruiz-Babot G, McGaugh EC, Nicholson JG, Gualtieri A, Gaston-Massuet C, Nostro MC and Guasti L (2019) Stem Cells, Self-Renewal, and Lineage Commitment in the Endocrine System. Front. Endocrinol. 10:772. doi: 10.3389/fendo.2019.00772

INTRODUCTION

Stem cells are endowed with the ability to self-renew and differentiate into various organ-specific cell types. They are mainly active during embryogenesis where complex autocrine, paracrine, and endocrine interactions govern their fate, proliferation, and gradual differentiation toward highly organized tri-dimensional organs. A growing number of evidence indicates that populations of stem cells are retained in most post-natal tissues (somatic or adult stem cells), where they exert essential functions throughout life, namely tissue maintenance/self-renewal, remodeling/plasticity in response to physiological demands as well as repair. By definition, adult stem cells have the ability to self-renew, however their differentiation potential is restricted to the array of specialized cell types corresponding to the organ in which they reside. The plasticity of the endocrine organs has been recognized only recently, and our understanding has been propellered by (i) the use of specific genetic mouse models, (ii) gene-discovery approaches for endocrine disorders, and (iii) reprogramming strategies to obtain functional endocrine cells. The acquired knowledge of the biology of endocrine organs is not only important for our understanding of pathological processes, but also for the potential application of cell-based therapies or restoration of stem cell function.

In this review, the role of stem cells in the endocrine system will be covered, from the perspective of tissue development and their function in tissue maintenance and organ plasticity. Recent data showing potential to harness the properties of stem cells for clinical applications is also reviewed.

THE PITUITARY GLAND

Endocrine Function in the Pituitary Gland

The pituitary gland is a small endocrine organ connected to the hypothalamus and together they form the hypothalamo-pituitary axis (HPA), which regulates vital physiological functions such as growth, reproduction, lactation, metabolism, and stress-responses (1). The pituitary gland can be separated into adenohypophysis (anterior pituitary), including both the anterior lobe (AL) and intermediate lobe (IL) derived from oral ectoderm, and the neurohypophysis (posterior pituitary) also known as the posterior lobe (PL) derived from neural ectoderm. In rodents the two lobes remain distinct and are separated by a cleft, with an epithelial lining known as the marginal zone (MZ). The PL is populated by the axonal termini of hypothalamic magnocellular neurons which release anti-diuretic hormone and oxytocin into the blood circulation. The AL develops from oral ectoderm and harbors five cell types: lactotrophs, producing prolactin (PRL); somatotrophs, which release growth hormone (GH); corticotrophs, which synthesize adrenocorticotrophic hormone (ACTH); thyrotrophs, secreting thyroid-stimulating hormone (TSH); and finally, gonadotrophs, which release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A further population of hormone secreting cells, melanotrophs, are found in the IL and are responsible for the synthesis of melanocyte-stimulating hormone (MSH).

Key Pathways Guiding Pituitary Gland Development

The development of the pituitary gland can be separated into three sequential steps, cell specification, cell lineage commitment and terminal differentiation (**Figure 1**). In mice, pituitary organogenesis begins at embryonic day (e) 8, with a thickening of a region of the oral ectoderm, known as the hypophyseal placode (HP) within the anterior neural ridge (ANR) and adjacent to the ventral diencephalon (VD). By e9, an epithelial invagination of the oral ectoderm, centered at the HP occurs forming a rudimental pouch known as Rathke's pouch (RP) (2). This process is directed by physical contact with the overlaying region of the VD known as the infundibulum, which eventually gives rise to the hypothalamic median eminence, the pituitary stalk and the PL (3). By e10.5 the infundibulum begins to evaginate toward the RP and tightly regulated apoptosis separates the RP from the underlying oral ectoderm (4). The lumen of the RP is surrounded by a highly proliferative epithelial layer of pituitary stem/progenitor cells (PSCs) (5). This cell population undergoes a rapid expansion between e11.5 and e13.5 during which the majority of endocrine cell precursors are generated (6). As these RP progenitors gradually exit the mitotic cycle, they express cell cycle inhibitors such as $p57^{KIP2}$ and $p27^{KIP1}$ and lose their epithelial characteristics in order to give rise to distinct pituitary cell types (7). By e14.5 PSCs are committed to one of the three endocrine lineages (expressing transcription factors T-box Factor 19, Pituitary (Tpit) (8), or POU domain, class 1, transcription factor 1 (Pit1)(9) or Steroidogenic Factor 1 (Sf1) (10), and as they begin to differentiate they migrate ventrally and laterally away from the RP lumen, forming the bulk of the AL, with the dorsal progenitors of the RP forming the IL. The residual luminal space of the RP, known as the cleft, and the periluminal MZ constitutes a stem cell niche where multipotent PSCs are maintained into adulthood (11–13). Terminal differentiation culminates shortly after birth when, in rodents, the pituitary gland undergoes a sustained perinatal period of proliferation and growth (14).

Patterning of the developing embryo and induction of the pituitary primordium are regulated by a complex array of sequentially expressed signaling molecules and morphogens. Together, these act to demarcate different regions of the VD (15) and control the developmental induction of the pituitary gland. Bone morphogenetic protein-4 (Bmp4) is expressed and secreted from the VD from e8.5; there it functions as an essential extrinsic requirement for RP formation, and its expression is maintained in the infundibulum up to e14.5 (16–19). Through induction of T-box transcription factor 2 (Tbx2), Bmp4 represses the expression of the morphogen sonic hedgehog (Shh) (20) and opposing gradients of Bmp4 and Shh define the infundibular region of the VD ensuring correct positioning of the RP. From e10.5 Bmp2 is expressed in the developing RP and is essential for RP maintenance and progenitor proliferation, before expression is lost by e14.5 (6, 16, 17). Fibroblast growth factor (Fgf)- 8, -10, and -18 are also expressed in the developing VD, appearing shortly after Bmp4 and maintained until e14.5 (17, 19, 21). Fgfs secreted by the VD activate the MAPK signaling pathway to promote the maintenance and proliferation of the dorsal region of the RP (6). Shh expression in the VD depends on SRY-box transcription factor 2 (Sox2) and Sox3 (22), and ensures the correct patterning of the region. The reciprocal inhibition of Shh and Bmp4 is required for correct infundibular positioning and induction of the RP. Consequently, ablation of Shh expression in the VD has been shown to cause altered expression of Bmp4, Wnt5a, and Fgf8 and a complete arrest in pituitary formation from the early stages of development (23, 24). Within the RP itself, Shh signaling is involved in progenitor proliferation as revealed by conditional deletion of its downstream transcriptional repressors Gli2 and Gli3 (25). Notch signaling in pituitary development appears important for infundibular morphogenesis, as mice null for the known target of Notch, Hairy and Enhancer of Split-1 (*Hes1*) have reduced evagination and disrupted development of the posterior lobe (26–29). Within the RP, Notch signaling is initially widespread and later restricted to the MZ (29, 30). There it promotes progenitor proliferation and maintenance (31), suppresses melanotroph and corticotroph differentiation (32) and promotes the emergence of the Pit1 lineage through integration with the transcription factor Homeobox Protein prophet of Pit1 (Prop1) (29, 33).

Wnt5a is expressed in both the VD and RP from e9.5 to e12.5 and is necessary for correct VD patterning, and indirectly for RP induction via non-canonical pathway (34). Wnt4 also signals via the non-canonical Wnt pathway, is expressed exclusively in the

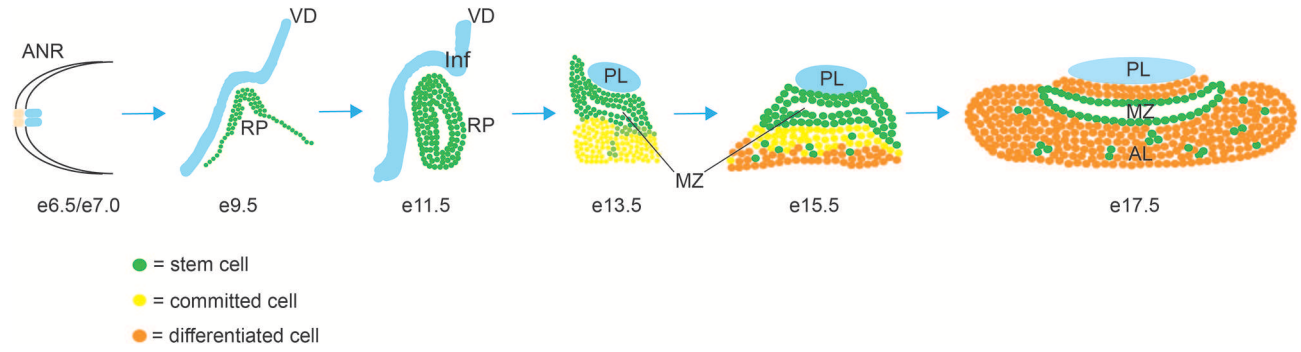

FIGURE 1 | Morphogenesis of the mouse pituitary gland. Abbreviations: AL, anterior lobe; ANR, anterior neural ridge; IL, intermediate lobe; Inf, infundibulum; MZ, marginal zone; PL, posterior lobe; RP, Rathke's Pouch; VD, ventral diencephalon.

RP, and appears to function in cell commitment since its deletion reduces the expression of Pit1 resulting in fewer somatotrophs, lactotrophs, and thyrotrophs (34). Canonical Wnt/β-catenin also plays an important role in pituitary development, and conditional gain or loss of function studies of β-catenin within the VD showed its role in regulating the expression of Fgf8, necessary for normal RP development (35). Within the RP, β-catenin has a role in Pitx2 activation stimulating progenitor proliferation (36) and later binds Prop1 and is necessary for the emergence of Pit1 lineage of endocrine cells (37). Further downstream of Wnt signaling, the transcription factor binding partners of β-catenin, Transcription factor Tcf3, Tcf4, and Lymphoid Enhancer Binding Factor 1 (Lef1), also play a role in pituitary development. Tcf3 acts as a repressor of the Wnt/β-catenin pathway in the anterior forebrain (38) and is essential for the development of the HPA in both human and mice (39). Tcf4 genetic ablation leads to an increase in early progenitor proliferation with increased and prolonged expression of Prop1, which can lead to aberrant tissue growth and tumor formation if not down-regulated (40–42). Genetic ablation of Lef1 does not have similarly pronounced effects, but its inhibition reduces Pit1 expression indicating a potential role as a repressor of pituitary differentiation (37, 43). The Wnt/β-catenin pathway is important in PSC proliferation and maintenance and deregulation of this pathway lead to stem cell-derived pituitary tumors. Activating mutations in β-catenin drive adamantinomatous craniopharyngioma both in mouse and humans (44, 45) and PSC Sox2^{+} cells have been shown to be the tumor initiating cells that are responsive to oncogenic β-catenin.

In addition to the role of morphogens and signaling pathways, the spatiotemporal expression patterns of transcription factors during pituitary development have also been extensively studied, particularly in the context of congenital forms of hypopituitarism (**Figure 2**) (46). The paired-like homeodomain transcription factor Homeobox Expressed in ES cells 1 (Hesx1) functions as a transcriptional repressor through its interaction with the transcriptional corepressor Transducin-Like Enhancer of Split 1 (Tle1) and is an important regulator of forebrain development (47). It is also crucial for early pituitary development, with Hesx1$^{-/-}$ mice showing multiple clefts and over proliferation (48). Importantly, in the RP Hesx1 represses Prop1 expression until e13.5 (48) when it is reciprocally downregulated by the Prop1/β-catenin complex (37). Hesx1 also acts as a repressor of the Wnt pathway, and it has been suggested that de-repression of the Wnt pathway in the anterior neural plate and RP underlies the phenotype of Hesx1$^{-/-}$ mice (48, 49). The closely related Sine Oculis homeobox (Six) transcription factors Six3 and Six6 are expressed in both the VD and RP, with Six6 expression maintained in the adult pituitary. Knockout studies have revealed that both transcription factors are involved in the regulation of progenitor proliferation, with Six6 acting a repressor of the cell cycle inhibitor p27KIP1 (50), and Six3 serving as a repressor of Wnt/β-catenin signaling (49). The paired homeodomain proteins, Pitx1 and Pitx2, are two additional important regulators of pituitary development expressed in the RP where they function redundantly in the maintenance of RP progenitors (4) and later play a role in thyrotroph function (51). Three different members of the LIM-homeodomain transcription factors (Lhx2, 3 and 4) are expressed during pituitary development. Lhx2 is expressed throughout the RP and VD, and appears to function in formation of the infundibulum, but is not involved in cell differentiation (52). By contrast Lhx3 and Lhx4 are expressed from e9.5 in the RP and are redundantly required for progenitor maintenance, and later at e14.5 Lhx4 is downregulated whilst Lhx3 expression is required for endocrine differentiation and maintained into adulthood (53). As stated above, Sox2 and Sox3 are expressed in the VD where they activate the expression of Shh (22) and of Six3/Six6 proteins (54, 55). Sox3 loss of function mutations can result in mild hypopituitarism (56), as can Sox2 haploinsufficiency (57). In both *Sox3*$^{-/-}$ and *Sox2*$^{+/-}$ mice the RP is bifurcated, and at least for *Sox3*$^{-/-}$ mutants this has been associated with expanded Bmp4 and Fgf8 domains in the VD (57, 58). This is likely a consequence of downregulation of Shh (22) and perhaps Six3/6 also (54, 55). Prop1 represents the earliest pituitary specific marker; it is first expressed at e10, and maintained throughout development in the Sox2^{+} progenitor cells, before rapid post-natal downregulation in all but a few Sox2^{+} PSCs (59–61). Prop1$^{-/-}$ mice have reduced Pit1 expression, and prolonged *Hesx1* expression resulting in the loss of somatotrophs, lactotrophs, and thyrotrophs (59, 60, 62). An

important role of Prop1 is the regulation of the epithelial-to-mesenchymal transition as progenitor cells migrate away from the residual RP lumen and begin to undergo differentiation. In the absence of Prop1, progenitors fail to populate the anterior lobe resulting in a dysmorphic pituitary gland by e14.5 (63, 64).

Progenitor endocrine cell lineage commitment is defined by the expression of three essential transcription factors Pit1, Tpit, and Sf1 (**Figure 2**). The process of differentiation relies on the activity of at least two epigenetic regulators, the histone demethylase Lsd1 (65), and the zinc finger protein Insm1 (66). Pit1 expression is activated by Prop1, in complex with β-catenin (37) and is required for the differentiation as well as the expansion and survival of lactotrophs, somatotrophs and thyrotrophs (67, 68). Somatotrophs are further specified by Neurod4 (29), and the Notch ligand Delta-Like homolog 1 (Dlk1) (69). In contrast lactotrophs are predominately specified by estrogen signaling (70). Thyrotrophs can first be identified by the expression of the transcription factor Forkhead Box L2 (Foxl2) and then α-Glycoprotein Subunit (αGSU) (19). Both are also expressed in gonadotrophs. Subsequently, Gata2 is expressed which can activate the expression of Chromogranin-A (Cga) (71). Gonadotrophs are broadly similar to thyrotrophs in terms of their expression of lineage commitment markers but can be differentiated by their expression of Gonadotropin Releasing Hormone Receptor Gnrhr (72) and later Sf1 which promotes the expression of Cga, Fsh and Lh (73). Corticotrophs and melanotrophs emerge from the Tpit (Tbx19) lineage (74, 75) which are further defined by their expression of the transcription factors Neuronal Differentiation 1 (NeuroD1) (76) and Paired Box 7 (Pax7) (77), respectively.

Stem Cells in the Developing and Adult Pituitary Gland

The past decade has seen a great deal of interest in the characterization of PSCs and their function through development to the maintenance of the adult gland, under normal physiological conditions, periods of endocrine stress or in pituitary disease (78–83). They are primarily identified by their expression of Sox2, which drives rapid proliferation in the lumen of RP during early development (84). By e13.5 the surge in pituitary precursor proliferation subsides and Sox9 is expressed alongside Sox2 in a subpopulation of PSCs (85). The AL also harbors a secondary stem cell niche with clusters of $Sox2^+$ PSCs scattered through the parenchyma (46, 86). Functional analysis of PSCs from these two different niches did not reveal obvious differences (87). Intriguingly these two disparate populations of PSCs appear to be physically interconnected to form a three-dimensional network, an architectural feature that hints at some, as of yet undescribed, concerted function (88).

Building upon early *in vitro* studies (12), more recent *in vivo* lineage tracing studies have demonstrated the multipotency of $Sox2/Sox9^+$ embryonic and adult PSCs and their contribution to tissue homeostasis (85, 89). Intriguingly, under normal physiological conditions adult PSCs are highly quiescent and largely inactive (15, 17, 19, 23), which may reflect the low tissue turnover rate of adult pituitary cells, relative to tissues with more active stem cell pools (24); this questions the notion of tissue maintenance as their function (17), particularly since major depletion of adult PSCs did not affect tissue homeostasis (90). More likely their primary function is to provide the organ with plasticity and some regenerative capacity. Genetic ablation of different endocrine cell populations induces PSCs activation and replenishment of ∼60% of the targeted hormone secreted cell type (25–28). However, this regenerative capacity is limited, as it tails off rapidly with age (28) and there is at least some contribution from endocrine cell proliferation and trans-differentiation (26). Perhaps more importantly, PSCs are also able to respond to physiological demand under periods of endocrine stress: for instance, experimental adrenalectomy leads to increases in $Sox2^+$ PSCs-derived corticotrophs and gonadotrophs (19, 29). Interestingly, if instead corticotrophs were depleted gradually, then the progenitor pool was not activated (30), suggesting that the loss of signal from the end organ may be required for PSCs mobilization (29). During pregnancy, the number and activity of lactotrophs rise in an expansion partly driven by estrogen (31) and indeed treatment of male mice with oestradiol causes a sharp rise in $Sox2^+$ PSC proliferation, a finding also observed in experimental gonadodectomy (19). The heightened activity of PSCs during the neonatal expansion of the gland and production of new endocrine cells is also clear, and they show increased proliferation as well as multipotent differentiation capacity (91). The potential involvement of PSCs in the subtler changes to the gland that occur during puberty and sexual maturation is logical, but as of yet unproven.

Stem Cells and Regenerative Medicine in the Pituitary Gland

Recent advances in the *in vitro* recapitulation of pituitary development highlight the potential of cell-based therapies to revolutionize the treatment of hypopituitarism, which is defined by the failure to secrete one or more pituitary hormones, and typically requires lifelong hormone replacement therapy. Pioneering work by Suga et al. reported the induction of self-organizing RP-like structures from mouse ES-cells, which contained corticotrophs and somatotrophs and were capable of rescuing systemic glucocorticoid level in hypopituitary mice (92). Mimicking pituitary development, their protocol involves the induction of adjacent layers of non-neural head ectoderm and hypothalamic neuroectoderm, which a follow up study showed was also applicable to human embryonic stem cells (ESCs) (93). Using an alternative approach, Dincer et al. were able to induce a placodal fate in adherent hESCs cultures, ultimately producing functional corticotrophs, that secreted ACTH after subcutaneous implantation in mice (94). Preliminary attempts at generating pituitary organoids from adult mouse PSCs have been performed, though to date, these have lacked the degree of self-organization, and functional hormone release achieved by their ESCs-derived counterparts (95). Interestingly, in all strategies ACTH-secreting cells are the predominant differentiated endocrine cell type produced. Future work will likely focus on the targeted generation of other hormone secreting cells, and move

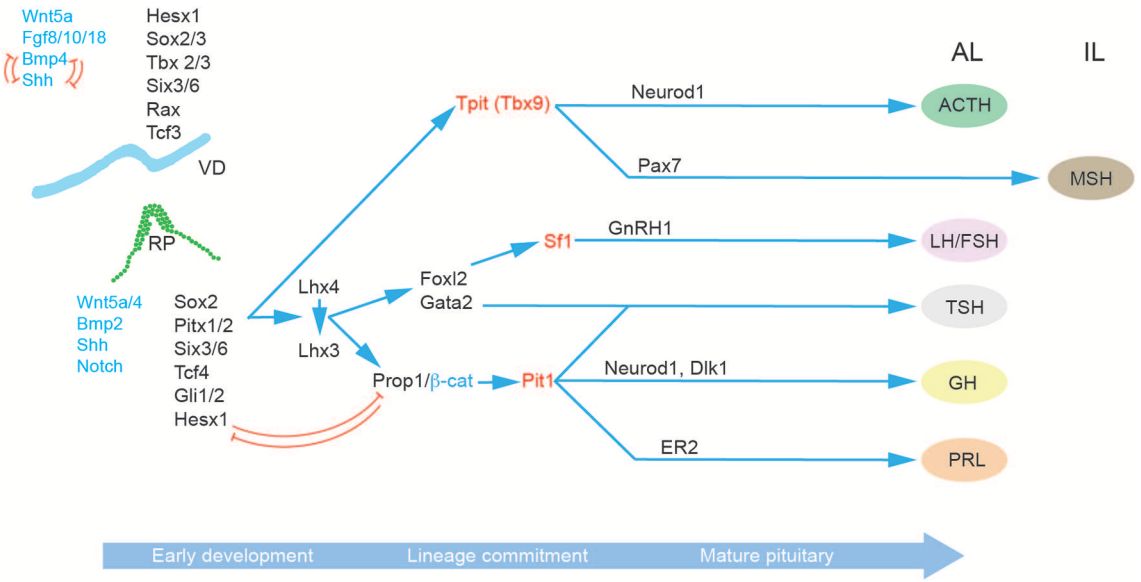

FIGURE 2 | Molecular regulation of pituitary gland development. A succession of transcription factors (black) and signaling molecules (blue) determine the establishment of RP and the subsequent lineage specification and differentiation in the progenitor cells of the developing pituitary hormone-secreting cell types characteristic of the mature anterior pituitary gland: corticotrophs (ACTH), gonadotrophs (FSH and LH), thyrotrophs (TSH), somatotrophs (GH), and lactotrophs (PRL). The key lineage commitment makers are highlighted in red. Arrows indicate upstream relationships in molecular signaling pathways, not necessarily direct activation. Red T-bar arrows denote repressive relationships. Abbreviations: ACTH, adrenocorticotropic hormone; AL, anterior lobe; FSH, follicle-stimulating hormone; GH, growth hormone; IL, intermediate lobe; LH, luteinizing hormone; MZ, marginal zone; PL, posterior lobe; PRL, prolactin; RP, Rathke's pouch, VD; Ventral diencephalon.

toward orthotopic transplants to investigate the degree to which transplanted cells can integrate into the regulatory circuitry governing physiological hormone secretion.

ADRENAL CORTEX

Endocrine Function and Key Pathways Guiding Adrenal Cortex Development

The adrenal cortex is essential for life. It is the primary site of steroid synthesis, producing glucocorticoids under the control of the HPA and mineralocorticoids under the control of the renin-angiotensin-aldosterone system (RAAS). Glucocorticoids regulate glucose metabolism, inflammation, immune responses, muscle and skeletal mass as well as cognition, well-being and memory, while mineralocorticoids control extracellular fluid volume and sodium homeostasis, and hence have an important influence on blood pressure.

The adrenal cortex originates from a group of cells within the dorsal coelomic epithelium at ∼e9.0 in mice and 3–4 weeks in humans (**Figure 3**). These cells form the so-called adrenogonadal primordium (agp) and express the master regulator of adrenocortical differentiation and function, namely Steroidogenic factor-1 (Sf1, encoded by Nuclear Receptor Subfamily 5 Group A Member 1 -*Nr5a1*) (96). $Sf1^+$ cells delaminate from the coelomic epithelium and invade the overlying mesonephric mesenchyme. The agp then separates forming the adrenal anlagen migrating dorsomedially and the gonadal anlagen, which settles dorsolaterally. Genetic and molecular evidence have demonstrated that the transcription co-factor Cbp/P300-Interacting Transactivator 2 (Cited2) interacts with the transcription factor Wilms Tumor 1 (Wt1) to stimulate expression of Sf1 in the agp prior to the separation between gonadal and adrenal primordia (97). The adrenal primordium is then invaded by migratory neural crest- and Schwann cell precursors-derived cells that will form the neuroendocrine medulla (see section on adrenal medulla). Subsequently the gland becomes encapsulated by mesenchymal cells. The cortex is composed of fetal adrenal cells that are established before the outer definitive adrenal population emerges between the capsule and fetal adrenal. Functional zonation is completed around birth. A crucial lineage relationship between fetal adrenal cells and adrenal capsular cells to the differentiated adrenal cortex was determined using specific Cre lines permitting the identification of cells that have at some time actively expressed *Nr5a1* under control of the fetal adrenocortical-specific enhancer (*FAdE*), an essential element in driving and maintaining Sf1 expression in the fetal cortex. These experiments indicated that a subset of capsular cells are indeed descendants of fetal adrenocortical cells that once expressed Nr5a1 (98).

Stem Cells and Self-Renewal in the Adrenal Cortex

The adrenal cortex undergoes a self-renewal process and important paracrine effectors supporting a dynamic centripetal streaming of adrenocortical cells have been identified with the use of specific mouse transgenic models (99). Adrenocortical self-renewal in the experimental animal relies on the differentiation

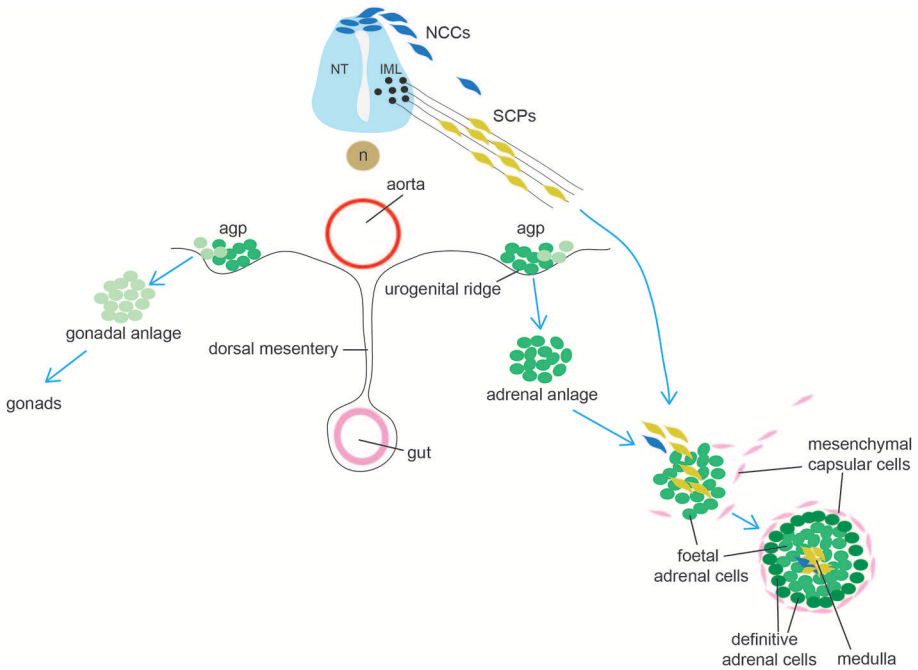

FIGURE 3 | Schematic representation of adrenal gland development. Cells from the adrenogonadal primordium (agp) form the adrenal and gonadal anlage. The adrenal anlage is invaded by migrating medullary progenitors who derive from early migrating neural crest-derived cells (NCCs, a minority in mice) and from late migrating Schwann cell precursors (SCPs). Concomitantly, the adrenal is encapsulated by mesenchymal cells. During late embryogenesis, definitive adrenal cells appears and will substitute fetal adrenal cells. IML: intermediolateral column (IML); NT, neural tube; n, notochord.

of at least two cell populations of progenitor cells, located in capsular and subcapsular compartments (**Figure 4**). It was shown that Shh is expressed in $Sf1^+$ but relatively undifferentiated cortical cells in the subcapsular region of the mouse (100, 101) and rat (102) adrenal starting from e12.5 and e13.5, respectively. Capsule cells transduce the Shh signal, and lineage-tracing studies have shown that $Gli1^+$ capsular cells delaminate into the cortex, lose their responsiveness to Shh, and become $Shh^+/Sf1^+$ progenitor cells; they then proceed to become fully mature steroidogenic cells forming the distinct histological and functional layers: zona glomerulosa (ZG, secreting aldosterone and expressing aldosterone synthase, encoded by *Cyp11b2*) and zona fasciculata (ZF, secreting glucocorticoids, expressing 11βhydroxylase, encoded by *Cyp11b1*) (100). Capsular $Gli1^+$ cells and subcapsular Shh^+ cells are therefore two interconnected types of adrenocortical progenitor cells; recently however it has been shown that the relative impact of capsular and subcapsular progenitor cells in generating new steroidogenic cells is extremely unbalanced post-natally with cortical Shh progenitor cells being preponderant in generating steroidogenic cells compared to the capsular Gli1 population (103).

Another player in the gland self-renewal is paternally expressed Dlk1/Preadipocyte Factor-1 (Pref1), a cleavable single-pass transmembrane protein and a member of the Notch/Delta/Serrate family. A number of experimental evidence suggest that Dlk1 may be involved in adrenocortical differentiation: (i) Dlk1 is expressed in $Shh^+/Sf1^+$ progenitor cells in rat adrenals (104), (ii) Dlk1 regulates *Gli1* levels in H295R cells, possibly through the secreted ectodomain Dlk1 and in a β1-integrin dependent fashion (104), (iii) its expression was found to be inversely correlated to the differentiation status of the ZG following remodeling of RAAS rats (104), (iv) its potential cross-talk with subcapsular Fgf signaling, as *Fgfr2IIIb* knock-out mice showed hypertrophic capsule and absence of capsular Dlk1 expression (105), and (v) its rapid disappearance after adrenal enucleation in rats and reappearance once zonation is restored (106). These data suggest that Dlk1 might be a negative regulator of adrenocortical differentiation, similarly to its well-established role in inhibiting adipogenesis (107). Interestingly, while Dlk1 is expressed in the subcapsular region of rat (104) and human (108) adrenals, it is mostly expressed in capsular cells in mice (105, 109). It is not currently known whether Dlk1 is co-expressed with $Gli1^+$ progenitor cells in the capsule, or whether Dlk1 and Gli1 mark two different populations.

The lineage relationship between fully differentiated ZG and ZF cells during post-natal life and during regeneration was established with the development of a specific mouse model where Cre recombinase was targeted to the *Cyp11b2* genomic locus (110). Genetic lineage tracing with these mice demonstrated that ZG cells can lineage convert to ZF cells in a Sf1 dependent manner, and these cells can mark the whole ZF in a period of 12 weeks, suggesting a relatively slow self-renewing process in the mouse adrenal cortex. However, as Cyp11b2 knock-out mice are still able to generate ZF cells, alternative and/or facultative cell sources active in sustaining ZF self-renewal (and therefore crucial in preserving homeostatic cortisol levels)

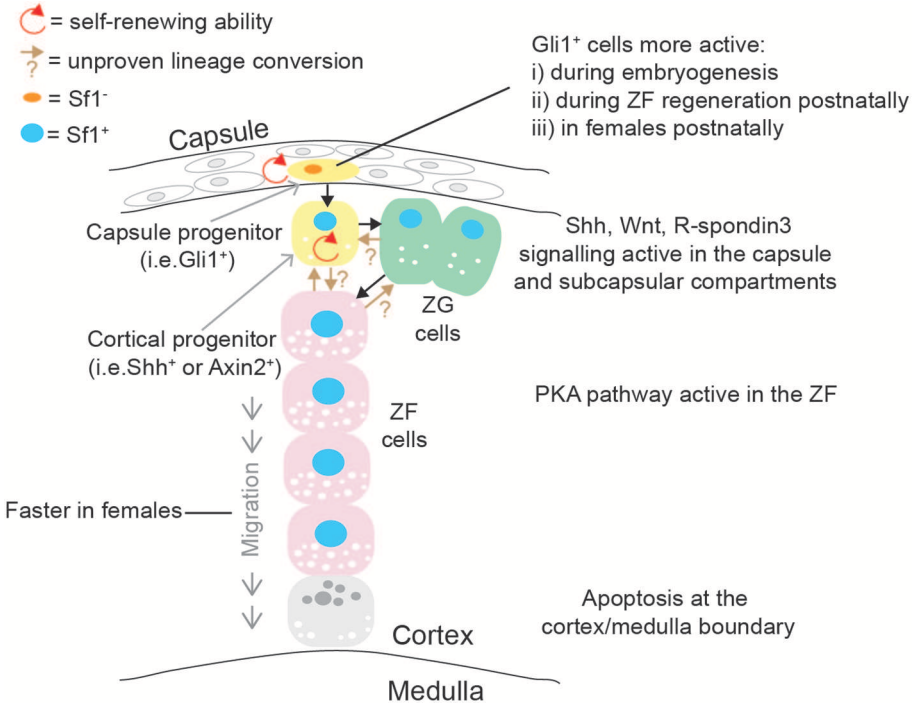

FIGURE 4 | Schematic representation of post-natal adrenal cortex centripetal streaming and self-renewal in mice. Gli1$^+$ cells in the capsule can give rise to Sf1$^+$/Shh$^+$ cortical cells: both are self-renewing adrenocortical progenitor cell populations. Shh$^+$ cells can become ZG cells, and ZG cells can lineage convert to ZF cells, which migrate centripetally. Direct differentiation between Shh$^+$ cells and ZF is probably occurring in parallel. These differentiation events are governed by pathways mostly active in the capsular/subcapsular region, while apoptotic figures are observed at the cortex/medulla boundary in senescence cells.

must be present, one example of such could be a subset of capsular/subcapsular progenitors able to directly differentiate toward a ZF identity.

Compelling evidence of the importance of other pathways in adrenocortical growth, self-renewal and zonation, has also been provided; for example, targeted disruption of β-catenin in Sf1$^+$ cells resulted in an impairment of adrenal cortex development and maintenance in mice; this phenotype was even more pronounced when a Cre transgene was expressed at high levels, resulting in adrenal aplasia (111). Conversely, constitutive β-catenin activation induced ZG adrenal hyperplasia which ultimately led to adrenal cancer development in mice (112). Wnt-responsive cells were found to be Shh$^+$ progenitor cells as well as differentiated, steroidogenic cells of the ZG, but not the ZF, and rarely cells that were actively proliferating. *In vitro* experiments also demonstrated that stimulation of β-catenin signaling caused decreased corticosterone release; this was corroborated by not only a reduced expression of steroidogenic genes such as *Cyp11a1*, *Cyp11b1*, *Star*, and *Mc2r*, but also by a diminished Sf1 expression and Sf1 occupancy on steroidogenic promoters. Interestingly, Coiled-Coil Domain Containing 80 (Ccdc80) was found to be a novel β-catenin-regulated gene in adrenocortical cells, and secreted Ccdc80 could partially phenocopy suppression of steroidogenesis induced by β-catenin, in a Sf1-independent fashion (113). Wnt4 is key activator of the pathway in the cortex and knock-out experiments in mice demonstrated that capsular R-spondin3, a secreted protein and a known positive regulator of Wnt/β-catenin pathway, induces Wnt4 and Shh expression within steroidogenic cells in the subcapsular compartment (114) and that its action is strongly antagonized by protein kinase A (PKA) activation, resulting in inhibition of ZG differentiation. PKA stimulation was able to increase inactivating and decrease activating β-catenin phosphorylation in adrenocortical cells *in vivo*. Therefore, it was suggested that PKA activation in the ZF is a key driver of Wnt inhibition and lineage conversion of cells to a ZG identity. The same authors provided evidence that constitutive PKA activation was able to inhibit β-catenin-induced ZG adrenal hyperplasia and subsequent tumorigenesis *in vivo* (115). Constitutive PKA activation, which was achieved by genetic deletion of the critical component Protein Kinase cAMP-Dependent Type I Regulatory Subunit Alpha (Prkar1a) was also found to be crucial for conversion of ZF cell to a zona reticularis (ZR)-like phenotype, seemingly via lineage conversion of the innermost ZF cells; interestingly this process was found to be sexually dimorphic as testicular androgens were shown to increase adrenocortical Wnt signaling (antagonizing PKA), leading to slower adrenocortical cell turnover and delayed ZR appearance whereas gonadectomy sensitized males to hypercorticism and ZG-like formation (116). More recently, a thorough study of adrenocortical self-renewal in mice shed more light on this sexually dimorphic phenomenon: genetic lineage tracing was achieved using Axin2 mouse model;

Axin2 has been shown to reliably act as a readout for Wnt-responsive cells, and, as such, it is a frequently-used marker of functional stem cells. By comparing male and female mice, they found that female mice had significantly higher proliferation as well as turnover than males; moreover, in females but not males, the capsule Gli1$^+$ population was found to be more active in generating new steroidogenic cells post-natally. Interestingly, proliferation rates, cortex turn-over and recruitment of capsular Gli1$^+$ cells was enhanced in males following orchiectomy, suggesting that androgens might inhibit full recruitment of some adrenal cortex stem cell compartments; this was further corroborated by showing inhibition of Gli1$^+$ cells activation in ovariectomised females treated with dihydrotestosterone. This data is important as it might explain the biology behind the higher incidence of adrenal diseases in females (117).

Important factors involved in adrenal cortex differentiation and self-renewal under physiological conditions have also been discovered by assessing mutation and changes in gene expression in adrenocortical tumors. Two examples are the histone methyltransferase Enhancer of Zeste Homolog 2 (Ezh2), the most deregulated epigenetic factor in adrenocortical carcinomas (118) and the transmembrane E3 ubiquitin ligase zinc and ring finger 3 (Znrf3), a known Wnt inhibitor which is frequently inactivated in adrenocortical carcinomas (119). Ezh2 was found to be an important epigenetic factor ensuring the unidirectionality of differentiation events from ZG to ZF. Targeted inactivation of Ezh2 in mouse adrenal cells was achieved through the use of a Sf1Cre line crossed to a floxed Ezh2 allele; these mice had hypoplastic adrenal glands and developed primary glucocorticoid insufficiency (low corticosterone, high ACTH, normal aldosterone in female mice) with blunted ZF differentiation (decreased *Cyp11b1* mRNA expression), suggesting that Ezh2 is a key regulator of ZF differentiation and identity. This suggestion was proved by further experimental data showing that Ezh2 not only programmed adrenocortical cells to respond to ACTH via PKA signaling, but also inhibited accumulation of capsular/pericapsular Gli1$^+$ and Wt1/Gata4$^+$ spindle-like pericapsular cells. Interestingly, these Gli1$^+$ and Wt1/Gata4$^+$ fibroblastic-like cells, commonly seen in aged or gonadectomised mice of specific backgrounds, were found to be derived from steroidogenic (Sf1$^+$) cells through a mechanism involving dedifferentiation, rather than direct amplification of capsular cell populations (120).

Znrf3 was found to be expressed in both ZG and ZF cells in mice; adrenocortical-specific loss of Znrf3, achieved through the use of both Sf1Cre and Cyp11b2Cre lines crossed to a floxed Znrf3 allele, developed adrenal hyperplasia in the ZF in a ACTH-independent manner with loss of normal adrenocortical architecture; this phenomenon was found to be dependent on Wnt signaling as genetic inactivation of Znrf3 together with Porcupine (a key enzyme required for Wnt ligands maturation and activity) displayed normal adrenal cortex architecture and reduced ZF hyperplasia. The authors also found that Wnt4, normally highly expressed in the ZG with a decreasing gradient into the outer ZF, lost this characteristic expression pattern and instead displayed moderate-level expression throughout the entire ZF. Interestingly, this pattern was also altered for β-catenin protein expression, as well as *Axin2* mRNA, strongly suggesting that loss of Znrf3 leads to increased Wnt/β-catenin in the ZF promoting hyperplasia (121).

Stem Cells and Regenerative Medicine in the Adrenal Cortex

There is an undeniable case for stem cell regeneration therapy in adrenal insufficiency, however it is still in its infancy. Initial experiments showed the ability to obtain a steroidogenic lineage when Sf1 was forced-expressed in hESCs (122). Since then, others have reported the conversion of mouse and human ESCs, mesenchymal stem cells and inducible pluripotent stem cells (IPSCs) into adrenocortical-like cells, all by over-expressing Sf1 [reviewed in (123)].

Our laboratory has devised a technology for the generation of steroidogenic-like cells via reprogramming of skin-, blood- and urine-derived cells in humans. Reprogramming was achieved via forced expression of Sf1 through lentiviral delivery, together with the activation of the protein kinase A (PKA) pathway and in the presence of luteinising hormone releasing hormone (LHRH). These reprogrammed cells had ultrastructural features resembling steroid-secreting cells, expressed steroidogenic enzymes and secreted steroid hormones in response to physiological and pharmacological stimuli. They were viable when transplanted into the mouse kidney capsule and intra-adrenal. Importantly, the hypocortisolism observed in cells derived from patients with adrenal insufficiency due to congenital adrenal hyperplasia was rescued by expressing the wild-type version of the defective disease-causing enzymes. This study provided for the first time an effective tool with many potential applications to study adrenal biology and pathobiology in a personalized manner and opened up avenues for the development of precision therapies (124). The main obstacle to a clinical application of the strategies described above is the absence of a protocol which allows derivation of (i) proper adrenocortical-like cells from pluripotent stem cells or somatic cells without overexpression of Sf1, and (ii) a cell population able to self-renew similarly to the cortex.

ADRENAL MEDULLA

Endocrine Function of the Adrenal Medulla

The adrenal medulla is the inner part of the adrenal gland and is mainly responsible for the synthesis and secretion of catecholamines, such as epinephrine (adrenaline) and norepinephrine (noradrenaline), both derived from the aminoacidic tyrosine and stored in vesicles prior to secretion. The main cell type of the adrenal medulla is the chromaffin cell (or pheochromocytes), named as such because of the affinity of catecholamines for chromium salts. The adrenal medulla is highly innervated by preganglionic sympathetic fibers. Epinephrine and norepinephrine are responsible for the execution of the fight-or-flight response of the sympathetic nervous system; such response involves (i) an increase in blood pressure via binding and activation of α1 receptors on vascular smooth muscle cells (resulting in vasoconstriction and increased blood flow to muscles and brain); (ii) an increase in the heart

rate and contractility, (iii) a relaxation of smooth muscles in the airways (via β2-adrenoreceptors, to increase breathing), (iv) an increase in glycaemia via activation of the glycogenolysis pathway concomitant to stimulation of glucagon secretion via β2 receptors and decreasing of insulin secretion via α2 receptors in the Islets of Langerhans.

Key Pathways Guiding Adrenal Medulla Development

Until very recently, chromaffin cells were thought to be direct derivates of neural crest, with a stream of neural crest-derived cells migrating and committing to a common sympathoadrenal lineage ending up in the vicinity of the dorsal aorta, where they would proliferate and continue migrating either ventrally (cells forming the adrenal medulla) or dorsally (cells forming the sympathetic ganglion) (125, 126). In 2017 Furlan et al., using genetic lineage tracing approaches and genetic ablation, convincingly showed that the majority of chromaffin cells derive from a specific cell type, termed Schwann Cell Precursor (SCP) (127) (**Figure 3**). SCPs are the earliest well-defined glial-like cell population during peripheral nervous system development (the radial glial being their corresponding identifiable precursor in the central nervous system) and known multipotent stem cells which can differentiate and generate different cell types, such as the parasympathetic nervous system (128, 129). SCPs themselves are a neural crest derivates which have undergone an extensive change in gene expression with many glial-associates genes (which are also expressed in Schwann cells) being activated. SCPs appear in mouse at ∼e12.5; later in development, they are also able to generate the so-called immature Schwann cells, which differentiate to form Myelin- and Remak- (non-Myelin) Schwann cells. SCPs have an intimate association with neurons and their processes and are extremely dependent on axonal signals for both migration, survival and differentiation, a feature that is not shared by neural crest cells, which migrate more freely. Over time, it has become clear that SCPs could generate direct derivates which were of a different lineage to Schwann cells, such as endoneurial fibroblasts (130), parasympathetic ganglia (128, 129), melanocytes (131), and mesenchymal cells giving rise to odontoblasts and tooth pulp cells (132). Furlan et al. traced SCPs using neural crest and glial-specific inducible Cre lines [Sox10 and the myelin component Proteolipid Protein 1 (Plp1)]. Injection of tamoxifen at e11.5 followed by analysis at e17.5 showed that at least half of chromaffin cells in the adrenal medulla derived from SCPs. The contribution of nerve-associated SCPs was further corroborated by genetic ablation of SCPs with diphtheria toxin subunit A, resulting in a significant depletion of chromaffin cells which were able to migrate to the adrenal medulla. Moreover, the dependency of adrenal medulla formation on SCPs migration along nerves was elegantly demonstrated by achieving specific ablation of preganglionic motor neurons, resulting again in a strong (78%) reduction of chromaffin cells, with the remaining chromaffin cells presumably derived from earlier neural crest migrating cells. Another key finding from this study is the demonstration of an early lineage segregation of sympathoblasts and chromaffin cells, which were until recently considered to originate from a common sympathoadrenal progenitor (133, 134).

Stem Cells and Regenerative Medicine in the Adrenal Medulla

The question of whether stem/progenitor cells with regenerating abilities persist in the post-natal adrenal medulla has not been extensively investigated. Initial *in vitro* studies showed that cells with progenitor characteristics could be enriched from bovine (135) and human (136) medullary extracts; these cells could also generate spheres expressing progenitor cell markers such as Nestin (a type IV intermediate filament protein expressed in multipotent neural stem cells), CD133, and Notch1. Subsequently, by using a Nestin–GFP transgenic mouse model, it was shown that $Nestin^+$ cells (accounting 6% of medullary cells) were negative for both Tyrosine-hydroxylase and chromogranin A (two markers of differentiated chromaffin cells), suggesting that Nestin was not expressed by mature chromaffin cells. Isolated Nestin-GFP cells were also able to generate spheres, which were able to differentiate into chromaffin cells and neurons. This was also confirmed *in vivo* where mice were subjected to repeated immobilization stress; again, the progeny of $Nestin^+$ cells, investigated using an inducible nestin–Cre mouse line, was found to include cells with glial, neuronal, and chromaffin identity (137). Chromaffin-like cells have been recently derived from hESCs via a multistep protocol involving first differentiation toward neuroectoderm-like caudal neural progenitors via TGFβ and GSK3β inhibition followed by establishment of neural crest stem/progenitor cells neurospheres in the presence of Fgf2 and Bmp2. Further treatment of these neurospheres with Bmp4 or with dexamethasone plus phorbol 12-myristate 13-acetate (PMA) induced a strong up-regulation of markers of mature chromaffin cells, such as tyrosine hydroxylase and Phenylethanolamine N-methyltransferase (138). The generation and culture of functional chromaffin-like cells could be employed in the field of regenerative medicine, specifically in cases of neuroendocrine/neurodegenerative diseases, and also for pain management.

THYROID GLAND

Endocrine Function in the Thyroid Gland

The thyroid is a butterfly-shaped gland located in front of the trachea. Its main function is to regulate body metabolism by producing thyroid hormones T4 and T3 from iodine. Thyroid tissue is composed by two cell types: follicular cells, responsible of thyroid hormones secretion, and parafollicular cells (or C cells), which secrete the hormone calcitonin, involved in calcium regulation. The thyroid gland is controlled by the pituitary gland through secretion of TSH which stimulates the thyroid gland to produce more hormones.

Key Pathways Guiding Thyroid Gland Development

Follicular cells arise from the thyroid anlage, a group of foregut endodermal cells located on the midline of the posterior

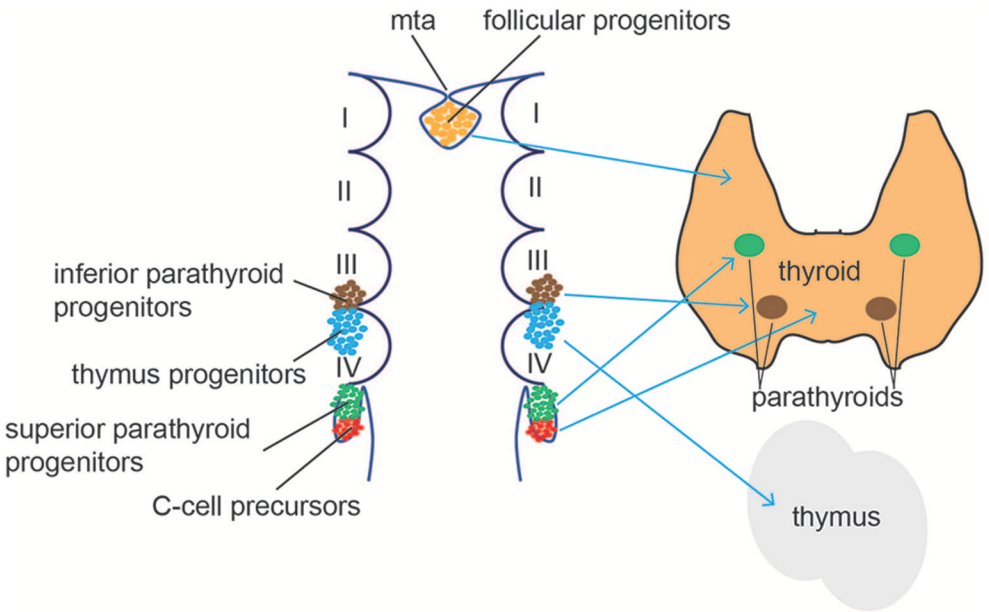

FIGURE 5 | Thyroid/Parathyroid development. Follicular thyroid progenitor cells (orange) derive from the midline thyroid anlage (mta), an endodermal tissue in the floor of the pharynx just caudal to the 1st pharyngeal arch. The superior parathyroid glands (green) originate from the 4th brachial pouch while the inferior parathyroid (blue) and the thymus develop from the 3rd brachial pouch. C cells (red) differentiate from the ultimobrachial body, below the 4th brachial pouch.

mouth cavity, while parafollicular cells differentiate from the ultimobranchial bodies, a structure derived from the fourth pharyngeal pouch in the developing neck (**Figure 5**). Recent reports demonstrate that parafollicular C cells develop from pharyngeal endoderm and not neural crest cells, as previously suggested (139, 140). Using a dual mouse lineage tracing strategy, Johansson and collaborators provided direct evidence that C cells derive from Sox17-expressing endodermal progenitors and not from Wnt1-expressing neural crest-derived progenitor cells (141). Both cell types migrate from their original sites to form the definitive thyroid gland (142, 143). During this process, thyroid anlage cells bud by proliferation and invade the surrounding mesenchyme. The thyroid primordium bifurcates bilaterally and migrates toward the larynx and proximal trachea, a process accompanied by intense thyroid progenitor proliferation. Once the left and right thyroid lobes are formed, functional cellular differentiation takes place, which in humans occur after the eleventh week of gestation (144).

During early specification of thyroid cells, exclusive cooperation of the transcription factors Haematopoietically-Expressed Homeobox Protein (Hhex), NK2 Homeobox 1 (Nkx2-1, also known as thyroid transcription factor, Ttf1), Pax8 and Forkhead Box E1 (Foxe1, also known as thyroid transcription factor 2, Ttf2) have been shown to be essential (145, 146). Indeed, genetic deletion of these transcription factors individually resulted in severe thyroid hypoplasia or lack of thyroid formation (147–151). Hhex plays a role in maintaining total progenitor cell numbers in the budding epithelium, while it is not required for thyroid precursor specification (145). Nkx2-1 is not essential for initial specification of the thyroid gland, but is required for the development and morphogenesis. Nkx2-1$^{-/-}$ mice develops a thyroid rudiment which degenerates through apoptosis (152), highlighting its primordial role in pharyngeal endoderm-derived tissues. Pax8 acts as a regulator of thyroid precursor survival. Similar to Nkx2-1$^{-/-}$ mice, Pax8$^{-/-}$ animals show a progressive degeneration of thyroidal primordium (145). Finally, Foxe1 plays a role in migration of thyroid precursor cells. Although the thyroid primordium is formed, progenitor cells in Foxe1-null animals remain attached to the pharyngeal floor whereas in wild-type embryos they are detached from the pharynx cavity and begin to migrate (149). Altogether, the coordinated expression and regulation of these key transcription factors in a timely manner drives the generation, expansion and migration of progenitor cells to form the definitive thyroid tissue.

Stem Cells and Self-Renewal in the Thyroid Gland

The first indirect evidence that the thyroid gland was an organ endowed with extremely slow self-renewal potential was provided in 1974 through observation of follicular cell proliferation (153). Subsequently, cell population kinetics was studied *in vivo* in dog thyroids via bromodeoxyuridine incorporation and *in vitro* in human thyroid slices, suggesting a complete turnover of ∼8.5–14.4 years (154). These and other studies in the 1980s led to the hypothesis of the existence of an unknown number of resident adult stem cells in the thyroid governing this slow self-renewal process (155). Initial efforts to isolate thyroid stem cells in mice showed the existence of a small pool of cells expressing the stem cell markers Oct4, nucleostemin and the ATP binding cassette (ABC)-dependent

transporter ABCG2 (the latter endowing cells with the ability to efflux the vital dye Hoechst 33342, also referred to as side population), while expressing low levels of differentiated thyroid markers such as thyroglobulin, TSH receptor, thyroid peroxidase, Pax8, or thyroid transcription factor 1 (Titf1). The same authors also demonstrated that ABCG2-expressing cells were located in the interfollicular space of the thyroid gland but not in cells lining the follicles (156).

In humans, another evidence of the existence of thyroid stem cells were reported by Thomas et al. (157); histologic staining and cultured cells derived from goiters (abnormal enlargement of the thyroid gland), showed a subset of cells expressing the stem cell marker Oct4, and the early endodermal markers Gata4 and Hepatocyte Nuclear Factor 4α (HNF4α) markers while they were negative for the differentiated cell marker thyroglobulin. Interestingly, these markers were found not to be expressed in the differentiated rat thyroid cell line FRTL5 cell line, while they were expressed in undifferentiated thyroid carcinoma cell lines.

Lan et al. isolated adult thyroid stem cells as side population from human goiters by employing Hoechst 33342 staining (expression of ABCG2) followed by fluorescence-activated cell sorting (FACS). Those cells, which accounted for 0.1% of the total cell population, showed stem cell morphological characteristics (smaller in size and higher nucleus to cytoplasm ratio compared with differentiated cells) and expression profiles compatible with an undifferentiated population, and were able to differentiate *in vitro* into thyroid cells upon TSH treatment. Interestingly, spheres established from dissociated thyroids were able to grow *in vitro* in a medium enriched with Egf and bFgf but without TSH, and contained a 50-fold enrichment of side population cells. When stem cells isolated from these 3D structure (named thyrospheres) were grown as monolayer or embedded in collagen, differentiation under the influence of TSH was observed (expression of Pax8, TG, NIS, TSHr, and TPO as well as 125 iodide uptake in response to TSH). These data proved the ability of adult human goiters-derived thyrospheres to differentiate into functional thyroid cells (158). In 2008, Fierabracci and collaborators generated spheroids in culture from human healthy thyroids; their thyrospheres could self-replicate *in vitro* and generate thyroid hormones upon differentiation conditions (159).

Fgfs and Bmps are essential signaling pathways for thyroid cell fate induction. Revest and collaborators reported lack of thyroid glands in Fgfr2b-deficient mice (160). The same phenotype was reported on Fgf10 knockout mice (161), suggesting that Fgf10 could act as a Fgfr2b ligand during thyroid development. Other Fgfs, like Fgf2 and Fgf8, have been involved in thyroid development (162). *In vitro* studies using mouse embryonic stem cells supports the evidence of FGF signaling in differentiating thyroid cells. Longmire and collaborators showed that Fgf2 and Bmp4 are required to generate functional thyroid cells from human and mouse ESCs/ iPSCs (163), reinforcing the notion that these signaling pathways are important during development of the thyroid glands.

Stem Cells and Regenerative Medicine in the Thyroid Gland

Studies in thyroid regeneration after partial thyroidectomy (PTx) showed that the central areas of both lobes act as the proliferative centers (164). Microarray analysis performed after PTx reveal increased expression of embryonic development pathways, suggesting potential dedifferentiation events or activation of resident stem/progenitor cells. Interestingly, levels of serum T4 hormone, which were decreased after PTx, recover to normal after a week. Accordingly, increases in TSH were detected after PTx to stimulate the gland to produce more T4. In fact, TSH is known to play a role in promoting undifferentiated progenitor/stem cells to transform into mature thyroid follicular cells (158, 165).

Zhang and collaborators have postulated a model for the origin of thyroid carcinoma from adult progenitor cells based on their cell of origin and the levels of differentiation (166), however the low turnover of thyroid gland cells make it difficult to study the relationship between normal and thyroid cancer stem cells.

Several groups have generated thyroid progenitor and mature functional thyroid cells from both mouse and human pluripotent stem cells (163, 165, 167–169). Pioneering work by Arufe and collaborators showed the ability of mouse ES cells to differentiate toward thyroid follicular cells when cultured in serum-free medium supplemented with TSH (165). In 2012, evidence of *in vivo* functionality was demonstrated using mouse ES-derived three-dimensional thyroid follicular cells. Differentiated cells, obtained through transient overexpression of the transcription factors Nkx2-1 and Pax8, were able to restore thyroid hormone plasma levels once implanted into athyroid mice (170).

Modulation of Tgfβ, Bmp and Fgf signaling pathways lead to the generation of primordial thyroid progenitor cells from mESCs (163), that could be further matured to functional, transgene-free thyroid follicular organoids able to secrete thyroid hormones and rescue hypothyroid mice after transplantation (168). Interestingly, iPSCs-derived human thyroid progenitor cells were obtained from healthy donors and patients with hypothyroidism (168). More recently, functional iPSCs-derived human thyroid follicular cells showed the ability to express thyroid proteins and secrete thyroxine *in vitro* (169).

PARATHYROID GLANDS

Endocrine Function in the Parathyroid Glands

The parathyroid glands are four small glands that produce and secrete parathyroid hormone (PTH) into the bloodstream. Located behind the thyroid gland, parathyroid glands control bodily calcium levels, playing a crucial role in regulating nervous and muscular systems, bone calcium release and calcium reabsorption in the kidney.

Key Pathways Guiding Parathyroid Glands Development

The parathyroids are endoderm-derived tissues that form from the third and fourth pharyngeal pouches in humans (171),

before migrating to the ventral midline of the pharyngeal and upper thoracic region (**Figure 5**). Studies in mice demonstrated a common origin of parathyroid and thymus cells in early organogenesis. The parathyroid-thymus primordia separate around e12.5 in mice during the ventral migration, a process mediated by cell adhesion molecules and Bmp4 signaling (172, 173). Expression of the transcription factor glial cells missing 2 (Gcm2) is essential for parathyroid specification. $Gcm2^{-/-}$ mice lack parathyroid glands and develop primary hypoparathyroidism (174) and human Gcm2 mutations have been associated with dysregulated parathyroid hormonal levels (175, 176). Gcm2 expression and patterning in the developing parathyroid gland is tightly controlled by Shh signaling (177, 178). Shh controls the expression of the transcription factors Tbx1 and Gata3 that, together with Gcm2, restrict the parathyroid cell fate of the third pharyngeal pouch (179). Indeed, $Shh^{-/-}$ mice showed smaller, aparathyroid primordia, due to the inability to activate Gcm2 expression. Moreover, Shh was found to be active in both dorsal endoderm and the adjacent neural-crest derived mesenchyme. Bain and collaborators showed evidence that Shh signals from both tissues promote parathyroid specification and organogenesis (180).

Stem Cells in the Parathyroid Glands

Resident adult stem cells in the parathyroids have been poorly characterized. Human parathyroid-derived stem cells (hPDSCs) were isolated from surgically removed parathyroid glands via enzymatic digestion (181). *In vitro*, selected clones of hPDSCs showed characteristic of adult stem cells as they: (i) could differentiate toward osteogenic, chondrogenic and adipogenic lineages using appropriate induction media, (ii) were positive for mesenchymal stem cell markers and negative for hematopoietic and endothelial markers, (iii) and showed telomerase activity and self-renewal capacity.

Hyperparathyroidism usually occurs due to clonal parathyroid hyperplasia or adenomas of the gland (182, 183). Parathyroid tissue from 20 patients with hyperparathyroidism showed clonal cellular expansion of resident stem cells in both malignant and benign parathyroid tumors, assessed by immunohistochemistry and FAC-sorting for the tumorigenic stem cell makers CD44/CD24 (184). The authors suggested the involvement of a population with stem cell markers in the development of parathyroid hyperplasia.

Stem Cells and Regenerative Medicine in the Parathyroid Glands

Differentiation of parathyroid-like cells from pluripotent stem cells has been achieved *in vitro* using mESCs. Bingham et al. reported the generation of parathyroid hormone (PTH)-secreting cells expressing both intermediate endoderm progenitor markers (Cxcr4, Eya1, Six1, and Pax2) and parathyroid-specific markers (glial cell missing-2 [Gcm2], CCL21, calcium sensing receptor [CaSR], and PTH) (185).

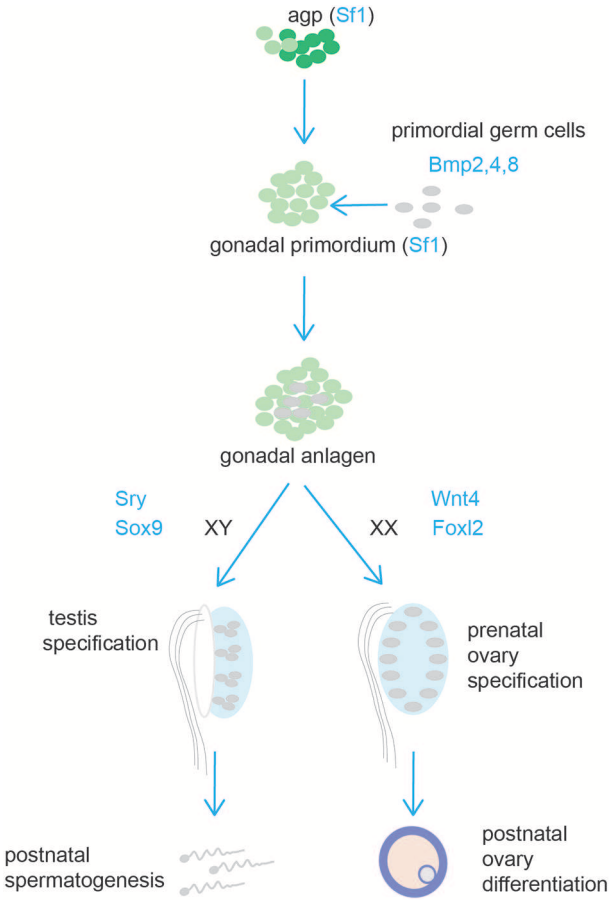

FIGURE 6 | Schematic representation of testis and ovary development. Cells from the adrenogonadal primordium (agp) form the gonadal anlagen. The gonadal anlagen is invaded by migrating primordial gem cells that derive from the region of the forming hindgut. Expression of Sry/Sox9, and Wnt4/Foxl2 determine gonad differentiation into testis or ovaries, respectively.

GONADS

Shared Developmental Stages of the Gonads

The gonads and the adrenal cortex originate from the agp (see above, adrenal cortex section). Gonadal primordia develop as paired thickenings of the coelomic epithelium known as the urogenital ridge (**Figure 6**). Initially, the mammalian gonads develop identically in both female and male embryos. The early mammalian gonad is in fact an undifferentiated primordium composed of bipotential precursor cells that can follow one of two possible fates to become either a testis or an ovary. In mice, development of the urogenital ridge starts at around e11 and continues until e11.5-12.0 when sexual differentiation begins. Primordial germ cells (PGCs) (the precursors of oocytes and spermatozoa in the ovaries and testes, respectively) do not arise within the ridge but migrate from an entirely separate source; at around e7, PGCs are seen in mice in the region of the forming hindgut. The appearance of PGCs is concomitant

with increase in the activity of Bmp2, Bmp4, and Bmp8. Early studies showed that ablation of Bmp4 (186) and Bmp2 (187) in mouse embryo resulted in lack and severe reduction of PGCs number, respectively.

Between around e9.0 and e11.5, PGCs migrate to the genital ridge (188, 189). During migration and after settling in the gonad the PGCs divide mitotically, and their number increases rapidly. By e13.5 the genital ridge contains thousands PGCs from an initial population of 10–100 in mice (190).

TESTIS

Key Pathways Guiding Testis Development

In the male gonad, PGCs give rise to T1-prospermatogonia and enter G0 mitotic arrest, a state in which they remain until after birth (191). T1-prospermatogonia resume proliferation during the first week after birth when they become T2-prospermatogonia and migrate to the seminiferous tubules' basement membrane. These cells give rise to the first round of spermatogenesis concomitant with the establishment of the initial pool of Spermatogonial Stem Cells (SSCs) that maintain spermatogenesis throughout post-pubertal life (192).

Differentiation of testis is marked by polarization of gonadal somatic Sertoli cells that form epithelial aggregates around germ cells. This process leads to the reorganization of the gonad into two compartments: the tubular testis cords (referred to as seminiferous tubules after birth), which consist of Sertoli cells and germ line cells, and the interstitial space between the cords, which contains Leydig cells (producing testosterone under the action of LH) and vasculature. Peritubular myoid cells surround Sertoli cells and deposit a basal lamina at the periphery of the tubular structures (193). Post-natally, Sertoli cells form tight junctions with each other that compartmentalize the seminiferous epithelium into basal and adluminal compartments.

In mammals, testis differentiation depends on gonadal expression of the Y-linked gene *Sry*, a transcription factor initiating Sertoli cell differentiation. By e11 in mice and 41–44 days post-ovulation in humans, *Sry* is detected specifically in Sertoli cells (194). Its expression is restricted between e11 and e13 in mice, whilst it is maintained at low levels during gestation in humans. Interestingly, testis-cord formation occurs between e12.5 and e13, a little later than when *Sry* is detected. Differentiation of testis seems dependent on a critical threshold of Sry-expressing cells. About 10% of Sertoli cells were found not express Sry in testes of chimeric XX↔XY embryos, while gonads composed of <30% Sry-expressing cells developed as ovaries (195). Experiments with transgenic mice demonstrated that the early male marker Sox9 is up-regulated by the transient expression of Sry specifically in Sertoli cell precursors (196). More recently, this was confirmed by the generation of a mouse antibody against Sry (197). Further studies revealed that Sry binds to multiple elements within Sox9 enhancer in mice and it does so along with Sf1 (198). The activation of a network of genes downstream of Sox9 then promotes male development while simultaneously blocking the genes that drive ovarian development [reviewed by (199)].

The fact the Sf1 is essential for gonadal development is widely accepted (200). During the early phase of proliferation (e11.5-12.0) Sertoli cells (and interstitial cells) derive from the division of cells expressing Sf1 of the coelomic epithelium. Sf1 is subsequently downregulated (or completely lost) in the coelomic epithelium and proliferation continue in $Sf1^{-}$ cells at and below the coelomic epithelium to produce only interstitial cells (201).

The importance of Sry in testis development is highlighted by numerous mutations causing sexual-development disorders (202), yet little is known about its regulation. Nevertheless, three key transcription factors, Gata4, Friend of Gata protein 2 (Fog2) and Wt1, have been implicated in the transcriptional or post-transcriptional regulation of the gene [reviewed by (202)].

Stem Cells and Self-Renewal in Testis

Spermatogenesis, the process that throughout the life of males produces sperm, represents a typical example of a supported stem cell system. Spermatogenesis occurs in the seminiferous tubules where spermatogonia that reside on the basement membrane undergo self-renewal divisions and proliferate to form spermatogonial clusters. In rodents, three types of spermatogonia have been identified, namely Type A, intermediate and B. Type A cells are the most undifferentiated and have been classified by morphological analysis into Asingle (As, isolated cells), Apaired (Apr, chain of two connected cells), or Aaligned (Aal, chain of 4, 8 or 16 or more cells), that remain connected by intercellular bridges due to incomplete cytokinesis (**Figure 7**). According to the prevailing theory in the field, known as the 'As model', spermatogonial stem cells (SSCs) are As cells (roughly 0.03% of the total number of spermatogonia) that divide into two daughter Apr spermatogonia which further divide into Aal spermatogonia (203, 204). Aal spermatogonia are the source of primary spermatocytes that will enter meiosis and further develop into haploid spermatids and sperm (205). Nevertheless, further studies have revealed that morphology alone is not sufficient to characterize spermatogonial cells. Undifferentiated spermatogonia were firstly identified as being negative for the surface receptor Kit (206, 207). However, more recent studies have revealed a more heterogeneous characterization of undifferentiated spermatogonia and several markers can now be used to identify SSCs. Comparison of gene expression by whole-mount double-staining of seminiferous tubules revealed that the transcription factor Plzf (promyelocytic leukemia zinc-finger) (208, 209) and the calcium dependent cell-cell adhesion glycoprotein E-Cadherin (210) have identical expression patterns and are present in all A spermatogonia (211). In contrast, the cell surface receptor Gfrα1 and the transcription factor Nng3 showed a more heterogeneous expression, where As, Apr and Aal can be stratified into Gfrα1 single-positive, Gfrα1/Ngn3 double positive, and Ngn3 single-positive. The shorter chains of cells have a greater probability of being Gfrα1 single-positive while longer chains tend to be Ngn3 single-positive (211–213). Moreover, the m-RNA binding protein Nanos C2hc-Type Zinc Finger 2 (Nanos2) promotes the male fate while suppressing meiosis in embryonic XY germ cells (214). Recently, pigs with heterozygous and homozygous mutations in Nanos2 were generated using the CRISPR/Cas9 system. Males pigs had an impaired development

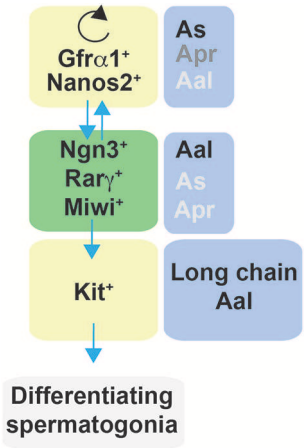

FIGURE 7 | Schematic representation of factors affecting self-renewal and differentiation of spermatogonia in mice. Spermatogonia are classified as Asingle (As), Apaired (Apr), and Aaligned (Aal) according to the number of cells contained in a syncytium. In steady-state, a subset of Gfrα1/Nanos2 expressing cells function as stem cells with the ability to self-renew their population. Gfrα1$^+$ spermatogonia have the ability to generate cells that lose the expression of Gfrα1 and become Ngn3$^+$/Rarγ$^+$/Miwi2$^+$, which can retain the stem cell potential but mostly become Kit$^+$ cells, and therefore are committed to terminal differentiation. In regenerative contexts, Ngn3$^+$/Rarγ$^+$/Miwi2$^+$ can regain Gfrα1 expression contributing to the self-renewing pool.

of testis, specifically homozygous Nanos2 knockout had no germ cells in the presence of intact seminiferous tubules (215). Nanos2 was found to be almost exclusively expressed in As to Apr cells, whereas Nanos3 is detectable in most undifferentiated spermatogonia (As to Aal) (214). This heterogeneity of gene expression has suggested functional heterogeneity within the same cluster of cells (i.e., As, Apr, Aal) (**Figure 7**).

Lineage tracing, live imaging and pulse labeling studies have revealed that differentiation of spermatogonia is more complex that previously described and have led to a revision of the traditional "As model." In steady-state, a subset of Gfrα1$^+$ cells resides on the top of the hierarchy (211) and function as stem cells with the ability to self-renew their population while maintaining a constant number of Gfrα1$^+$ spermatogonia (216). Moreover, Gfrα1$^+$ spermatogonia were shown to continually interchanged between As, Apr and Aal spermatogonia through a combination of incomplete division and syncytial fragmentation. At the same time, all categories of As, Apr, and Aal Gfrα1$^+$ spermatogonia had the ability to generate cells that lose the expression of Gfrα1 and become positive for Ngn3. Ngn3$^+$ cells, independently from the chain length (including As cells), are destined for differentiation and become Kit$^+$ which undergo several further rounds of cell division and are committed to terminal differentiation. Eventually all Kit$^+$ cells are derived from Ngn3$^+$ cells (211, 213). Interestingly, these studies also demonstrated that Ngn3$^+$ cells retain the capability of regaining GFRα1 expression, fragmenting into single cells or shorter syncytia (through breaking of intercellular bridges), and contributing to the long-term stem cell pool. This reversion is rare in homeostasis but becomes more frequent during regeneration, for example after tissue insult by a cytotoxic reagent or transplantation (211, 217). Therefore, Ngn3$^+$ spermatogonia have been referred to as "potential stem cells" (211, 217). In this context, further studies have identified other subpopulations of undifferentiated cells that contribute to the self-renewing pool. Carrieri et al. identified a novel population of Ngn3$^+$ spermatogonia that express the Piwi protein Miwi2, which was shown by cell ablation to be crucial for efficient regenerative spermatogenesis after injury (218). More recent studies have further characterized germline stem cells; for example, Gfrα1$^+$ cells comprise subpopulations that express the transcription factor Pancreatic and duodenal homeobox 1 (Pdx1) (219), the Erb-B2 Receptor Tyrosine Kinase 3 (Erbb3) (220), Inhibitor of differentiation 4 (Id4) (221) and Shisa family member 6 |(Shisa6) (222).

Spermatogonia reside within specialized microenvironments - referred as "niches" - in the basal compartment of seminiferous tubules. A undifferentiated spermatogonia, including Gfrα1$^+$ subpopulation, localize preferentially to the area adjacent to the vasculature network of arterioles and venules that accompanies interstitial cells (216, 223, 224). On making the transition into differentiating spermatogonia, they migrate out of these areas and disperse over the entire basal compartment of the seminiferous epithelium (225). Interestingly, live-imaging revealed that Gfrα1$^+$ cells intersperse between Ngn3$^+$ and Kit$^+$ spermatogonia and are in constant movement in the basal compartment where they actively migrate between Sertoli cells (216). Such a microenvironment can be designated as an open stem cell niche.

Although the molecular mechanisms governing the maintenance and fate of A undifferentiated spermatogonia are yet not fully understood, Sertoli cells are widely regarded as key contributors to the maintenance and differentiation of SSCs, being the main source of the Glial -derived neurotrophic factor (Gdnf) (the ligand for GFRα1 receptor complex), and Fgf2 (226). For example, *in vivo* overexpression and loss-of-function models show that the dosage of Gdnf regulates accumulation/depletion of undifferentiated spermatogonia (227), and *in vitro* stimulation with Ggnf leads to proliferation of GFRα1$^+$ cells (212). Gdnf-mediated proliferation of SSCs involves regulation of Src family kinases, Yes, Lyn and Fyn. Gdnf activates Src family kinases, which further stimulate the phosphoinositide 3-kinase (PI3K)/Akt pathway (228) and up-regulates N-Myc expression to promote SSCs proliferation (229). More recently, further studies revealed that Gdnf production is regulated by the canonical Notch pathway (191, 230) via the transcriptional repressors Hes1 and Hey (231). Fgf2 was shown to expand GFRα1$^+$ cells, although these cells had a distinct phenotype from Ggnf. Fgf2 expanded a retinoic acid receptor γ (Rarγ) expressing subset of cells showing Fgf2 function to be more appropriate for spermatogonial differentiation (226). It is known that retinoic acid (RA), which is synthesized from Vitamin A, is required for spermatogonial differentiation (232, 233). The generation of Kit$^+$ spermatogonia was blocked in the testes of Vitamin A deficient mice and reinitiated after administration of Vitamin A. Lineage-tracing analysis revealed that Ngn3$^+$ cells (but not Gfrα1$^+$), which

specifically express Rarγ, transit to Kit^+ cells rapidly and efficiently in response to RA (234). Fgf2 signaling is dependent on Map2k1 pathway activation to drive SSC self-renewal via upregulation of the transcription factor Ets variant 5 (Etv5) and transcriptional repressor B-cell CLL/lymphoma 6, member B (Bcl6b) (235). Another study indicated that Fgf2 may regulate SSCs proliferation *in vitro* via phosphorylation on Akt and Erk1/2 pathway (236). Finally, it should be mentioned that the activation of the Wnt/β-catenin pathway is thought to drive the transition from $Gfrα1^+$ to $Ngn3^+$ spermatogonia, and signaling is likely initiated by Wnt6, which is uniquely expressed by Sertoli cells (222, 237, 238). Evidence for the importance of Sertoli cells as supporting/regulatory cells also comes from *in vivo* knockout experiments, which identified Sertoli cell specific genes, for example Connexin 43 (cx43), Swi-independent 3a (Sin3a), cytochrome P450 enzymes (Cyp26b1), and Ets related molecule (Erm), some of which play a role in the above pathways, that are essential in supporting germ cell proliferation and/or survival (239–242) and normal spermatogenesis (243).

Another factor which is important for the maintenance of the SSCs pool is oxygen availability. The microenvironment where SSCs reside can be described as being low in oxygen (or hypoxic), a condition that induces the activation of transcription factor hypoxia inducible factor 1α (HIF1α) and can inhibit cell differentiation (244). Staining of adult testis revealed the expression of HIF1α in the stem cell niche along the basement membrane of the seminiferous tubules, while the signal diminishes as cells differentiate, implying a possible role of Hif 1α in germ cell development (245).

Another important question is the extent to which the knowledge acquired using rodents can be applied to humans. Human spermatogonia are characterized by their nuclear morphology and staining with haematoxylin as Adark and Apale spermatogonia (246). Adark spermatogonia are thought to function as reserve stem cells, whilst Apale spermatogonia are progenitors of spermatocytes. Nevertheless, their identity, self-renewal and differentiation abilities are just beginning to emerge. Prepubertal human spermatogonia showed expression of genes important in mouse SSCs regulation (247). Immunohistochemistry on tubule sections revealed human spermatogonial cells share some (i.e., GFRα1) of the markers found in rodents (248). More recently, three independent groups revealed using single-cell RNA-sequencing in human testis clear evidence for heterogeneity and identified distinct cell clusters including SSCs (249–251). These findings provide a starting point for further studies, such as the evaluation of SSC frequency and assessment of SSC activity (252).

Leydig cells, the testosterone-producing cells of the adult testis, derive from stem Leydig cells, spindle-shaped cells that lack steroidogenic cell markers (253). Once formed, Leydig cells rarely die or divide. Nevertheless, their depletion in conditions such as ethane dimethanesulfonate is followed by the appearance of new, fully functional adult Leydig cells (254, 255), which are thought to arise from precursors stem cells (254). Very recently, it was shown in male rats that Fgf-homologous factor-1 (Fhf1 or Fgf12), an intracellular protein, is abundant in Leydig cells and that injection of Fhf1 resulted in Leydig cells regeneration from precursor stem cells in rats where Leydig cells were pharmacologically ablated (256).

In contrast, one study reported that complete ablation of Sertoli cells *in vivo*, either in fetal life (e16.5) or post-natal life, did not lead to repopulation of the testis with new Sertoli cells, indicating Sertoli cells do not possess regenerative capacity and no stem Sertoli cells are present in adult testis (257).

Stem Cells and Regenerative Medicine in the Testis

In recent years, the pluripotency characteristics of SSCs has emerged. For example, the generation of pluripotent embryonic stem like cells was established from neonatal mice testis (258). Similarly, in humans SSCs yielded human testis-derived embryonic stem-like cells (htESLCs) (259, 260); htESLCs were shown to differentiate *in vitro* into derivatives of all three germ layers including neural, epithelial, osteogenic, myogenic, adipocyte, and pancreatic lineages (261). Therefore, SSCs are considered a feasible source for applications in regenerative medicine.

Adverse effect of cancer treatments in men include long-term infertility. If cancer occurs after puberty sperm cryopreservation is the simplest and the most effective method to preserve fertility, nevertheless in prepubertal patients this is not an option. The self-renewal and differentiation abilities of SSCs make these cells a promising tool in the treatment of infertility. To this end, cryopreservation of testicular tissue before chemo-therapy and later autotransplantation of SSCs could theoretically be used to restore fertility. In this context promising results have been obtained in animals. Already in 1994, Brinster and Zimmermann showed that male mice stem cells injected into seminiferoustubules repopulated sterile testes and donor recipients produced mature spermatozoa (262). Human germ cells xenotransplanted to testes of busulfan-treated mouse (with suppressed spermatogenesis) testes survived for at least 6 months and proliferated during the first month after transplantation, however no human-differentiating spermatogonia were identified (263). Similarly, spermatogonia in the testis of a prepubertal boy were shown to migrate to the basement membrane of the mouse recipient seminiferous tubule and were maintained as germ cells (247). Human testicular cells from adult men were isolated, maintained and proliferated *in vitro* for longer than 20 weeks. In 4 out of 6 men, even after prolonged *in vitro* culture, xenotransplantation to mice demonstrated the presence of functional SCCs (264). Importantly, testicular cells from a 6.5- and 8-year-old boys were cultured *in vitro* for at least 15.5 weeks (265). Elhija et al. established a 3D agar culture system which was able to induce germ testicular cells from mice to generate morphologically normal spermatozoa (266). Sato et al. reported the use of an *in vitro* organ culture method that supported complete mouse spermatogenesis (267, 268); subsequently this methodology was used to generate viable sperm, which through micro-insemination resulted in healthy offspring (269). Although it might take a while before the first clinical trial

of SSCs autotransplantation is granted, these pre-clinical data are promising.

OVARY

Key Pathways Guiding Ovary Development

In females, PGCs divide by mitosis with incomplete cytokinesis until around e13.5 in mice and 11–12 weeks in humans producing germ cell cysts (also called germ cell nests) (**Figure 8**) (270). Mitotic division ends and germ cells enter meiosis-I and arrest in the diplotene stage of prophase-I eventually becoming oocytes (271). Germ cell cysts start undergoing breakdown (starting at around e18 until post-natal day 5) to produce primordial follicles (primordial follicle pool) consisting of a single oocyte surrounded by pre-granulosa cells (272–274). At this time, the ovary is reorganized into morphological compartments, the cortex (containing primordial follicles) and the medulla. During a process called folliculogenesis primordial follicles further develop to become potential fertilizable eggs at sexual maturity. During a first phase (the preantral phase), primordial follicles mature into primary and secondary follicles. In a second phase (the antral or gonadotropin-dependent phase) granulosa cells secrete follicular fluid generating fluid-filled antral follicles. After the onset of puberty, activation and further maturation of follicles lead to oocytes ovulation. Just before ovulation, oocytes complete the first meiotic division and begin the second meiotic division which is completed only after fertilization.

Factors that determine ovarian specification include members of the Wnt/β-catenin pathway. Expression of Wnt4 is firstly detected from e10 onwards. When sex specific differentiation begins, Wnt4 is downregulated in males and continues to be expressed in females. Ovaries of $Wnt4^{-/-}$ appeared masculinized (absence of Müllerian duct and development of Wolffian duct) indicating that Wnt4 is a determinant of the female gonad (275). A mutation in the human R-spondin1 (*RSPO1*) gene, was shown to be responsible for female-to-male sex reversal. Moreover, the same study reported that Rspo1 is expressed specifically in XX gonads of mice during the critical stage (e13–15) of gonad differentiation (276). Female $Rspo1^{-/-}$ mice showed male phenotypic features similar to what observed in $Wnt4^{-/-}$ mice (277). Another factor involved in ovarian determination is the transcription factor Foxl2, which is detected in female mice as early as e12.5. $Foxl2^{-/-}$ female mice revealed Foxl2 is required for ovarian follicle formation (278, 279). Moreover, $Wnt4^{-/-}Foxl2^{-/-}$ double knockout ovaries resulted in the formation of testis tubules and harbored well-differentiated spermatogonia (280).

Stem Cells and Self-Renewal in Ovary

For many years, the mammalian ovary was thought to contain at birth a fixed non-renewing pool of oocyte-containing follicles, that are depleted to exhaustion with aging (281). However, in 2004, Johnson et al. (282) challenged this notion. The group counted the number of atretic (degenerating follicles) and non-atretic (healthy) follicles in ovaries of mice. Based on the number of degenerating follicles at any given time under normal conditions they predicted complete exhaustion of the follicle reserve by young adulthood. Nevertheless, the number of non-atretic follicles declined less than expected. Thus, they speculated that germ line stem cells are present in the post-natal ovary of mice. Not surprisingly, this study ignited a debate on the possibility of post-natal neo-oogenesis in mammals (283, 284), and prompted follow-up investigations. Convincing evidence of the presence of female germline stem cells (FGSCs) [also known as oogonial stem cells (OSCs) (285)] in the mammalian ovary was provided for the first time in 2009 by Zou et al. Firstly, putative FGSCs were identified in neonatal and adult mice ovaries by dual immunofluorescence analysis of BrdU incorporation and mouse vasa homolog (Mvh, a germ-cell marker). Subsequently, FGSCs were isolated from neonatal (nFGSCs) and adult (aFGSCs) mice ovaries by two-step enzymatic digestion and immunomagnetic isolation of Mvh-positive cells. These cells were maintained in culture for months and expressed markers of germline cells and proliferation. Furthermore, when GFP labeled aFGSCs were transplanted into ovaries of infertile mice (sterilized by pre-treatment with cyclophosphamide and busulfan), histological evaluation after 2 months showed that ovaries had many oocytes at all stages of development, including GFP-positive oocytes, suggesting that oocytes can be regenerated in sterile recipient females by transplantation of FGSCs. Ultimately, the transplanted mice produced offsprings that had the GFP transgene (286). Following this study, within a short period of time, similar data were generated. A wealth of literature reported the isolation of mitotically active germ cells from adult animals mainly using magnetic-assisted cell sorting or FACS and subsequent culture of the isolated cells (287–295) [also reviewed by (285)]. Importantly, by the use of FGSCs intragonadal transplantation-base approaches, these studies also confirmed the functional capacity of mouse FGSCs to restore ovarian function and produce offsprings (287, 294, 296).

For a few years, possibly partly due to the lack of appropriate methodology (297), the question of whether FGSCs actually contribute to oocytes during *de novo* folliculogenesis in female adult mice under physiological conditions remained unresolved (298–300). Finally, compelling evidence was provided by the use of a tamoxifen-inducible system that traced Oct4- expressing cells permanently marked with enhanced yellow fluorescent protein (EYFP) in post-natal mouse ovaries. This line of evidence proved the existence of active ovarian germ stem cells *in vivo* and their function in replenishing the primordial follicle pool under physiological conditions (301). Soon after, this result was confirmed by inducible ablation of premeiotic germ cells undergoing differentiation into oocytes driven by the promoter of Stimulated by Retinoic Acid gene 8 (Stra8). With this approach, the study demonstarted that new oocytes are formed in ovaries during adult life and that some of these oocytes contribute directly to the pool of oocytes used for natural reproduction (302).

Aside from the numerous animal studies that have populated the literature since the traditional view of a finite pool of oocytes was challenged (282), human investigations have also emerged. A significant progress in the field was made when viable Mvh^{+} cells were isolated from human ovarian cortical tissue and maintained

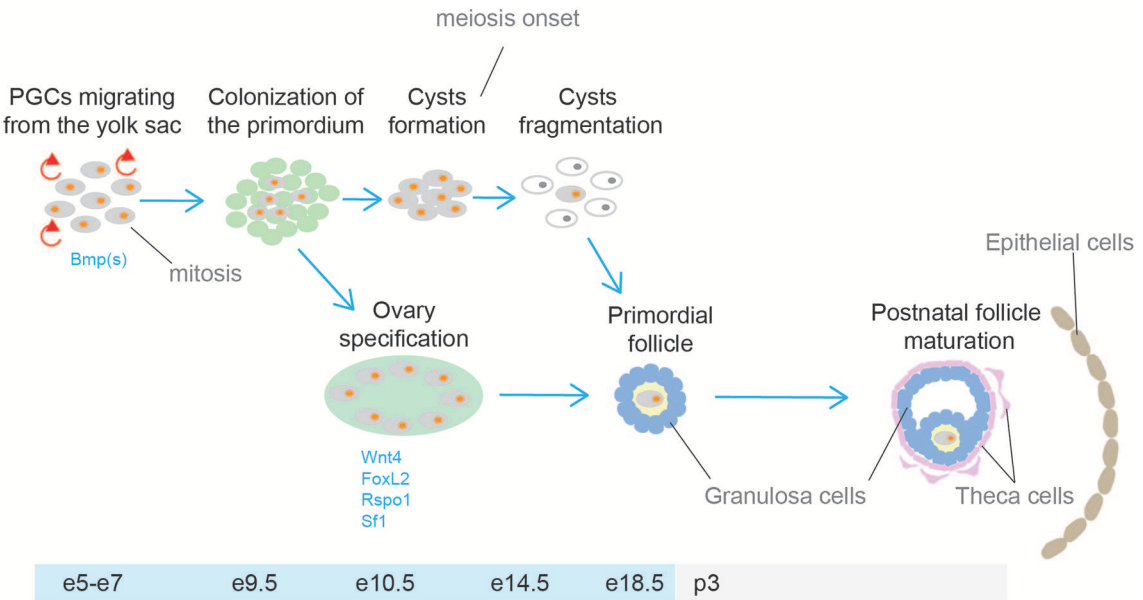

FIGURE 8 | Schematic representation of ovary development. Primordial germ cells (PGCs) colonize the gonadal primordium and undergo mitotic division with incomplete cytokinesis producing cysts. Subsequently, germ cell cysts undergo breakdown to produce primordial follicles, consisting of a single oocyte surrounded by pre-granulosa cells. During sexual maturation, primordial follicles develop further eventually becoming potential fertilizable eggs at sexual maturity.

in vitro where they spontaneously generated oocytes as confirmed via morphological and gene expression analyses and attainment of haploid status. Mvh^{+} cells isolated from adult human ovaries were stably transduced with a GFP expression vector, injected in adult human ovarian cortical tissue biopsies and then xenografted into female mice where formation of follicles containing GFP-positive oocytes was observed (303). Similar results were independently obtained by other groups (304–307).

With multiple laboratories now confirming the existence and functional characteristics of FGSCs, new studies have recently emerged in the attempt to investigate their biological activities and regulatory mechanisms (i.e., self-renewal, differentiation, apoptosis). Zhang et al. reported that Cadherin 22 (Cdh22), a member of cadherin family, is required for FGSCs self-renewal via different mechanisms, including interacting with the Jak–Stat and β-catenin signaling pathways (308). In a follow-up study, the same group showed that Cdh22 interacts with Pik3 to phosphorylate Akt3, which enhanced the expression levels of N-Myc and members of the cyclin family to promote self-renewal. Moreover, Gdnf was also shown to be essential for FGSC self-renewal via a more complicated mechanism: Gdnf-Gfrα1 activates Akt3 via PI3K or Src family kinase (Sfk), and Sfk upregulates its target genes, Bcl6b, Etv5, and Lhx1. Nevertheless, Src, the key intermediate factor for SSCs, was not the functional molecule of Sfk family in the Gdnf signal network of FGSCs (309).

The origin of FGSCs has been debated for years. Soon after their first pubblication, Johnson et al. suggested bone marrow as a potential source of female germ cells (310). However, a later study showed, by the use of transplantation and parabiotic mouse models, no evidence that bone marrow cells, or any other normally circulating cells, contribute to the formation of mature ovulated oocytes both in the steady state and after induced ovarian damage (311). A follow-up investigation by Lee at al. reported conflicting conclusions. Transgenic mice with germline-specific expression of GFP underwent bone marrow transplantation (BMT) after injection with busulfan and cyclophosphamide. BMT rescued fertility, but all offspring derived from the recipient germline (312). More recently, positive results came from injection of human bone marrow–derived stem cells (BMDSC) into mice with chemotherapy-induced ovarian damage. BMDSC treatment resulted in production of higher numbers of preovuolatory follicles, metaphase II oocytes, 2-cell embryos, and healthy pups (313).

While much research of stem cells in ovary has focused on FGSCs, indication of normal somatic stem cells has also been provided. The work by Honda et al. showed evidence in newborn mice ovaries of putative thecal stem cells with the ability to self-renew and differentiate *in vivo* and *in vitro*. These putative thecal stem cells formed characteristic anchor-independent round colonies, and, after stimulation, started to differentiate and show characteristic signs of steroidogenesis. Moreover, after transplantation into ovaries these putative thecal stem cells showed aggregation immediately adjacent to developing follicles and in both theca interna and externa during folliculogenesis (314). Using BrdU incorporation and doxycycline inducible histone2B-green fluorescent protein pulse–chase techniques, Szotek et al. identified a putative somatic stem/progenitor cell in the ovarian surface epithelium (OSE) in the adult mouse ovary. Interestingly, Virant-Klun et al. isolated and characterized putative ovarian stem cells obtained from the OSE of the adult

human ovary in women with no naturally present oocytes and follicles. Small round cells (2–4 μm) with a bubble-like structure that expressed early embryonic developmental markers were separated and cultured *in vitro* where they proliferated, with some cells reaching a diameter of ∼20 μm after 5–7 days (315, 316). Since their discovery, somatic stem cells in the ovary have been of particular interest as these cells may be responsible for ovarian cancer during adult life as well as neo-oogenesis [reviewed by (317)].

Stem Cells and Regenerative Medicine in the Ovary

The finding of FGSCs in adult human ovaries promts the question whether these cells can be utilized somehow to enhance, prolong or restore fertility in women. Although this might seems a far-fetched scenario, reproductive biologists are already working toward this goal. One possiblity is a procedure known as "Autologous germile mitochondrial energy transfer (AUGMENT)," which involves the use of patient matched FGSCs mitochondria to invigorate oocytes of women with a history of poor egg and embryo quality (318). Another option is based on autologous oocytes transplantation approaches to prolong or restore ovarian function. This would include the development of techniques designed to reconstitute human ovarian tissue which would allow the production of functional eggs from FGSCs entirely *ex vivo* (285). While there is a long way ahead, these techniques would offer women faced with fertility challenges a unique opportunity for bearing a genetically-matched child.

ENDOCRINE PANCREAS

Endocrine Function in the Pancreas

The pancreas contains both an exocrine and endocrine component, with the endocrine system accounting for ∼5–15% of the total pancreas. The exocrine pancreas is composed of acinar cells that secrete digestive enzymes into the pancreatic duct and assist with digestion. The five main cell types of the endocrine pancreas are located within clusters of cells known as the islet of Langerhans, which include: glucagon-producing α-cells, insulin-producing β cells, somatostatin-producing δ cells, ghrelin-producing ε cells and polypeptide-producing PP cells. The α, β, δ, and PP cells play critical roles in maintaining physiologic blood glucose levels, while ε cells play a role during fetal development, but they are virtually absent in the adult pancreas. During periods of elevated blood glucose, which occurs after food ingestion, β cells release insulin which signals to the liver, adipose tissue and skeletal muscle to increase glucose uptake. Conversely, during periods of low blood glucose, α cells secrete glucagon, triggering hepatic glycogen breakdown and glucose secretion into circulation. Somatostatin is known to inhibit both insulin and glucagon secretion, and PP inhibits glucagon release in low-glucose conditions. Together, through the concerted release of these hormones, blood glucose levels are able to remain within a physiologic range (319).

Key Pathways Guiding Pancreas Development

During embryonic development, the pancreas emerges from the endoderm, a primordial germ cell layer that gives rise to the digestive and respiratory tracts and their derivative organs. Pancreas development begins around e9.5 in the mouse, at which time the dorsal bud emerges from the Pdx1-expressing region of the posterior foregut, followed by the ventral bud at e10.0 (320–322). Following a 180-degree rotation around the duodenum, the dorsal and ventral buds fuse to form a single pancreatic anlage. The pancreatic epithelium begins to protrude and undergoes extensive remodeling and formation of a web-like structure, or plexus (320). During this time, the surrounding mesenchyme secretes factors such as Fgf10 and Egf, which are critical for pancreas differentiation and proliferation (320, 323, 324). During plexus remodeling, signaling from the surrounding mesenchyme and polarization of epithelial cells lead to the formation of regions with distinct developmental potential: the tip contains the multipotential pancreatic cells (MPCs) and the trunk contains bi-potent endocrine/ductal progenitors (**Figure 9**). The multipotent progenitors express Pdx1, Pancreas Associated Transcription Factor 1a (Ptf1a), NK6 Homeobox 1(Nkx6-1), Carboxypeptidase A1 (Cpa), Myc, and Sox9 and provide a source of cells that can become endocrine, ductal and acinar cells (325–332). Cells of the trunk that undergo endocrine and ductal commitment continue to express *Nkx6-1,* a transcription factor required for β cell development, but lose expression of *Ptf1a*, a transcription factor that becomes restricted to acinar cells (333, 334). As cells commit to the endocrine lineage, the pancreatic epithelium and mesenchyme get connected to the vasculature and become less hypoxic, HIF1a (a marker of hypoxia) expression decreases and cells of the epithelium upregulate *Ngn3*, a basic loop helix transcription factor marking all endocrine progenitors (335–337). In order for cells to undergo endocrine differentiation and upregulate Ngn3 expression, Notch signaling must be downregulated (338, 339). In addition to Notch inhibition, recent work by a number of groups have demonstrated that inhibition of Wnt, Tgfβ and Hippo (through the downregulation of its effector Yes Associated Protein, Yap) signaling further enhances human endocrine differentiation (**Figure 9**) (340–342). The mechanism by which endocrine cells form the islet of Langerhans had been thought to occur as a result of delamination of individual endocrine cells, followed by their subsequent coalescence. This paradigm has recently been challenged by Sharon et al., who proposed that islets form from peninsula-like structures (340, 343–345). In this model, Sharon et al. demonstrated that endocrine cells maintain cellular contact during islet formation: α cells are believed to initially emerge from the trunk region to form the peripheral cells of the islet, followed by the emergence of β cells, which maintain contact with the α cells, in order to form the islet core. At least in the mouse, the final size of the organ is dictated by the number of progenitors that arise during embryonic development and contrary to other organs, such as the liver, the pancreas has very limited proliferative potential in adults (346).

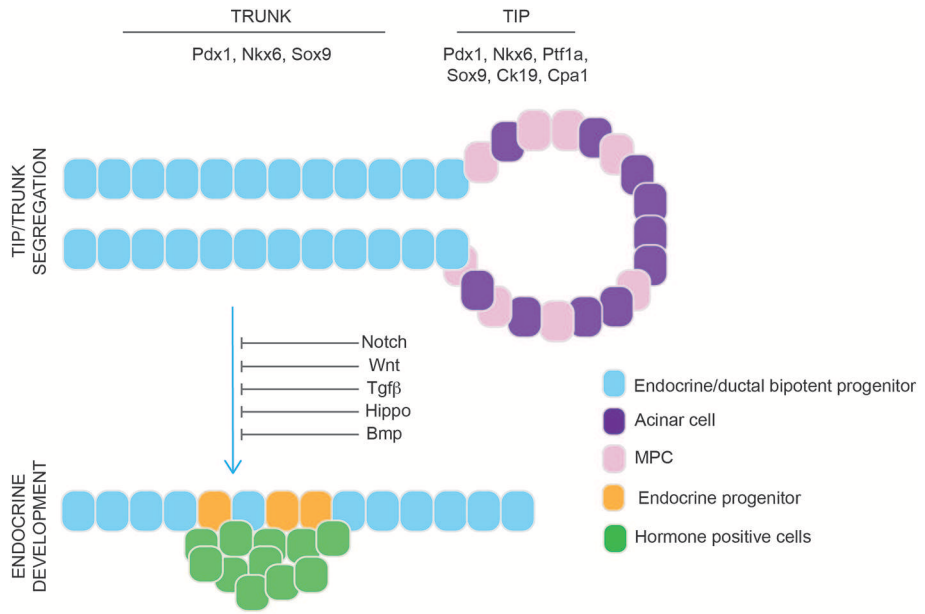

FIGURE 9 | Schematic representation of murine pancreatic development. Multipotential Pancreatic Cells (MPC) (pink) and acinar cells (purple) are located at the tip of the pancreatic epithelium. The trunk contains endocrine/ductal bipotent progenitors (light blue) that migrate out of the epithelium and differentiate to endocrine progenitor cells (orange) which give rise to hormone positive cells (green cells). Endocrine commitment is driven by inhibition of Notch, Wnt, Tgf-β, Hippo, and Bmp signaling pathways.

β Cell Regeneration in the Pancreas

The proliferative capacity of the endocrine pancreas gradually decreases after birth, with β cells showing minimal evidence of proliferation and turnover (347). However, β cell mass increases during pregnancy, suggesting that an increase in β cell mass can occur under physiological conditions (348). Therefore, understanding the mechanisms guiding β cell regeneration has been of particular interest as this knowledge could potentially be leveraged to intentionally increase β cell mass as a treatment for diabetes.

The main mouse models used to study pancreatic regeneration, which have been eloquently reviewed (349), include: pancreatic duct ligation, partial pancreatectomy (removal of 50–75% of the pancreas), chemical induced pancreatitis, and β cell ablation models caused by drug administration, such as alloxan or streptozotocin (349). Partial pancreatectomy (Ppx) has historically been the most common model to study regeneration as it leads to both acinar and islet cell regrowth, making it an interesting model for β cell regeneration (350, 351). Although ductal cells have been identified in some Ppx models to be the source of acinar and β cell regeneration, lineage tracing studies suggest that pancreatic regeneration occurs through self-renewal, where acinar cells generate new acinar cells and β cells generate new β cells (352–355). Using an insulin lineage tracing mouse model to label terminally differentiated β cells, in combination with a Ppx mouse model, Dor et al. identified that the main source of β cell generation is through self-renewal (352). Supporting this idea, using a DNA analog-based lineage tracing method in order to detect each round of cell division, Teta et al. demonstrated that β cells come from pre-existing β cells and not a source of stem/progenitor cells in the adult pancreas (356). Recent publications have shown that β cell heterogeneity exist within the islet, with some β cells having been identified as being more proliferative and immature than other β cells. The heterogeneity that exists could explain the ability of some β cells to be capable of self-renewal, while the less proliferative β cells cannot (357–359).

If, however, β cell regeneration occurs through the proliferation of existing β cells, self-renewal would not explain β cell regeneration in mouse models of type 1 diabetes where near-complete β cell ablation occurs. Interestingly, in a mouse model containing a transgene for an insulin promoter and diphtheria toxin (DT) receptor sequence that can result in up to >99% ablation of β cells following DT treatment, β cell regeneration was shown to occur as early as 15 days post-DT treatment. In this model, using lineage tracing to label glucagon-producing α cells prior to DT-treatment (360), β cell regeneration from α cells was demonstrated. The ability of α cells to transdifferentiate to β cells introduces the idea that endocrine cells retain plasticity, which has been the basis for efforts to identify compounds that could modulate α to β transdifferentiation, but so far with no success (361–363).

Additionally, other studies suggest that insulin expressing cells are in fact the stem cells of the pancreas, being able to generate other exocrine and endocrine tissues (364). More recent work identified pancreatic cells within an islet-depleted cell population, such as ductal tissue, that can generate insulin-expressing cells following transplantation in mice, suggesting a non-endocrine

progenitor-like population exists that can also generate insulin producing cells (365).

Overall these studies indicate that the type of stress caused by pancreatic injury and/or the resulting environment may dictate the source of β cell regeneration, thereby adding to the difficulty in deciphering the mechanisms of β cell regeneration in a natural and physiological manner in humans.

Stem Cells and Regenerative Medicine in Pancreas

In addition to generating β cells through regeneration, using cadaveric donors or human pluripotent-stem cells (hPSCs) offers another source of β cells for therapy. Human cadaveric islets and whole pancreas transplantation have been performed for patients with type 1 diabetes and have demonstrated the ability to normalize glycemia. However, the requirement for numerous donors for each patient, potential requirement for a subsequent transplant, and lack of donors have made hPSC-derived β cells a more compelling source of cells for the treatment of diabetes. The most efficient differentiation protocols to date attempt to recapitulate key stages of pancreas development *in vitro*, including: (1) definitive endoderm formation, (2) posterior foregut patterning, (3) Pdx1 induction, (4) pancreatic progenitor generation ($Pdx1^+$/$Nkx6\text{-}1^+$ cells), (5–6) endocrine commitment ($Ngn3^+$ cells), and (7) β-like cells differentiation ($Nkx6\text{-}1^+Cpep^+$ cells) (340, 341, 366–370). Pancreatic progenitors offer an appealing source of cells for transplantation as they give rise to all cells of the pancreas following transplantation in mice and can normalize glycemia in an streptozotocin-induced diabetic mouse model of diabetes (367, 368, 371). Supporting the use of hESC for the treatment of diabetes, ViaCyte^{TM} has launched several clinical trials to test the safety of pancreatic progenitor transplantation in humans (NCT02239354, NCT02939118, NCT03162926, NCT03163511). Outcomes of these initial clinical trials will provide knowledge that will be the basis of future hPSC-derived pancreatic transplantations. Although PPs have demonstrated the ability to normalize glycemia in mice, generating β cells *in vitro* from hPSC may allow for a more efficient means to normalize glycemia and contain a more committed endocrine population that would not give rise to other cells of the pancreas, such as acinar cells. Therefore, generating hPSC-derived β cells *in vitro* could provide a cell product that would be more efficient for diabetes therapy. In 2014, two groups identified protocols to generate $Nkx6\text{-}1^+$/serum-C-peptide $(Cpep)^+$ cells from hPSC *in vitro*, and although the hPSC-derived β-like cells could release insulin in response to a glucose challenge, further maturation only occurred following transplantation in mice (372, 373). More recent publications have claimed the generation of more functional β cells from hPSC *in vitro*. However, efficiencies of these published protocols remain poor, with some protocols requiring fluorescence-activated cell sorting using a transgenic INS:GFP reporter cell line, and protocol reproducibility has yet to be confirmed (341, 342, 370). Although signaling pathways guiding human β cell differentiation have been identified in these reports and have helped push the field forward, generating a population of cells that is therapeutically relevant will require extensive improvements in the efficiency, purity, reproducibility, and functionality of hPSC-derived β-like cell directed differentiation protocols.

AUTHOR CONTRIBUTIONS

LG and KM contributed to sections on adrenal cortex, adrenal medulla, and gonads. GR-B contributed to sections on thyroid and parathyroids. CG-M, JN, and AG contributed to the section on pituitary. MN and EM contributed to the section on pancreas.

FUNDING

The authors' research was funded by Barts Charity (MGU0436 to LG and KM), Biotechnology and Biological Sciences Research Council (BB/L002671/1 to LG), Rosetrees Trust (M335-F1 to LG), Canadian Institute of Health Research (CIHR to MN), Toronto General and Western Hospital Foundation, Ontario Institute for Regenerative Medicine (OIRM to MN), Medicine by Design (to MN), Banting and Best Diabetes Centre (BBDC to MN), Medical research Council MRC-CRTF (to JN), Action Medical Research (to CG-M), AFM-Telethon (to LG and GR-B), and H2020-MSCA-IF-2018 (to GR-B).

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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 Endocrinology

REVIEW
published: 19 November 2019
doi: 10.3389/fendo.2019.00782

Cancer Stem Cells and Neuroblastoma: Characteristics and Therapeutic Targeting Options

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The majority of embryonal tumors or childhood blastomas derive from pluripotent progenitors or fetal stem cells that acquire cancer stem cell (CSC) properties: multipotency, self-renewal ability, metastatic potential, chemoresistance, more pronounced levels of drug transporters, enhanced DNA-damage repair mechanisms, and a quiescent state. Neuroblastoma (NB) is considered a neuroendocrine tumor and is the most common extracranial neoplasm in children. NB pathogenesis has frequently been associated with epigenetic dysregulation and a failure to implement a differentiation program. The origin, characteristics, and isolation of the CSC subpopulation in NB are still incompletely understood, despite the evidence that this cell subset contributes to disease recurrence and acquired resistance to standard therapies. Here, we summarize the literature regarding the isolation and characterization of CSCs in NB over the past decades, from the early recognition of the expression of stem cell factor (SCF) or its receptor c-KIT to more recent studies identifying the ability of G-CSF and STAT3 to support stem cell-like properties in NB cells. Additionally, we review the morphological variants of NB tumors whose recent epigenetic analyses have shed light on the tumor heterogeneity so common in NB. NB-derived mesenchymal stem cells have recently been isolated from primary tumors of NB patients and associated with a pro-tumorigenic role in the tumor microenvironment, enabling immune escape by tumors, and contributing to their invasive and metastatic capabilities. In particular, we will focus on epigenetic reprogramming in the CSC subpopulation in NB and strategies to target CSCs in NB.

Keywords: cancer stem cells, neuroblastoma, anti-cancer therapies, STAT3, mesenchymal stem cells

OPEN ACCESS

Edited by:
Michaela Luconi,
University of Florence, Italy

Reviewed by:
Erika Peverelli,
University of Milan, Italy
Tullio Florio,
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *05 August 2019*
Accepted: *28 October 2019*
Published: *19 November 2019*

Citation:
Veschi V, Verona F and Thiele CJ (2019) Cancer Stem Cells and Neuroblastoma: Characteristics and Therapeutic Targeting Options. Front. Endocrinol. 10:782. doi: 10.3389/fendo.2019.00782

EMBRYONAL TUMORS AND PLURIPOTENT PROGENITORS

Embryonal tumors (ETs) can be divided into ETs of the central nervous system (CNS) in infants, such as medulloblastoma, medulloepitheliomas, atypical teratoid/rhabdoid tumors, primitive neuroectodermal tumors (PNETs) that occur in brain and spinal cord, neuroblastoma of CNS, embryonal tumors with multilayered rosettes, and other ETs or childhood blastomas that are derived from embryonal or fetal stem cells, such as neuroblastoma, retinoblastoma, rhabdomyosarcoma, nephroblastoma-Wilms' Tumor, ependymoma, and hepatoblastoma (1).

Their origin from pluripotent progenitors confers a high level of heterogeneity to these tumors, which is often the major hurdle for effective treatment. Recently, it has been demonstrated that by using a human pluripotent stem cell (hPSC)-derived tumor model in various ETs, particularly atypical teratoid/rhabdoid tumors (AT/RT), the presence of an embryonic stem cell (ESC)-like signature is associated with histology and poor prognosis of these tumors (2). These findings suggest that the activation of an early embryonic program in the hPSC progenitors may govern the genetic and epigenetic changes leading to the alteration of the differentiation potential of ETs.

In the next paragraphs, we will review one particular ET, neuroblastoma (NB) tumor, and its origin from pluripotent stem cells. We will first define cancer stem cells (CSCs) and their principal characteristics that contribute to chemoresistance in NB, such as plasticity and dormant phenotype, and we will then focus on the origin and isolation of CSCs in NB.

CSCs and Chemoresistance

CSCs are a subpopulation of neoplastic cells characterized by asymmetrical division: a CSC divides into two daughter cells, of which one will remain a CSC (self-renewal) while the other is competent to develop into a differentiated neoplastic cell. These heterogeneous differentiated cells form the bulk of the tumor (3). Chemotherapy is an effective treatment modality to reduce or control tumor growth for many cancer patients. Many of the early chemotherapeutic drugs were cytotoxic molecules whose mechanism of action included blockade of DNA synthesis, interference with nuclear or cytosolic microtubule dynamics, or inhibition of enzymes involved in metabolism (4, 5). However, studies have shown that cancer cells, while initially sensitive, may develop resistance due to the overexpression of multidrug resistance proteins (MDR), drug efflux pumps, the accumulation of mutations in drug target genes (topoisomerase, TP53), and/or the activation of anti-apoptotic pathways (6, 7). Resistance to cytotoxic drugs may also be attributable to a subpopulation of tumor cells that is inherently not sensitive to the cytotoxic drug and the fact that many cancer stem cells have a low proliferative rate (8, 9). A recent study on a genetically engineered mouse model of glioblastoma showed that quiescent CSCs survived treatment with temozolomide and differentiated into highly proliferative cells capable of regenerating the tumor (10). Thus, CSCs may develop resistance to chemotherapy, and thus strategies using only a single cytotoxic treatment may fail, reinforcing the concept that multimodal or targeted approaches are necessary. A recent review (11) gives a more detailed description of the characteristics of CSCs and the genetic, epigenetic, and metabolic mechanisms that contribute to their chemoresistance.

Plasticity and Dormant Cells: CSC Characteristics of Chemoresistance

Plasticity is the capacity of cells to acquire different phenotypes. This characteristic has been shown during the terminal differentiation of adult tissues, which undergo physiologic or pathologic chronic stress conditions, and maintain the ability to change their phenotype. Plasticity is important for tissue regeneration and response to environmental stimuli, but it can be exploited by cancer cells. Cellular phenotypic changes are not only intrinsic to the cancer cell during tumor progression but can be influenced by the tumor microenvironment (signaling molecules, stromal cells, vascular supply, and therapeutic agents). The phenome of a cell is the sum of all its phenotypic traits and may arise due to alterations in the epigenetic thresholds caused by genetic alterations driving tumor progression (12). They may be associated with the induction of a more aggressive cancer cell or a subpopulation resistant to chemotherapeutic drugs, able to escape the immune system and with metastatic potential.

The contribution of plasticity to tumor initiation has been described in terms of two principal models. The first suggests that different tumor types arise from different cancer cells. For instance, cancers that occur in liver can be distinguished in hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) and may derive from the hepatocytes or from the cholangiocytes, respectively. The second hypothesizes that the same original cancer cell may promote different malignant cancer phenotypes through a plasticity process due to genetic alterations and dynamic epigenetic changes (13). Intrinsic cellular plasticity has also been considered as a contributor to cancer dormancy, a mechanism fundamental to the metastatic process.

The time period during which patients do not show any symptoms before cancer relapse is known as cancer dormancy. Field experts have elaborated two general models of dormancy. The first, tumor mass dormancy, is characterized by an interruption of overall tumor growth due to the balance between proliferation and cell death, which may be associated with angiogenic and immunologic dormancy (14–17). The second category, known as cellular dormancy or solitary cell dormancy, refers to the capacity of a single cancer cell to temporarily arrest its cell cycle (18–20). Plasticity and dormancy are two fundamentally interconnected biological states in cancer stem cells, and they affect the CSC chemo-resistance and metastatic potential through metabolic reprogramming, epigenetic alterations, and complex interactions with the immune system.

Of note, in metastatic NB tumors, aggressive cellular clones with CSC characteristics derived from different metastatic sites retain plasticity and adaptive stemness after sequential changes in culture conditions *ex vivo*. These clones undergo dynamic and reversible stem cell-related molecular transitions in response to tumor microenvironment stimuli, as demonstrated by a comprehensive study of epithelial-mesenchymal transition (EMT)- and stemness-associated molecules based on functional and transcriptomic data (21). The continuous molecular rearrangements in these metastatic clones may have determined the heterogeneity of high-risk NB tumors and the occurrence of poor clinical behavior. Interestingly, it has been demonstrated that *in vitro* switching between two cellular phenotypes maintaining stem-like properties could be responsible for chemoresistance and functional heterogeneity of NB. These two cellular states of the murine, Neuro2a, and human, IMR-32 and SK-N-SH, NB cell lines show different capabilities in terms of anchorage-dependent or independent growth and distinct molecular signatures upon different culture conditions *in vitro*.

This can be defined as a new form of "reversible adaptive plasticity," supporting the dynamic nature of NB CSCs and their flexibility to interconversion between different cellular states (22). These recent findings are not surprising considering that the plasticity of NB cells was discovered a long time ago, but previous findings were mostly related to their ability to differentiate along the fetal ganglionic lineage between the neural crest stem cell progenitors and the adrenal medullary precursor cells (23).

Another mechanism of chemoresistance in NB cells is the acquisition of a dormant phenotype that may last decades and may account for late relapse, as was reported in a clinical case report of a patient affected by metastatic NB after 52 years of apparent dormancy (24, 25). MYCN oncogene contributes to the maintenance of stemness in NB CSCs, contributing to their plasticity and chemoresistance. In fact, because of their dynamic response to the tumor microenvironment, CSCs can escape from anti-tumor therapies by entering a dormant state, defined as the G0/G1 quiescent phase of the cell cycle (26).

ORIGIN AND ISOLATION OF CANCER STEM CELLS IN NEUROBLASTOMA

Neuroblastoma (NB), one of the most common childhood extracranial solid tumors, derives from embryonic neural crest cells. NB frequently occurs along the chain of sympathetic neural tissue along the spinal cord and also in the adrenals, since the adrenal medullary cells are neural crest-derived. The most aggressive tumors display amplification of the MYCN oncogene and TERT rearrangements, which is associated with poor survival, even in localized disease. NB has also been considered a neuroendocrine tumor as it derives from neural crest cells (nervous system) and is often localized in hormone-producing organs such as the adrenal gland (endocrine system). This particular origin or localization and its heterogeneity and failure to differentiate, represent the main characteristics of NB and are thought to contribute to the lack of efficacy of multimodal therapeutic approaches. Thus, identifying the properties of the most aggressive NB cancer stem cell (CSC) or tumor-initiating cells (TICs) may pave the way to promising therapeutic strategies. In the next paragraph, we will summarize the history of the isolation of NB CSCs (**Figure 1**).

What Is Already Known About the Origin and Isolation of CSCs in Neuroblastoma?

Some 30 years ago, studies indicated the presence of a subpopulation of stem cell-like progenitors or tumor-initiating cells in NB tumors. Stem cell factor (SCF) and its receptor c-kit are simultaneously expressed in several NB cell lines and tumors (27). SCF and c-kit is a key signaling pathway that is involved in many fundamental biological processes, such as gametogenesis, hematopoiesis, and melanogenesis, and has also been shown to promote cancer growth in tumors such as acute myeloid leukemia, small cell lung carcinoma, and breast carcinoma (28, 29). By treating NB cell lines (SK-N-BE and SH SY5Y) with an anti-c-kit antibody, tumor growth but not differentiation was reduced, thus implicating an autocrine regulatory loop of SCF/c-kit in the growth of NB cells (27). The trophic role of SCF ligand on neural crest cells has been demonstrated by studies on human and animal models with an induction of the outgrowth of the c-kit positive neurites. However, the role of c-kit in NB differentiation is unclear despite findings that c-kit mRNA expression levels were variably expressed in different NB cell subsets. Although NB cells are sensitive to imatinib mesylate (gleevac), a small molecule inhibitor of protein tyrosine kinases including c-kit, it was unclear whether this was a generalized decrease in NB cell growth or resulted from the inhibition of a NB stem-like cell (30).

The Biedler lab first noted heterogeneity in NB cell lines with the isolation of morphologic variants from NB cell lines (31) that had distinct gene expression profiles and were capable of interconversion. N-type (neuroblastic) sympathoadrenal neuroblasts have short neuritic processes, S-type (substrate-adherent) cells had glial or Schwannian-like characteristics, and I-type (intermediate) cells express features of both N- and S-type cells (**Figure 1**). They found the I-type cells represent the most aggressive subpopulation within a tumor and could give rise to N- and S-type NB cells (31, 32). I-type NB cells show properties of stem-like cells, including the expression of stem cell markers such as CD133 and c-kit, and advanced stage NB tumors had higher levels of expression of cells with I-type characteristics than tumors from lower stages. Transcriptional profiling (33) of the I-type cells compared with N- or S-type cells revealed elevated levels of stemness markers including: *Notch*, whose overexpression impairs the neural/glial differentiation maintaining the stem cell and the malignant potential of these cells; PIGF2, the placental growth factor that induces angiogenesis; GPRC5C, which is highly expressed in embryonic stem cells and TRKB and LNGFR, which have been shown to increase the survival, invasiveness, and chemoresistance of this subpopulation (34, 35). Thus, the I-type NB cells have stem cell-like features, with both the potential for self-renewal and for multi-lineage differentiation or bi-directional differentiation into N- or S-type NB cells. This supports the hypothesis that a stem cell or tumor-initiating cell contributes to the growth of NB tumors (32). All of these three components have been found in primary NB tumors and/or isolated from bone marrow aspirates, suggesting that the proportions of these different cell types may play a role in affecting the clinical behavior of the NB tumor. In a small selected cohort of primary NB tumors, a higher fraction of cells with I-type markers was found in tumors from patients that eventually relapsed as compared to tumors that were progression-free (32).

The tumor microenvironment can influence tumor stemness, and in particular, it has been demonstrated that hypoxia enriches stem-like populations and increase their invasive capacity in NB. Specifically, upon the exposure to an "injured conditioned medium" derived from bone marrow stromal cells exposed to hypoxia and oxidative stress, a highly tumorigenic fraction of cells (functionally defined by side population = SP) were found to express high levels of the stemness marker Oct4 and migrate to this conditioned medium *in vitro* and to hypoxic zones in *in vivo* xenograft models. The SP represent a subset of cells

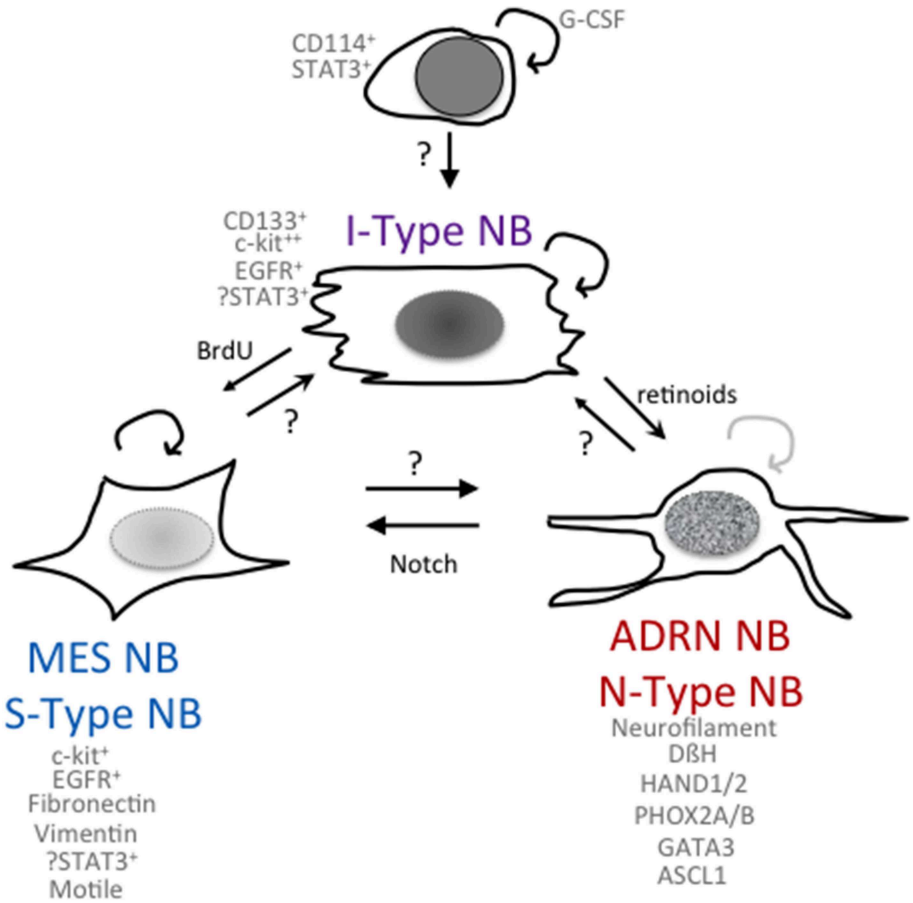

FIGURE 1 | Phenomes of NB cells with cancer stem cell or tumor-initiating properties. Schematic of potential relationships among NB cells with differing phenotypes and functionalities.

isolated from several different tumors endowed with CSC-like properties. The ability of this SP fraction to migrate to the hypoxic/ischemic region of NB tumor suggests that the hypoxic tumor microenvironment may represent the ideal niche for these cells and also for the cancer stem cell (CSC) fraction dynamically subjected to alternative phases of acute and chronic hypoxia, which mimic stress, or injury conditions (36).

These early studies on stem cell properties in NB were limited by their reliance on NB cells that had been adapted to cell culture for many years, and it was unclear how relevant they were compared to a patient's primary, chemo-refractory, or relapsed tumors. David Kaplan's research team and others isolated NB cells from primary tumors and bone marrow metastases and first maintained them in defined media. They used both molecular markers and functional assays to show that advance stage NB tumors contain a high frequency of tumor-initiating cells (TICs), cells with cancer stem cell functionalities. They noted differences between TICs isolated from NB tumors from patients with high- and low-risk clinical parameters and identified CD24 and CD34 as potential markers expressed by TICs that enabled xenograft tumor formation at a lower precursor frequency. In particular, sphere-forming cells derived from high-risk NBs exhibited a higher frequency of self-renewal and capacity to form metastatic tumors in murine xenograft models, even when 10 cells were implanted at an orthotopic location (37). To understand whether there were differences in chemosensitivity, they performed a high-throughput small-molecule screen using these TICs. Two compounds were shown to selectively inhibit NB TICs (DECA-14 and rapamycin) at nanomolar concentrations *in vitro* and to dramatically reduce tumor growth *in vivo*. DECA-14 is a dequalinium analog, C-14 linker, which is an antimicrobial agent frequently used in over-the-counter medicinal products such as throat lozenges or mouth wash. Interestingly, these compounds were ineffective on normal pediatric stem cells (skin-derived progenitors or SKPs) that share similar neural crest stem cell markers and have stem cell-like properties like NB TICs but are not tumorigenic. These results indicated that specific drugs could be developed that selectively inhibit a CSC population while potentially sparing a normal stem cell compartment. This is an important consideration when one considers the targeted treatment of stem cells in pediatric cancers, where it is essential to minimize effects on normal development (38). More recently, the role of c-KIT in NB tumor cells has been revisited with the finding that high levels of c-kit in NB tumors are associated with poor

patient prognosis. Using primary NB tumor TICs or NB cell lines in culture, a transient population (about 5%) of NB cells express high levels of c-KIT, and these cells were found to have a higher growth rate than c-kit-low cells. *In vitro*, selected high c-kit-expressing cells re-establish in NB cell lines or NB-TIC in culture. The finding that vascular endothelial growth factor/prokineticin-1 signaling, a niche factor important in the development of enteric neural crest cells, is important for maintaining c-kit $^+$ NB cells suggests that the microenvironment of a tumor cell affects its aggressiveness. A downstream target of activated c-kit is mTOR, which may provide a mechanistic link to the selective activity of rapamycin in inhibiting NB-TICs (39).

Neural crest development is a finely regulated process that orchestrates the equilibrium between pro-stemness and pro-differentiation signals. The STAT3 signaling pathway is required for correct neural crest development, and its activation, mediated by the granulocyte colony-stimulating factor (G-CSF), has been implicated in the tumorigenesis of neural crest-derived tumors (40). Based on findings that G-CSF also played a role in the survival and differentiation of progenitor cells in the post-ischemic brain (41), the Shohet lab identified an enriched population of G-CSF receptor (G-CSFR/CD114) positive cells in chemotherapy-resistant or relapsed NB (42). These CD114$^+$ cells had a transcriptome similar to early neural crest progenitors, embryonic stem cells, and induced pluripotent progenitors. Their subsequent studies identified that G-CSF via activation of STAT3 promoted NB tumorigenicity and metastasis (43, 44), raising the possibility that small molecule inhibitors of the JAK/STAT pathway that were being developed for other cancers may selectively target these highly aggressive NB tumor cells. AZD1940, a relatively selective JAK/STAT inhibitor, was shown to inhibit NB tumor cell growth *in vitro* and decrease NB xenograft growth *in vivo* (45). However, small-molecule inhibitors specific for the JAK/STAT pathway have been difficult to develop, and many have significant activities against other kinases. A specific STAT3 targeted agent is AZD9150, a 16-oligonucleotide antisense molecule targeting the 3′ region of human STAT3 and inhibiting mRNA and protein production. Systemic administration limits its effectiveness in solid tumors, but a Phase I study did show inhibition of the target STAT3 and reduced tumor growth in Diffuse Large B-Cell Lymphoma (46). In preclinical studies in NB, AZD9150 selectively inhibited cytokine-activated STAT3 signaling yet showed only a modest 20% inhibition of NB cell line growth *in vitro*. Intratumoral injections showed that AZD9150 inhibited STAT3 expression yet had little effect on the primary growth of tumor xenografts. However, utilizing a secondary tumor formation assay for stem cell activity, it was found that AZD9150-treated tumor cells had a decreased ability to initiate secondary tumor formation. Limiting dilution analyses indicated that there was a 10-fold decrease in the precursor frequency of NB tumor-initiating cells from AZD9150-treated primary tumors compared to the frequency of tumor-initiating cells in untreated primary tumors (47). Although it is not clear that the NB TIC in this study is the same as the CD114$^+$ cell in previous studies, it does provide compelling evidence for the presence of a STAT3-expressing NB tumor-initiating cell.

The Role of miRNAs and lncRNAs in the Control of NB Cell Stemness and Differentiation

miRNAs have been implicated in the regulation of stem cell maintenance and neuronal differentiation (48, 49). In several tumors, such as colorectal cancer, breast cancer, and lung cancer, it has been demonstrated that miRNAs modulate the maintenance, proliferation, and reprogramming of CSCs by targeting the principal oncogenic signaling pathways, such as the notch, Wnt/β-catenin, PI3K/AKT, and JAK/STAT pathways (50). Also, in NB tumor, it has been shown that miR-25 modulates CSC stemness through interaction with Gsk3β and activation of the Wnt pathway (51). Moreover, Hsu et al. identified a signature of 25 miRNAs differentially expressed in CD114$^+$- vs. CD114$^-$-expressing NB cell lines. This CD114$^+$ miRNA signature is consistent with the miRNAs overexpressed in the transition of embryonic stem cell (ESC) to neuronal precursors (miR-106b, 21, 25, 30e, 598, 93), and the miRNAs repressed upon neuronal lineage differentiation (miR-25, 106b, 17, 18, 19, 20a, 143, 27) (42).

A number of miRNAs have been shown to regulate various aspects of NB cell differentiation, but a consensus as to which are critical regulators has yet to emerge. These include miRNA-449a, miR-10a, miR-10b, miR-204, miR-506-3p, and miR-124-3p, with miRNA-449a regulating the PKP4 and MFAP4 genes involved in cellular interactions and the control of neuritic extensions (52). MiR-10a is upregulated in the differentiated NB cell lineage after administration of all-trans-retinoic acid (ATRA) (53), and one mechanism may be miR-10b inhibition of Nuclear Receptor Corepressor 2 (NCOR2), a transcriptional co-repressor, which regulates the genes involved in differentiation of NB cells (54). MiR-204 decreases the expression levels of PHOXB2, which plays a central role in modulating the differentiation of NB cells (55). Zhao et al., using a functional high-content image screen, identified 14 miRNAs, including miR-506-3p and miR-124-3p, whose loss was associated with neurite outgrowth (56). Knockdown of the long non-coding RNA (lncRNA), Malat1, induces a reduction of neurite outgrowth and is associated with inhibition of the ERK\MAPK pathway, which blocks the differentiation of N2a NB cells (57).

MESENCHYMAL AND ADRENERGIC PHENOTYPES OF NEUROBLASTOMA

Some 10 years ago, epigenetic alterations, mainly promoter hypermethylation and inactivation of tumor suppressor genes, were recognized to play a major role in several deadly tumors, including colon cancer and glioblastoma. Recently, a large number of mutations have been found to affect the expression/function of a large variety of proteins involved in modifying chromatin. In addition, increasing evidence shows that super-enhancer-driven core regulatory transcriptional circuits drive lineage specificity during normal development and that malignant transformation may co-opt these circuitries to drive tumor formation (58, 59). NB and other pediatric tumors have a low mutational burden, suggesting that alterations in

their epigenetic regulation may be crucial mechanisms in tumor initiation and progression. This dysregulation of the epigenome may contribute to the heterogeneity and plasticity common in ETs and NB in particular. Several recent studies have dissected the NB epigenetic landscape, demonstrating a vulnerability to compounds that target epigenetic enzymes crucial for NB cell survival, proliferation, and differentiation, such as SETD8 and EZH2 (60–62).

Using patient-derived cell lines, Versteeg et al. have molecularly characterized two NB cell types, an adrenergic (ADRN) NB cell type and a mesenchymal (MES) NB cell type (**Figure 1**) (63), that are capable of interconversion and share properties with the N- and S-type NB cells first identified by Biedler and colleagues (see above). *In vitro* experiments on cell lines derived from the same patient showed different mRNA expression levels of the cancer stem cell marker CD133 (64, 65). $CD133^-$ cells propagated as semi-attached spheres and did not migrate, while $CD133^+$ cells grew attached, formed lamellipodia, and were able to migrate. Gene set enrichment analysis showed that $CD133^-$ cells present an adrenergic phenotype associated with high levels of PHOX2A, PHOX2B, and DBH, typical of classic NB cells, while the $CD133^+$ cells showed high levels of SNAI2, VIM (vimentin), and FN1 (fibronectin), which are typical mesenchymal cell markers (63). Using four isogenic cell lines, van Groningen et al. found 485 genes associated with an MES mRNA signature and 369 genes associated with an ADRN mRNA signature. These genes were used to evaluate 33 NB cell lines, and it was revealed that most NB cell lines clustered as ADRN or MES, although some had an intermediate phenotype between MES and ADRN. Interestingly, the signatures of the MES cells were similar to those of human neural crest-derived cell lines, suggesting that MES cells correspond to precursors of the adrenergic lineage (63). To confirm the relationship between MES and ADRN, van Groningen studied the trans-differentiation potential of the two cell types. $CD133^+$ and $CD133^-$ cells from the heterogeneous cell line AMC700B were sorted, and $CD133^+$ was found to lack clonogenic potential but within eight passages contained almost 50% $CD133^-$ cells. The trans-differentiation capacity of $CD133^+$ and $CD133^-$ cells *in vivo* was observed by subcutaneously injecting these cells into mice. Both $CD133^+$ and $CD133^-$ cells formed tumors in mice, although $CD133^-$ cells were more aggressive than $CD133^+$ (63). Overexpression of the homeobox transcription factor PRRX1 (63) or activation of notch in an ADRN NB cell induces transdifferentiation to an MES phenotype, which is more resistant to the cytotoxic agents used in treatment of NB (66). Specifically, van Groningen and colleagues have demonstrated that MES NB cells are more resistant *in vitro* to the commonly used drugs for the treatment of NB, doxorubicin, cisplatin, and etoposide, compared with ADRN NB cells. Furthermore, the number of $PRRX1^+$ MES NB cells increases in tumors treated with standard chemotherapy and in relapsed tumors *in vivo* (63). The downstream activation of notch signaling or the overexpression of PRRX1 may be responsible for the different sensitivity to drugs of the two cellular phenotypes. However, further studies are needed to elucidate the mechanisms involved in NB cell chemoresistance, particularly in the MES phenotype.

Evidence suggests that super-enhancers play a central role in cell identity (58, 59, 67). ChIP-seq analysis for H3K27ac and H3K4me3 histone modification was performed in four MES and five ADRN NB cell lines, including three isogenic cell pairs. Epigenetic bioinformatic analyses indicated that the nine cell lines clustered in two separated groups: MES and ADRN. The same clustering was found in the three isogenic pairs. Some 286 super-enhancers were associated with the MES type, and 276 super-enhancers were linked to the ADRN-type. The genes, such as DBH, CHGA, and DLK1, associated with the ADRN-specific super-enhancers were associated with known markers of adrenergic differentiation, while genes associated with MES-specific super-enhancers included WNT5A, IGFBP2, FN1, and IL13RA1. Induced pluripotent stem cell (iPSC) research has suggested that cell identity is imposed by a core set of TFs that mutually bind one another's super-enhancers, thus creating a feed-forward loop and resulting in very high expression of their associated gene (58, 59, 67). In NB, they found 20 MES TF genes with strong MES-specific super-enhancers, for example, *MEOX1*, *MEOX2*, *SIX1*, *SIX4*, *SOX9*, *SMAD3*, and *WWTR1* and 18 ADRN super-enhancer-associated TF genes, including *ASCL1*, *EYA1*, *GATA3*, *HAND1*, and *SIX*. These super-enhancer-associated TF genes are therefore candidate master gene regulators of the two cell types. These different cell types have differing functionalities, with the ADRN cells being more sensitive to cytotoxic drugs than the MES NB cells and MES cells being more migratory than ADRN cells.

A commonality among the NB cell types with stem cell-like behavior is their enrichment in tumors from chemotherapy refractory or relapsed patients. Thus, it is imperative to study the vulnerabilities of these different cell types. Several studies have shown that N-type or ADRN NB cells are more sensitive to cytotoxic agents than MES or S-type NB cells. However, the differential expression of caspase 8 by S-type but not N-type NB cells is associated with their sensitivity to TRAIL-induced apoptosis (68). Additionally, systemic administration of IFNγ to NB patients has been shown to induce caspase 8 in their tumors and IFNγ treatment of N-type NB cells *in vitro* also induces caspase 8, rendering many NB cell lines sensitive to TRAIL-induced apoptosis (69). Given the propensity of NB cells to transdifferentiate, it will be important to understand the signaling pathways that control these conversions in order to optimize treatment modalities and relapse.

THE ROLE OF MESENCHYMAL STEM CELLS

Mesenchymal Stem Cells in NB (NB-MSCs) Isolated From NB Tumor Mass

Recent evidence highlights the influence that cells in the tumor microenvironment play exert over tumor growth (70, 71) as well as how the tumor cells or secreted tumor factors play at distant tissues that may become sites of tumor metastases (72). Pelizzo et al. (73) proposed that mesenchymal stromal cells (MSCs) isolated from the NB tumor mass promote tumorigenesis by interacting with tumor cells and other stroma cells in the

complex network of the tumor microenvironment (**Figure 2**). Neuroblastoma mesenchymal stem/stromal cells (NB-MSCs) were defined as cells of the tumor microenvironment isolated from seven patients with diagnoses of NB. NB-MSCs showed a spindly morphology and entered into senescence faster than BM-MSCs (control). This, together with cell cycle analyses indicating that NB-MSCs show a higher number of cells arrested in G0-G1, suggests that cells are primed for "dormancy." Dormant tumor cells are characterized by G0/G1-phase arrest and resistance to chemotherapeutic drugs (74). Flow cytometric analyses showed that NB-MSCs have the same level of expression of stem markers Sox2, Oct3\4, and Nanog as BM-MSCs. This suggests that NB-MSCs retain pluripotent potential in the tumor microenvironment. Moreover, NB-MSCs and BM-MSCs share similar levels of expression of membrane markers typical of MSC cells, such as CD73, CD90, CD105, and HLA-I. Since MSCs have the potential to differentiate into many different cell types, Veschi et al. studied the differentiation potential of NB-MSCs (73). NB-MSCs were able to differentiate into osteocytes and chondrocytes but not into adipocytes. This data suggests that NB-MSCs are both morphologically and functionally altered.

MSCs regulate immunity by interacting with innate immune cells (including macrophages, NK cells, and dendritic cells) and adaptive immune cells (including B and T cells) (75, 76). The influence of NB-MSCs on the immune system was studied by co-culturing NB-MSCs with T lymphocytes obtained after treatment with phytohemagglutinin (PHA-P). NB-MSC exerted an anti-proliferative effect on immune cells compared with BM-MSCs. The differing developmental potential and functional effects on T-cells suggest that NB-MSCs have the potential to impact the tumor microenvironment in a manner distinct to BM-MSCs (71). Gene expression profile analysis revealed that NB-MSCs isolated from tumor samples derived from NB patients were enriched in EMT-associated genes compared to BM-MSCs. NB-MSCs showed up-regulated expression of CDH2 and MMP-9 genes. CDH2, also known as N-cadherin, favors trans-endothelial migration, and MMP-9, a crucial enzyme involved in extracellular matrix remodeling, promotes cell invasion and metastasis by collagens and fibronectin degradation. Of note, high expression levels of CDH2 and MMP-9 correlate with poor prognosis in NB tumors (77, 78). Moreover, NB-MSCs expressed higher levels of CXCR4 and reduced levels of CXCL12 compared

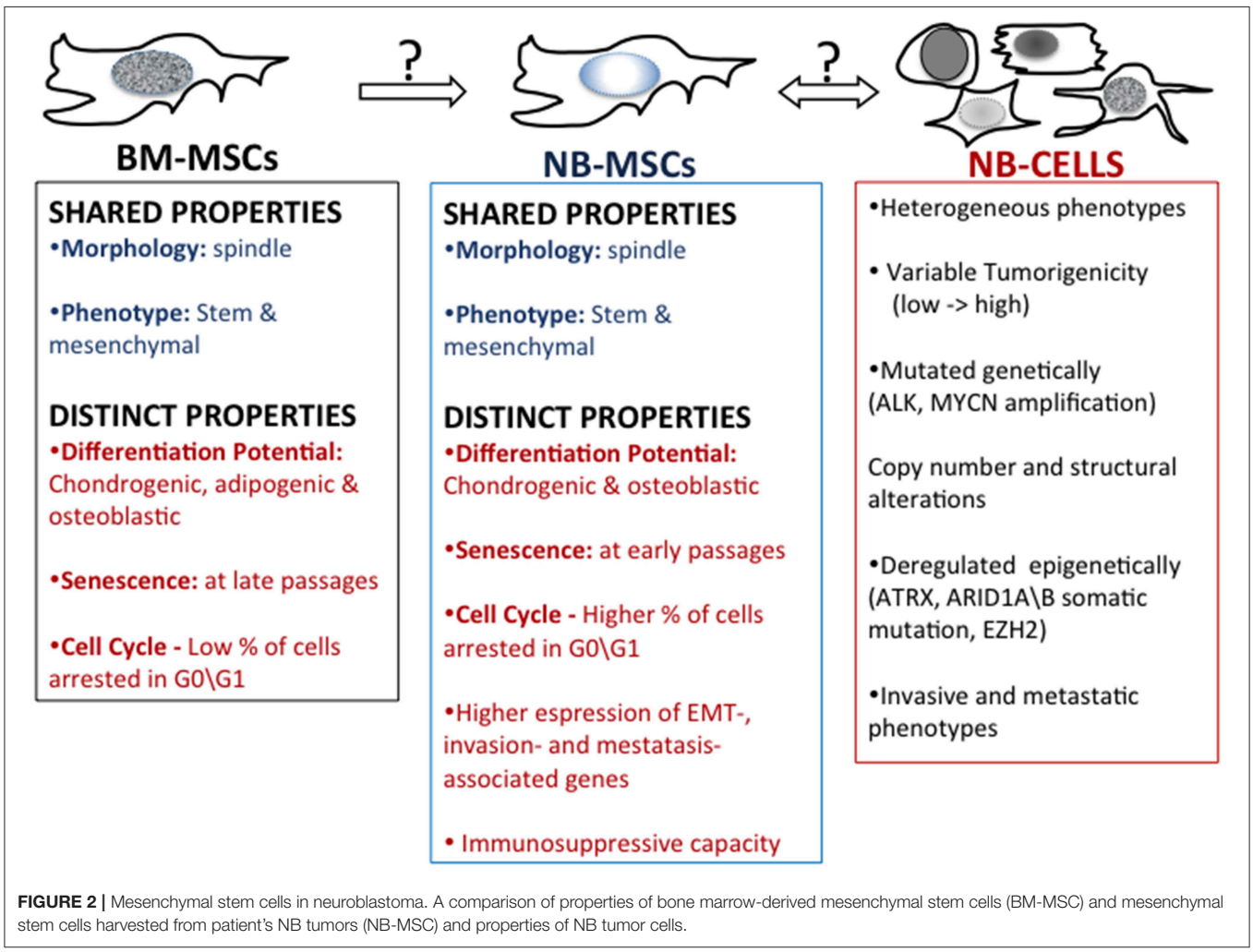

FIGURE 2 | Mesenchymal stem cells in neuroblastoma. A comparison of properties of bone marrow-derived mesenchymal stem cells (BM-MSC) and mesenchymal stem cells harvested from patient's NB tumors (NB-MSC) and properties of NB tumor cells.

to BM-MSCs, supporting the pivotal role of the CXCL12/CXCR4 axis in promoting NB invasiveness (73).

The tumor microenvironment and NB-MSCs play a central role in promoting the immune-escape, invasiveness, and metastatic propensity of NB cells. What role they play in the phenotypic switching of the MES and ADRN NB types remains to be delineated, as does the contribution of the tumor microenvironment to this process. Additionally, how the tumor impacts the complex networks regulating the possible switching between BM-MSCs-NB-MSCs and NB cells remains to be explored. Consideration of these events will be crucial to designing novel approaches targeted to the NB tumor cells and cells within its microenvironments that support its growth and suppress immune surveillance.

CONCLUDING REMARKS

ETs, due to their origin from pluripotent progenitors, are resistant to standard therapies. Novel insights into the molecular mechanisms and genetic and epigenetic changes characterizing the CSC subpopulation in ETs, and in NB in particular, have been provided over the last 30 years. These findings have shed new light on potential different therapeutic applications and strategies for targeting CSCs in NB, from the use of last-generation compounds as STAT3 inhibitors to the use of stromal cells as NB-MSCs as a tool for drug delivery. Additional efforts are needed, especially for the high-risk NBs, which may represent a switch from an embryonic-like condition to malignant metastatic disease due to the co-option of early embryonic programs. Given that epigenetic programs are crucial during differentiation and development, it is easy to understand the impact of epigenetic alterations in NB, which is fundamentally a neurodevelopmental disease due to a failure of neuroblasts to differentiate. NB has been definitely considered a "stem cell tumor." Several epigenetic modifications have been implicated in endowing stemness and self-renewal properties to NB CSCs. Moreover, recently developed epigenetic inhibitors, which may target crucial regulatory processes for the formation and maintenance of the CSC population, have entered clinical trials for pediatric tumors.

AUTHOR CONTRIBUTIONS

VV and CT conceptualized and wrote the manuscript. FV contributed to drafting the manuscript.

FUNDING

VV was a recipient of a fellowship funded by Associazione Italiana per la Ricerca sul Cancro (AIRC) under 5 × 1000 (9979) project. CT was funded by the Intramural Research Program of the National Cancer Institute, NIH.

ACKNOWLEDGMENTS

We apologize to colleagues whose work we have not been able to include due to space constraints.

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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 Endocrinology

MINI REVIEW
published: 26 November 2019
doi: 10.3389/fendo.2019.00773

Metabolic Regulation of Epithelial to Mesenchymal Transition: Implications for Endocrine Cancer

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The last few decades have witnessed an outstanding advancement in our understanding of the hallmarks of endocrine cancers. This includes the epithelial to mesenchymal transition (EMT), a process that alters the morphology and functional characteristics of carcinoma cells. The mesenchymal stem cell like phenotype produced by EMT allows the dislocation of cancer cells from the primary tumor site with inheritance of motility, metastatic and invasive properties. A fundamental driver thought to initiate and propagate EMT is metabolic reprogramming that occur during these transitions. Though there remains a paucity of data regarding the alterations that occur during EMT in endocrine cancers, the contribution of deregulated metabolism is a prominent feature. This mini review focuses on metabolic reprogramming events that occur in cancer cells and in particular those of endocrine origin. It highlights the main metabolic reprogramming outcomes of EMT, encompassing glycolysis, mitochondria oxidative phosphorylation and function, glutamine and lipid metabolism. Comprehending the metabolic changes that occur during EMT will help formulate potential bioenergetic targets as therapies for endocrine cancer metastasis.

Keywords: epithelial-mesenchymal transition, metabolism, endocrine cancers, mitochondria, metastasis

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *01 August 2019*
Accepted: *23 October 2019*
Published: *26 November 2019*

Citation:
Bhattacharya D and Scimè A (2019) Metabolic Regulation of Epithelial to Mesenchymal Transition: Implications for Endocrine Cancer. Front. Endocrinol. 10:773. doi: 10.3389/fendo.2019.00773

The preceding few decades have witnessed an outstanding advancement in our understanding of the hallmarks of cancer including the rare endocrine tumors. These include the signals that sustain their proliferative capacity, prevent apoptosis, enable replicative immortality, and induce angiogenesis (1). Another important consideration that has drawn significant attention is the epithelial to mesenchymal transition (EMT) of cancer cells that is thought to be required for their metastasis (2). The metastatic cascade of cancer cells involves loss of adhesion between cells, which results in their dissociation from the primary tumor and subsequently inheritance of a mesenchymal stem cell like phenotype. This is characterized by motility changes in cell to matrix interactions and plasticity to grow in different tissues (3). EMT results in tumor initiating cells (TICs) having properties similar to cancer stem cells (CSCs), which are associated with initiation, dissemination and recurrence of cancer (4). A fundamental driver thought to initiate and propagate EMT are metabolic alterations that occur during these transitions (5, 6). Though there is little information on the role of metabolism in EMT of endocrine cancers, an understanding in other cancers will provide some potential insight. This review will focus on how metabolic reorganization is an important regulator of EMT with particular regard to endocrine type cancers.

EMT INDUCES TICs

EMT occurs when tumorigenic epithelial cells acquire a mesenchymal stem cell like phenotype by undergoing transcriptional, epigenetic, and metabolic changes. This causes loss of cell to cell adherence, imparting motility to the cells which are free to migrate and invade tissues, a process known as metastasis. Normally EMT is a physiological process necessary for organogenesis during embryonic development and for wound healing (7). However, it is thought that epithelial cancer cells also undergo EMT, becoming TICs, escaping from the primary tumor to enter in the blood circulation as potentially invasive cancer cells. EMT renders stem cell like properties to cancer cells, evidenced by increased self-renewal and survival, anchorage independent growth, and loss of differentiated characteristics (2).

TICs derived from EMT share similar characteristics with CSCs such as metastatic potential, chemo resistivity, anti-apoptotic function, gene expression signature, and metabolic profile (4). To sustain differences in morphology and function, the metabolic needs of EMT cells are different from the epithelial tumor cells from which they were derived, but similar to CSCs. Association between EMT and the acquisition of stem cell like properties have been observed in few endocrine cancers such as pancreatic, prostate, thyroid, and pituitary cancers (8, 9). Shaul et al., identified 44 common metabolic genes in CSCs which are also upregulated during EMT (10). Some of the important genes that are common between CSCs and cells that have undergone EMT are the glycan synthesis genes that modulate cell to cell interactions and gene expression (11, 12), lipid synthesis genes (13) and other genes associated with cancer aggressiveness, cell migration, and metastasis (14, 15). Some of these metabolic genes are also closely associated with EMT of the endocrine cancers.

How the metabolic network is reprogrammed to influence EMT remains unclear. The shift from epithelial to the mesenchymal morphology and loss of cell-cell adhesion is orchestrated by various transcription factor families (EMT-TFs) such as Snail, Twist and Zeb (16). EMT-TFs are known to regulate the transcription of various metabolic genes of different bioenergetic pathways (17). Glycolysis, mitochondrial oxidative phosphorylation (Oxphos), glutamine and lipid metabolism are the main energy producing pathways that maintain cellular harmony, but their metabolic deregulation and reprogramming influence initiation and progression of EMT and metastasis (**Figure 1**).

GLYCOLYTIC REGULATION OF EMT

Unlike normal cells, cancer cells rely more on aerobic glycolysis, also known as the Warburg effect, to meet their elevated demand for energy during proliferation (18). In this regard, ATP is generated by a high rate of glycolysis followed by lactate production from pyruvate in the cytosol instead of pyruvate oxidation in the mitochondria, despite the presence of sufficient oxygen. The induction of genes associated with enhanced glycolytic flux also causes procurement of stem cell like properties in EMT (19–22). Indeed, the importance of aerobic glycolysis to EMT is characterized by the preponderance of deregulated glycolytic enzymes associated with cancer metastasis (23).

It is not clear how aerobic glycolysis favors EMT. One hypothesis is it provides a survival benefit against anoikis, which is a type of cell death that occurs when insufficient matrix attachment generates high levels of reactive oxygen species (ROS) to kill the cell (24). Normally anoikis would be a barrier to metastasis, but it is bypassed by EMT by critically decreasing oxidative metabolism via the Warburg effect to minimize production of ROS (25).

Despite the prominence of deregulated glycolysis in endocrine cancers there are few data available regarding its role in EMT. Proteomic analysis of endocrine pancreatic cells showed predominance of the Warburg effect and enhanced expression of factors involved in glucose metabolism (26). The potential implication for EMT is highlighted by metabolic profiling conducted on the exocrine pancreatic ductal adenocarcinoma (PDAC) that identified a subpopulation having a distinct glycolytic character. This subpopulation was strongly correlated with a stem cell like phenotype, indicative of EMT (27). Exposure of PDAC cell lines to known EMT inducers such as tumor necrosis factor-α and transforming growth factor-β resulted in conspicuous EMT accompanied by enhanced glycolysis and lactate secretion.

Dysregulation of glycolytic enzymes are evident in some endocrine cancers, but their role in EMT is unknown. For example, in pancreatic cancer cells, there is upregulation of the key enzymes of glycolytic metabolism and glucose transporters (28). Moreover, in different subsets of thyroid carcinoma, upregulation of hexokinase 2 (HK2), that phosphorylates glucose to form glucose 6-phosphate was observed (29). Intriguingly, in non-endocrine PDAC, HK2 is correlated with EMT and poor prognosis of the disease (30, 31). It has also been reported that breast cancer cells have augmented HK2 and its dose dependent inhibition by 2-deoxyglucose impede their EMT (32). Another glycolytic enzyme that is upregulated in many cancers is phosphoglucoisomerase (PGI). It mediates conversion of glucose 6-phosphate to fructose 6-phosphate and is associated with motility, migration, metastasis, and EMT in breast and lung cancers (33, 34). This is reflected by PGI mediated induction of EMT-TFs and increased metastatic potential in breast cancer cells (35). Although the role of PGI in EMT has been studied for many cancers, it is yet to be elucidated if it has a role in the endocrine tumor setting. Other glycolytic enzymes linked to metastasis and progression of endocrine type cancers are aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase (36–38).

Also, some cancer cells can facilitate a metabolic shift toward aerobic glycolysis by upregulating glucose metabolism by impeding gluconeogenesis. For example, in several cancers including endocrine pancreatic cancer cells, loss of fructose-1,6-bisphosphatase (FBP1) that catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, is associated with increased cancer stem cell like phenotype and metastasis (39–42). FBP1 has been shown to be a direct target of Snail and Zeb1 transcriptional repression that promotes an increase for invasiveness of cancers cells (39, 43, 44). Restoring FBP1

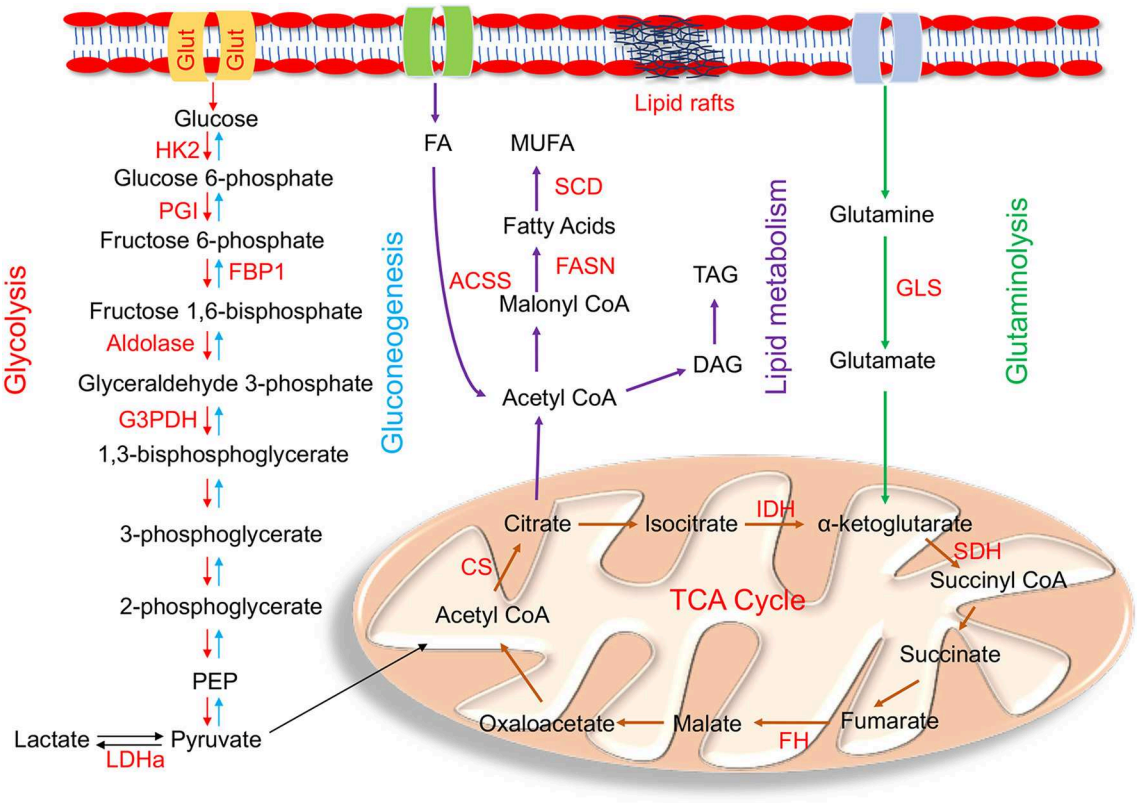

FIGURE 1 | Enzymes within their pathways that are implicated in metabolic reprogramming during EMT. Schematic diagram of enzymes (highlighted in red) within the metabolic pathways of glycolysis, gluconeogenesis, mitochondrial, glutamine, and lipid metabolism alluded in the text that are altered during metabolic reprogramming that occurs in EMT. The main glycolytic enzymes that are upregulated in EMT are HK2 (hexokinase 2); PGI (phosphoglucoisomerase); aldolase, G3PDH (glyceraldehyde-3-phosphate dehydrogenase), and LDHa (lactate dehydrogenase a). FBP1 (fructose-1,6-bisphosphatase), the rate limiting enzyme of gluconeogenesis, is downregulated during EMT. Mutations in the tri-carboxylic acid (TCA) cycle enzymes linked to EMT are IDH (isocitrate dehydrogenase); SDH (succinate dehydrogenase); FH (fumarate hydratase); and CS (citrate synthase). The key enzyme involved in glutaminolysis is GLS (Glutaminase). *De novo* lipogenesis key enzymes involved in lipid metabolism are ACSS (acyl CoA synthetase); FASN (fatty acid synthase); and SCD (stearoyl CoA desaturase). Cells undergoing EMT also have high TAG (Triacylglycerols) levels.

expression, reduced glucose uptake, glycolysis and lactate generation concomitant with increased mitochondrial Oxphos that suppressed EMT (45).

In addition to the glycolytic enzymes, EMT induction occurs through enhanced activity and expression of glucose transporters, Glut1 and Glut3. They are important proteins that regulate glucose uptake, enabling rapidly dividing cells to sustain aerobic glycolysis (22). High levels of Glut1 are characteristic of endocrine cancers including thyroid carcinomas, pancreatic and high grade serous ovarian cancers where inhibition of Glut1 impeded glycolysis mediated cancer progression (26, 38, 46). Similarly, Glut3 expression in non-small cell lung cancer is associated with increased glucose uptake, activation of EMT-TFs and tumor cell invasiveness (47).

Another important factor that is positively regulated with cancer invasiveness and EMT is lactate dehydrogensase a (LDHa), a rate limiting enzyme converting pyruvate to lactate during aerobic glycolysis. Invasive pituitary adenomas exhibit high levels of LDHa both *in-vivo* and *in-vitro* (48). In intestinal-type gastric cancer cell lines, silencing LDHa downregulates Zeb2 and the synergistic decrease of LDHa and Zeb2 decreased cancer invasion, metastasis and poor prognosis (49). Also, in bladder cell lines, high levels of LDHa stimulated EMT leading to migration and invasion of the tumor cells (21) and silencing Ldha inhibited tumorogenecity in pancreatic cells *in vivo* (50). Similar to most aggressive cancers, LDHa was overexpressed in endocrine type cancers such as follicular thyroid carcinoma and papillary thyroid carcinomas compared to non-cancerous tissues (51).

MITOCHONDRIAL REGULATION OF EMT

Although there is a considerable amount of evidence linking mitochondrial dysfunction and cancer, the role of mitochondria in EMT has only recently been expounded (**Figure 2**). Decreased mitochondrial Oxphos capacity is usually associated with EMT of many cancer types and thus associated with promoting aerobic glycolysis. A combined RNAseq and metabolomics profiling of 20 different solid cancers has shown that downregulation of mitochondrial proteins, especially those involved in Oxphos, are associated with metastasis and EMT (52).

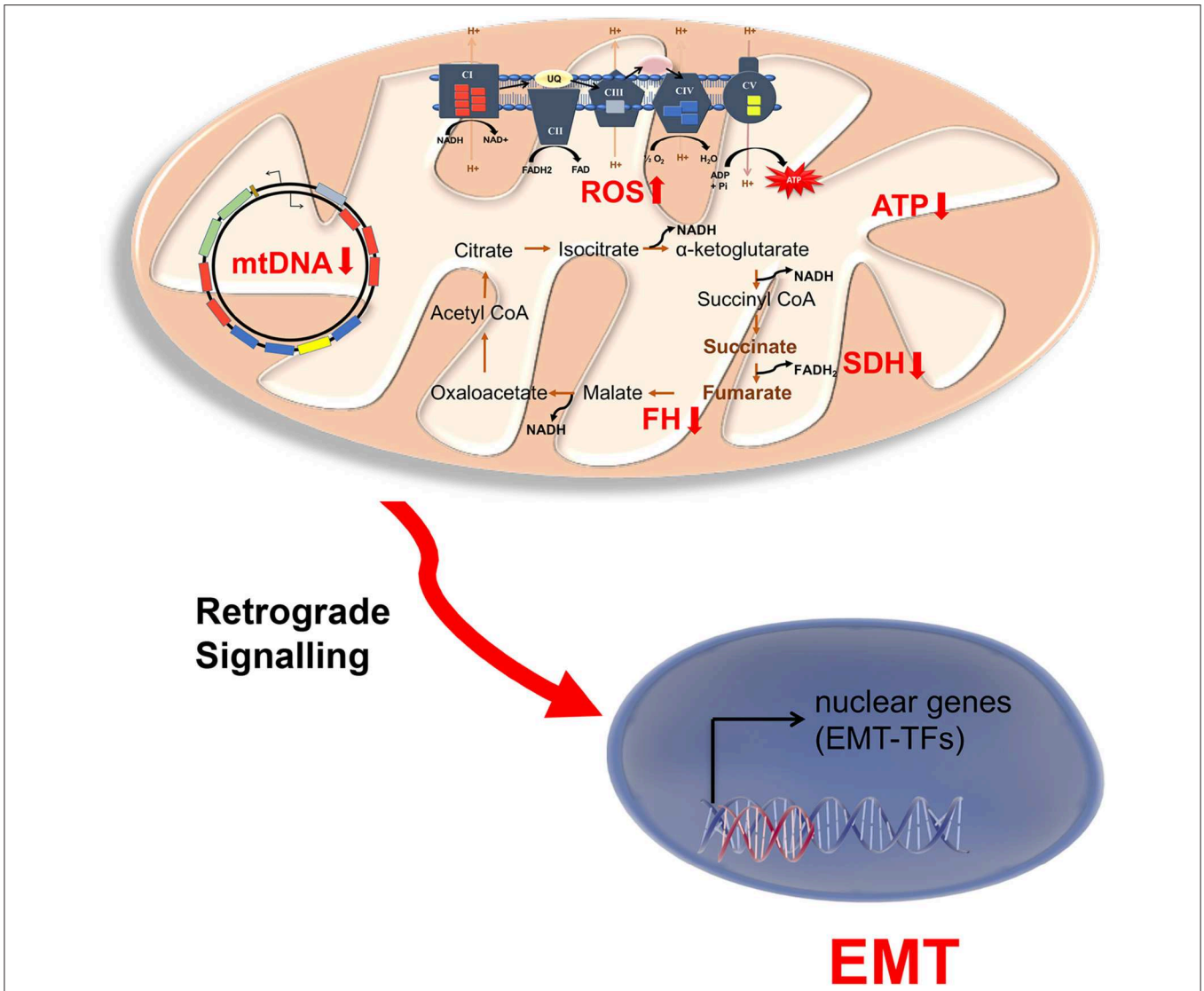

FIGURE 2 | Mitochondrial dysfunction in endocrine cancers. Schematic representation of mitochondrial disruptions associated with EMT in endocrine cancers. NADH and $FADH_2$ produced in the TCA cycle is utilized by electron transport chain (ETC) to produce ATP for Oxphos. As mitochondrial encoded genes are limiting for Oxphos, mutation in mitochondrial DNA (mtDNA) also causes low mitochondrial ATP and increased ROS (reactive oxygen species) production. The increase production of ROS and/or mutation in the TCA cycle enzymes succinate dehydrogenase (SCD) and fumarate hydratase (FH) result in accumulation of succinate and fumarate. These might be involved in retrograde signaling to activate nuclear EMT transcription factors, particularly in neuroendocrine cancers pheochromocytoma and paraganglioma.

The reduction of Oxphos to enhance EMT and metastatic progression is also associated with mutant and/or reduced levels of mitochondrial DNA (mtDNA) (53). Importantly, four out of five electron transport chain (ETC) complexes are made up of functional subunits encoded from mtDNA that are indispensable for ATP production from Oxphos. For example, mutation of mtDNA encoding Complex I subunits of the ETC increases the propensity of oncocytic thyroid cancers (54). The importance of mtDNA mutations to metastasis is highlighted by experiments using cybrid technology, to distinguish the contribution of mitochondrial genome to cancer metastasis (55). Cybrids carrying mtDNA mutations resulted in higher metastasis compared to controls with no mtDNA mutation (55). Reduced mtDNA content might cause mitochondria to nuclear retrograde signaling, whereby the mitochondrial dysfunction triggers nuclei to express genes that activate EMT and metastasis (56, 57). ROS, a mitochondrial byproduct of the ETC, which can damage and mutate mtDNA, was also shown to induce metastasis of tumor cells (58). Intriguingly, decreased ETC complex I and III activity were associated with ROS production in the endocrine thyroid oncocytic carcinomas (38).

The EMT-TF Snail has been shown to reduce ETC complex formation by targeting cytochrome c oxidase (Cox), a Complex IV enzyme responsible for transferring electrons for

mitochondrial respiration. Snail binds to the mtDNA promoter and downregulates the expression of three Cox subunits, COX6c, COX7a and COX7c, reducing the formation of Complex IV, thereby suppressing oxygen consumption and mitochondrial respiration (59).

A master regulator of mitochondrial biogenesis and enhancer of Oxphos, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc-1α), has been shown to either suppress or activate EMT and metastasis depending on cancer types and metabolic cues of the tumor microenvironment (60). In endocrine thyroid cancers, Pgc-1α has been found to be downregulated concomitant with increased glycolytic flux (61). However, pancreatic CSCs have high levels of Pgc-1α compared to differentiated pancreatic tumor cells, suggesting that the association between Pgc-1α and EMT is dependent on stages and metabolic plasticity of cancer (62). Indeed, prostate cancers that are highly heterogeneous, provide different landscapes for Pgc-1α functioning. In some prostate cancers, Pgc-1α via an ERRα dependent mechanism increases the overall oxidative metabolism and blocks EMT and metastasis (63). On the contrary, androgen mediated AMPK activation causes prostate cancer cell growth through Pgc-1α mediated increase in mitochondrial biogenesis, glucose oxidation and fatty acid oxidation (64). Similarly, in non-endocrine breast cancer cells Pgc-1α mediated mitochondrial Oxphos and biogenesis facilitates metastasis (65). Silencing Pgc-1α impaired the invasion and metastasis without affecting the proliferation of the primary tumor.

One of the important metabolic pathways in mitochondria is the tri-carboxylic acid cycle (TCA) that provides reducing agents for Oxphos and metabolites for various biosynthetic pathways. Evident in endocrine cancers are mutations of TCA cycle operational enzymes, which are linked to EMT (66). The neuroendocrine tumors, pheochromocytoma and paragangliomas are associated with mutation of succinate dehydrogenase (SDH) that converts succinate to fumarate (66). In particular, a mutation of SDHb a subunit of SDH, is thought to alter glucose and glutamine utilization and cause epigenetic modifications that results in EMT (67–69). Moreover, SDH mutation is hypothesized to cause consumption of extracellular pyruvate to maintain the Warburg effect conducive for cell growth and thus EMT potential (70). This is through pyruvate carboxylation for aspartate biosynthesis, which utilizes glucose derived carbons produced in glycolysis. SDHb related changes in pheochromocytomas and paragangliomas are coupled to bioenergetic reprogramming where decrease in complex II of the ETC caused compensatory increase in Complexes I, III and IV with concomitant decrease in ATP levels (71). SDHb mutations cause upregulation of metastatic genes and epigenetic silencing of cell adhesion protein, keratin 19, leading to EMT and rendering the tumor cells more aggressive and invasive (67, 72).

Mutation of another important enzyme of the TCA cycle, fumarate hydratase (FH), which converts fumarate to malate, is also implicated in metastasis of pheochromocytoma and paraganglioma. This is due to accumulation of high levels of fumarate and succinate that act as oncometabolites by enhancing epigenetic modification of DNA hypermethylation, ROS production and changes in the mitochondrial structure (66, 73–76). In particular, fumarate accumulation has been shown to inhibit α-ketoglutarate-dependent dioxygenases that are involved in DNA and histone demethylation. Recently, fumarate buildup in renal cancers has been shown to cause EMT by inhibiting Tet dioxygenase mediated demethyation of antimetastatic miR-200 which is a known activator of metastasis and EMT (77).

In the TCA cycle, isocitrate dehydrogenase that converts isocitrate to α-ketoglutarate by decarboxylation, is also associated with many cancers (78). An oncometabolite formed due to mutation of isocitrate dehydrogenase, 2D hydroxyglutarate, induced metastasis and EMT in colorectal cancers by increasing Zeb1 expression (79). Finally, EMT is also correlated with citrate synthase (CS) activity, which converts oxaloacetate to citrate. CS induces morphological and metabolic alterations resembling EMT in human cervical carcinoma cells. It might also play a role in endocrine pancreatic cancers that are characterized by increased CS activity (80, 81).

Apart from glucose, many cancer cells also rely on glutamine as a nutrient source (82, 83). Glutamine metabolism replenishes the pool of TCA cycle intermediates (anapleuresis) that might be exported out of the mitochondria (cataploresis) for biosynthesis of building blocks. Thus, the use of glutamine for anapleuresis is very important for cancer cell proliferation and sustainability. Many carcinomas have an upregulation of the enzyme glutaminase 1 (GLS1), that catalyzes the first reaction of glutaminolysis, glutamine to glutarate for anapleuresis (84–86). In prostate cancer, loss of GLS1 activity was associated with decreased rate of glucose utilization and cancer progression (87). The potential importance of GLS to EMT in endocrine cancers, is highlighted by its inhibition that blocked EMT progression and metastasis by repressing Snail in non-endocrine colon and breast cancers (86). Recently drugs targeting glutaminase activity has been successfully used in preclinical trials to impede the invasiveness of tumor cells, thus counteracting EMT (85, 88, 89). Contrary to the role of GLS1, some studies have shown that over expression of GLS2, the mitochondrial isoform of GLS1, reduces tumor progression, invasion and poor prognosis as in human hepatocellular carcinoma tissues (90). It has been shown to cause repression of EMT through downregulation of Snail both *in vitro* and *in vivo* (91). NMR based metabolic profiling revealed that compared to sporadic tumors, pheochromocytoma and paraganglioma had higher levels of glutamine indicating the influence of glutamine metabolism in pathogenesis of these endocrine cancers (66, 92).

LIPID METABOLISM AND EMT

In addition to aerobic glycolysis, *de novo* lipogenesis is augmented in many cancers, but little is known about the involvement of lipid metabolism in EMT. Lipidemic analysis on prostate cancer cells that had undergone EMT, showed increased triacylglycerols and fatty acid synthase (FASN) (93). FASN is an important enzyme in fatty acid synthesis catalyzing the NADPH-dependent condensation of acetyl-coenzyme A (CoA) and malonyl-CoA to produce palmitate. FASN, prominent

in various cancer types, can increase expression of epidermal growth factor receptor (ErbB) that promotes EMT of breast cancer cells and invasive ductal carcinomas (94). High FASN expression levels in pancreatic cancer and papillary thyroid carcinoma patients is associated with poor survival rate, but its importance to EMT is unknown (95, 96).

Overexpression of acyl-CoA synthetases (ACSs), which convert long chain fatty acids into acyl CoA and stearoyl CoA desaturase-1 (SCD), induced EMT and increased cellular migration and invasion in colorectal cancer (97). Their dysregulation for EMT in endocrine cancers has not been studied. However, metabolic stress such as hypoxia or caloric restriction, enhances ACS expression which is involved in the growth of pancreatic cancer cells (98). SCD, the rate limiting enzyme converting saturated fatty acids into monounsaurated fatty acids maintains cellular homeostasis by regulating their ratio. Dysfunctional SCD results in high levels of monounsaturated fatty acids that is observed in several endocrine cancers. Also, it is considered as a predictive marker for metastasis and possible EMT (99). Importantly, inhibition of SCD in prostate cancer blocked tumor gowth and survival (100).

The plasma membrane (PM) integrity also has a crucial role in mediating EMT. This is evident by marked differences in the lipid composition of PM between normal epithelial cells and the cells that have undergone EMT (101). Cancer cells undergoing EMT might be influenced by a number of signaling pathways that are activated by extracellular ligands or receptors, which are attached to the PM. Recent evidence has shed light on reorganization of the PM that caused destabilization of lipid raft domains, which imparts motility and metastatic properties to the cancer cells undergoing EMT (102). Stabilization of the lipid rafts have been made possible by pharmaceutical and nutritional interventions that result in metastasis (103, 104). The importance of PM composition affecting EMT is also reinforced by the influence of cholesterol, whereby altering cholesterol content of plasma membrane is associated with increased mesenchymal stem cell like phenotype (102). Indeed, depletion of cholesterol content in mesenchymal like tumor cells by statin reduced PM fluidity, cell motility and metastatic potential (105).

CONCLUSION AND PERSPECTIVES

EMT causes dissociation of cancer cells from primary carcinomas, which migrate and disseminate to distant sites. In this review, we have summarized how cancer cell metabolic reprogramming reflected by changes in glycolysis, mitochondrial Oxphos, glutamine and lipid metabolism are involved in EMT. For many cancer types it is not known if one or more metabolic pathways are necessary for EMT and metastasis, nor if they operate independently or together within a metabolic framework. Moreover, it has not been resolved if the same metabolic reprogramming in different cancer types have opposite effects on EMT and metastasis. Although, endocrine cancers are one of the most aggressive cancers types, there is a gap in research connecting its metabolic deregulations and EMT. Elucidation of the compromised metabolic targets will help in identifying potential therapeutic targets.

Finally, recent advancement in the understanding of EMT, has unveiled that metastatic cascade is multifaceted, where EMT is required for tumor initiation and invasiveness, but mesenchymal to epithelial transition (MET) is crucial for the later stages of metastasis, particularly during metastatic colonization. Despite considerable understanding in metabolic regulation of EMT, there is not much known about metabolic control of MET. As MET occurs at a distant site from primary tumors and are functionally different than cells that have undergone EMT, it is likely that these cancer cells have a completely different metabolic reprogramming.

AUTHOR CONTRIBUTIONS

AS and DB both conceived and designed the idea and wrote the paper. DB and AS collected the information for the manuscript.

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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 Endocrinology

REVIEW
published: 20 February 2020
doi: 10.3389/fendo.2020.00054

Experimental Evidence and Clinical Implications of Pituitary Adenoma Stem Cells

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Edited by:
Natalia Simona Pellegata, Helmholtz Center Munich, Germany

Reviewed by:
Sabrina Corbetta, University of Milan, Italy
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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 30 August 2019
Accepted: 28 January 2020
Published: 20 February 2020

Citation:
Würth R, Thellung S, Corsaro A, Barbieri F and Florio T (2020) Experimental Evidence and Clinical Implications of Pituitary Adenoma Stem Cells. Front. Endocrinol. 11:54. doi: 10.3389/fendo.2020.00054

Pituitary adenomas, accounting for 15% of diagnosed intracranial neoplasms, are usually benign and pharmacologically and surgically treatable; however, the critical location, mass effects and hormone hypersecretion sustain their significant morbidity. Approximately 35% of pituitary tumors show a less benign course since they are highly proliferative and invasive, poorly resectable, and likely recurring. The latest WHO classification of pituitary tumors includes pituitary transcription factor assessment to determine adenohypophysis cell lineages and accurate designation of adenomas, nevertheless little is known about molecular and cellular pathways which contribute to pituitary tumorigenesis. In malignant tumors the identification of cancer stem cells radically changed the concepts of both tumorigenesis and pharmacological approaches. Cancer stem cells are defined as a subset of undifferentiated transformed cells from which the bulk of cancer cells populating a tumor mass is generated. These cells are able to self-renew, promoting tumor progression and recurrence of malignant tumors, also conferring cytotoxic drug resistance. On the other hand, the existence of stem cells within benign tumors is still debated. The presence of adult stem cells in human and murine pituitaries where they sustain the high plasticity of hormone-producing cells, allowed the hypothesis that putative tumor stem cells might exist in pituitary adenomas, reinforcing the concept that the cancer stem cell model could also be applied to pituitary tumorigenesis. In the last few years, the isolation and phenotypic characterization of putative pituitary adenoma stem-like cells was performed using a wide and heterogeneous variety of experimental models and techniques, although the role of these cells in adenoma initiation and progression is still not completely definite. The assessment of possible pituitary adenoma-initiating cell population would be of extreme relevance to better understand pituitary tumor biology and to identify novel potential diagnostic markers and pharmacological targets. In this review, we summarize the most updated studies focused on the definition of pituitary adenoma stem cell phenotype and functional features, highlighting the biological processes and intracellular pathways potentially involved in driving tumor growth, relapse, and therapy resistance.

Keywords: pituitary, cancer stem cells, self-renewal, tumorigenesis, drug sensitivity

ADULT STEM CELLS VS. CANCER STEM CELLS

Tissue-resident adult stem cells are undifferentiated cells able to self-renew for long periods by asymmetric division, and to generate the bulk of differentiated cells within tissues to support organ repair and regeneration (1). Adult stem cells fate and functioning greatly depend on the stem cell niche, a specialized surrounding microenvironment which control stem cell homing and the balance between self-renewal and differentiation (2). In general, healthy stem cells show a stable diploid genome, slow growth rate or quiescence, granting their persistence for the lifetime of the organism, and express pattern of surface markers distinctive for stem cells in different organs (3). Before having a complete phenotype maturation, stem cells develop into partly differentiated progenitors or committed cells (4).

Tumors are characterized by cell heterogeneity including cell populations with stem-like features, whose identification have revolutionized the concept of cancer origin: according to the hierarchical model of tumorigenesis, tumors arise from a pool of self-renewing cancer stem cells (CSCs) which initiate and maintain tumor growth, and establish a differentiation hierarchy by generating non-tumorigenic differentiated cancer cell bulk. Stem cells, immature progenitors, or more differentiated progenies that develop oncogenic mutations, followed by the accumulation of multiple genetic, epigenetic and microenvironmental hits, acquire a fully transformed phenotype which, associated to typical stem cell features, gives origin to tumor specific CSCs (5). Importantly, hierarchical organization is reversible due to cell plasticity of differentiated cancer cells which can de-differentiate to regenerate CSCs and sustain tumor progression (6). CSCs have been identified in both hematopoietic and solid tumors, showing unlimited self-renewal and proliferative potential also in the absence of extracellular cues, pluripotency, expression of stemness markers, and aneuploidy. CSCs, as well as normal stem cells, have improved DNA-repair and detoxification mechanisms, and high activity of anti-apoptotic and pro-survival pathways. Thus, CSCs are able to resist to anticancer drugs determining chemoresistance, and supporting tumor progression and relapse (7). Migratory capacity is also a common feature of normal stem cells and it is functional to cell homing and tissue regeneration (8), whereas the high motility of CSCs favors their invasive and metastatic capacity. Differently from normal stem cells, during tumor progression CSCs build their own altered niche, recruiting neighboring stromal, mesenchymal, endothelial, and immune cells; this delicate microenvironment further protects and favors CSC maintenance and proliferation (9). Although normal stem cells and CSCs share key signaling pathways and transcription factors which regulate self-renewal and determine cell fate (Wnt/β-catenin, BMPs, Sonic hedgehog (SHH), Notch, Sox2, Oct4, Nanog, Bmi-1, PI3K/Akt cascade), in normal stem cells this signaling tightly depends on extrinsic growth factor signals, while in CSCs, dysregulation or hyperactivation of transcription factor-mediated pathways drives abnormal self-renewal and tumorigenesis, sustaining differentiation into highly proliferative cancer cells (10).

Normal stem cells can be experimentally identified and isolated according to the expression of specific stem cell antigens (e.g., SOX2, NANOG, OCT4, CD44) and the *in vitro* clonogenic and differentiation potentials; however, CSC characterization, beside these parameters, requires the establishment of the tumorigenic potential in *in vivo* models.

CSC-dependent intratumor cellular heterogeneity has been typically considered the basis for malignant tumor development, progression and spreading. However, in recent years, several studies reported the characterization of CSC-like subpopulation also in benign tumors [i.e., meningiomas (11) and pituitary adenomas (12)], highlighting the possibility that, similarly to normal organogenesis during development, tumorigenesis of all kinds of neoplasia, either benign or malignant, requires the activity of stem-like cell subpopulations (13).

AN OVERVIEW OF THE HUMAN PITUITARY GLAND

Pituitary is the main endocrine regulatory gland, which transmit hypothalamic signals to target organs through systemic hormone secretion. It is composed of anterior (adenohypophysis) and posterior (neurohypophysis) lobes with distinct embryological origin, morphology and functions; the endocrine center is adenohypophysis whose specialized cell types are identified according to the secreted hormone: corticotroph cells produce adrenocorticotropic hormone (ACTH); gonadotrophs, follicle-stimulating (FSH) and luteinizing (LH) hormones; lactotrophs, prolactin (PRL); somatotrophs, growth hormone (GH), and thyrotrophs, thyroid-stimulating hormone (TSH). Undifferentiated pituitary stem cells (PSCs) exist within anterior pituitary and give rise to the three main progenitor lineages, characterized by the expression of essential transcription factors for lineage commitment and terminal differentiation: (i) PIT1-positive PSCs differentiate into GH/PRL/TSH-expressing cells, (ii) TPIT-cells originate the ACTH-secreting subpopulation, while (iii) LH/FSH-secreting cells derive from SF1 lineage (14). Secretory cell types also include somato-lactotrophs which release both GH and PRL, and are considered less differentiated precursors of the mature cells releasing the specific hormone. Neurohypophysis receives peptide hormones (anti-diuretic hormone and oxytocin) via axonal terminals of neurons projecting from the hypothalamus, and releases them under the hypothalamic control.

Adenohypophysis also contains endothelial cells and pericytes, and non-endocrine S100β-positive folliculo-stellate (FS) cells which produce growth factors and cytokines regulating

Abbreviations: HES, Hairy Enhancer of Split; HEY, HES-related with YRPW motif; Smo, Smoothened; Ptch1, Patched; GLI, Gli zinc finger transcription factors; LATS1/2, large tumor suppressor homolog 1 and 2; YAP, Yes-associated protein; TAZ, Transcriptional co-activator with PDZ binding motif; TEAD, Transcriptional enhanced associate domain; FZD, Frizzled; LEF, Lymphoid enhancer factor; LGR, Leucine rich repeat containing G protein-coupled receptor; TCF7L1, Transcription factor 7 like 1; CXCR4/7, CX-chemokine receptors 4 and 7; TGFRB2, Transforming Growth Factor Beta-Receptor 2; TWIST1, Twist-related protein 1; ZEB1, Zinc-finger E-box-binding homeobox 1.

and sustaining hormonal cell activity by integrating paracrine signals (15). FS cells maintain pituitary homeostasis, favoring the maturation of stem/progenitor cells and have been considered as putative PSCs (16, 17). Indeed, the fine tuning of number and activity of secretory cell types in different physiologic conditions requires high plasticity of the pituitary gland and is based on the presence of stem and progenitor cells, which control cell turnover and differentiation (18).

Pituitary Stem Cells in Cell Turnover and Responses to Hormones

Adult pituitary gland plasticity grants continuous cell turnover (homeostasis), and dynamically adapts its activity to either physiological cues (during puberty somatotroph cell number increase, or during pregnancy and lactation an increase in lactotrophs is observed) or pathological damages, by increasing specific hormone production. This process involves the recruitment of non-hormonal pituitary stem/progenitor cells (16), which are characterized by the expression of stem cell markers (Oct4, Nanog), the growth as spheroids ("pituispheres"), and the ability to differentiate into pituitary secretory cells. For example, the activity of these subpopulations gives rise to the generation of waves of novel corticotroph and gonadotroph cells after adrenalectomy or gonadectomy (19, 20).

Putative mouse pituitary progenitors express the transcription factor Sox2 and are mainly localized dispersed within anterior pituitary parenchyma and in the marginal zone, a remnant of Rathke's cleft. Functionally, they grow *in vitro* as spheroids and are able to differentiate into all hormone-secretory cell types (21, 22). The major role of Sox2-expressing cells in adult mouse pituitary regeneration and plasticity has been further strengthened by the observation that these cells self-renew and naturally give rise to secretory cells throughout organism lifespan (23–25), even in response to selective ablation of mature endocrine pituitary cells (23, 26). Sox2 expression has been often associated with Sox9 and CD133 expression, as well as with the activation of developmental pathways, essential for stem cell homeostasis and embryogenesis. Although most studies have been carried out using adult mouse pituitaries, the similarity of the pattern of SOX2 expression in human pituitary, and the close structural resemblance of the stem niche from rodents to humans, allowed the extrapolation of the information from the animal model to humans (27).

Undoubtedly, multiple human stem cell populations depend on SOX2 activity for persistence and differentiation. However, in adult rat or mouse pituitary besides Sox2-expression, cells positive for Sox9, Nestin (28, 29), S100 (30, 31), E-cadherin, Oct4, Prophet of PIT1 (Prop1) (32, 33), Paired-Related Homeodomain Proteins 1 and 2 (Prrx1/2) (34–36), and glial cell line-derived neurotropic factor receptor-α 2 (GFRα2) (32, 35, 37), have been proposed as stem/progenitor candidates, with the expression of the different markers sometime overlapping within the same cell subset. Currently, only fragmentary evidence of stem-like properties of these cell subpopulations is available, being occasionally tested for the ability to undergo epithelial-mesenchymal transition (EMT) (38), to form colonies (13), and to retain multipotency (39). In rats, post-natal pituitary stem/progenitor cells were detected in the marginal zone and/or identified as GFRα2$^+$/Sox2$^+$/Sox9$^+$ cell clusters scattered within the parenchyma of anterior pituitary; hormonal modulation at the middle of gestation increases the number of cells expressing stem markers in the marginal zone, while at beginning of lactation differentiated markers were predominant in the parenchyma and correlated to changes in cell proliferation; this observation supports the hypothesis that the PSC niche actively drives physiological pituitary plasticity (40).

Among embryonic transcription factors correlated to stemness, Prop1 has been detected in adult PSCs to regulate EMT (32, 33, 38). In rat anterior pituitary, hormonal cell differentiation is associated with Prop1 and Sox2 downregulation during post-natal development (21, 29, 33, 41, 42). In addition, SOX2 and PROP1 double positive cells, also expressing embryonic PRRX1 and PRRX2, have been detected in adult human pituitary gland (34). Mouse Sox2/Prop1-expressing PSCs are characterized by the co-expression of GFRα2, β-catenin, E-cadherin, as well Sox9, and Oct4, spherogenesis ability *in vitro*, and low proliferative rate *in vivo* after birth, suggesting their undifferentiated, stem-like nature (32).

As observed for stem cells from different tissue, Sox2$^+$/Sox9$^+$/E-cadherin$^+$ PSCs have been detected within specific niches, which in adult mouse and rat anterior pituitary were identified in the marginal zone (21, 29, 32, 39, 41), and within the anterior lobe parenchyma (21, 25, 42–44). A similar organization and expression profile of PSCs has been described in the human gland (27, 32), although their regulation, likely involving soluble factors, cell surface proteins and extracellular matrices, is still not completely clear (31, 42, 44–47).

Among gene and protein signatures of the cells populating stem cell niches, the chemokine CXCL12 and its receptor CXCR4 are commonly detected. CXCR4 has been identified in different adult organs, including neuroendocrine tissues; in particular this receptor/ligand system is expressed in human normal anterior pituitary (48, 49), in both hormone-secreting (50) and non-hormonal cell types of humans and rodents (i.e., FS cells) (16, 51, 52). Post-natal mouse PSCs, isolated as side population (SP) cells in flow cytometry experiments, show Cxcl12/Cxcr4 expression (29) as well as S100-positive cells, likely comprising the Sox2$^+$-stem/progenitor cells detected in the rat anterior lobe (41, 52). CXCL12/CXCR4 chemokine system is crucial for CNS development, functioning, and stemness maintenance (53), being expressed in embryonic and adult CNS stem cells, and playing a role in pituitary stem-related plasticity (54). *CXCR4* is upregulated in putative PSCs (44, 52), and SP cells of mouse pituitary gland (34). In the stem cell niches CXCL12/CXCR4 axis acts as chemoattractant and trophic factor for several cell types *via* paracrine and/or autocrine mechanisms (55), and induces EMT in progenitors (47, 56); this activity was also described in pituitary (51), further suggesting an association between its expression/activity and the stem cell phenotype.

Moreover, tissue/organ regeneration after physical or immunological injuries implies the activation of PSCs (57, 58) as described in pathological conditions which frequently cause transient or permanent hypopituitarism (e.g., altered embryonic

MARKER	REFERENCE	MARKER	REFERENCE
Sca1-Nanog-Oct4	Chen et al., 2005	Oct4-Notch4-JAG2-CD133-Nestin	Xu et al., 2009
S100β	Lepore et al., 2005	CD133	Yonoue et al., 2011
Nestin	Gleiberman et al., 2008	CD133-Nestin	Chen et al., 2014
Sox2	Fouquier et al., 2008	Sox2-Nestin-CD44-CXCR4-KIT-KLF4	Martens et al., 2015
Sox2-Sox9-CD44-CD133-Sca1	Chen et al., 2009	Sox2-Oct4-NANOG-KLF4-CD73-CD90- CD105-Vimentin	Orciani et al., 2016
GFRα2-Prop1	Garcia-Lavandeira et al., 2009	Sox2-Oct4-Nestin- CD133-CXCR4	Wurth et al., 2017
Sox2-Prop1	Yoshida et al., 2009	Notch1-4-JAGGED1	Perrone et al., 2017
Sox2-Sox9	Rizzoti et al., 2013	Sox2-Oct4-KLF4-Prop1	Peverelli et al., 2017
Sox2-Prrx1/2	Higuchi et al., 2014	Sox2	Capatina et al., 2019

FIGURE 1 | Expression of stem cell-associated markers in adult pituitary stem cell (PSC) and pituitary adenoma stem cell (PASC) populations. Adult PSCs exist within normal anterior pituitary and have been characterized by the expression of a discrete number of stem cell markers, most of them showing overlapping expression with Sox2-positive cell subset. Evidence supporting the presence of pituitary tumor stem cells is also based on enhanced stem cell marker expression. The figure summarizes the main markers used to identify the stem cell phenotype and corresponding references.

formation, traumatic brain insults, tumor growth, or resection) (59). Since these clinical conditions constrain patients to a lifetime hormone replacement, a better understanding of the PSC regenerative potential and the mechanisms involved, could represent a therapeutic option for hypopituitarism. Transgenic mouse models were used to induce ablation of specific pituitary hormonal lineages to mimic pituitary injuries and study the regenerative properties of PSCs (60). For example, in *GHCre/iDTR* mice, diphtheria toxin (DT) treatment causes the elimination of GH-secreting cells. These experimental conditions trigger the activation of $Sox2^+$ cells giving origin to $Sox2^+/GH^+$ cell population (23), although this regenerative capacity of the pituitary is time- and age-limited (58). Similarly, the differentiation potential of PSCs was reported after lactotroph ablation (23). Conversely, using an ACTH-secreting cell ablation model, authors reported that self-duplication of residual mature cells, rather than PSCs, is the predominant source for corticotroph restoration/replacement in the adult (61).

More recently, *in vitro* 3D multicellular structures (organoids), which better recapitulate phenotype and functions of the original tissue (62), have been proposed as a new experimental way to investigate PSC biology and differentiation pathways upon injury-activated stimuli (63). Organoid cells, derived from normal and GH-depleted adult mouse pituitary, mainly express Sox2 and E-cadherin, as previously described for PSCs (21, 32) and retain Sox2 after expansion in culture, showing limited differentiation capacity. Organoids from cell cultures of injured *GHCre/iDTR* pituitary show cystic structure, low proliferative activity, immature pituitary phenotype and alterations in specific pathways (i.e., Wnt/Lgr) as compared to dense undamaged pituitary organoid models, thus representing a valuable tool to study the regulation of putative PSCs in both normal and activated conditions (63).

Overall, $Sox2^+$ cells, persisting throughout life and being able to differentiate into pituitary hormone-secreting lineages, represent the most widely validated *in vitro* PSC model; however, studies performed in different animal models did not report a univocal phenotype, and the existence of a single or distinct stem cell populations is not definitely proven. Therefore, the expression of multiple markers (Gfrα2-3, Prop1, Sox2, Oct4, Sox9, β-catenin, E-cadherin), outlined in **Figure 1**, might reflect *in vivo* heterogeneity of both PSCs and committed progenitor populations, indicating the existence of cell subsets in the pituitary, different for origin, phenotype, activation of transcription factors, or niche interactions, which display different functions in adult human organ plasticity. Further characterization of human adult PSCs will allow a better understanding of the physiological and pathological roles of these cell subsets.

CANCER STEM CELLS IN PITUITARY ADENOMA

A detailed characterization of CSC-like subpopulations was performed in the adamantinomatous craniopharyngioma, a

low-grade pediatric pituitary tumor originating from Rathke's pouch, which may display an aggressive clinical course (64). Moreover, the CSC hierarchical tumorigenesis model was also proposed to be at the basis of the development of other benign tumors, and, in particular, pituitary adenomas (PAs) (54, 65, 66). As performed in other tumors, identification and isolation of PA stem cells (PASCs) relies on immunostaining followed by FACS or image microscopy (immunohistochemistry or immunofluorescence) in order to detect surface stem markers in post-surgical pathology preparations or, more recently, in *in vitro* cell cultures enriched in stem-like subpopulations by flow cytometry or growth in stem cell permissive media. However, CSCs are mainly operationally defined, using functional assays such as assessment of cell proliferation or clonogenic activity (to detect sustained proliferative potential) and sphere formation assay (as an index of self-renewal ability), Nonetheless, since *in vivo* tumorigenic property still represents the main defining parameter for CSCs, mouse xenograft is the most reliable method for assessing the presence of CSCs in a cell culture. However, in PAs, cell senescence, involved in preventing the malignant features in these tumors, is believed to inhibit the development of PAs in mouse models (67), and indeed not all the studies were able to demonstrate this feature in mice, leading to the establishment of alternative animal models, i.e., tumor growth in zebrafish embryos (68).

The following paragraphs will highlight consensus and diverging reports on these features in PASCs and the implications for their definition. A comparative analysis of the data from the different studies are summarized in **Table 1**.

Isolation of Putative Pituitary Adenoma Stem Cells

Most reports describe the *in vitro* isolation of putative PASCs through the subpopulation selection using stem cell-permissive media originally used to isolate and cultivate neural stem cells. In particular, beside few differences, a common, fundamental feature is observed in all media formulations from the published studies: the absence of serum and the presence of growth factors, generally EGF and bFGF. Serum-free medium was already used to enrich in CSCs, cultures from several different solid tumors (79) and allowed to retain *in vitro* genotype and phenotype features similar to the original tumor (80). In almost all the studies to date published, these experimental conditions held to isolate PA cells able to grow in suspension as spheroids, a feature generally considered an *in vitro* index of self-renewal.

However, it has to be remarked that, to date, the success rate of this procedure is still far from 100%. In the study of Xu et al. two out of eight of the tested PAs gave rise to stem-like cell cultures (69). In another study, 69.6% of cultured non-functioning PAs (NFPAs) ($n = 46$) showed the formation of non-adherent spheroids after 2 weeks of culture in stem cell-permissive medium; sphere generation was significantly higher in aggressive tumors as assessed by cavernous sinus invasion (78). In our lab, we developed a similar protocol to isolate and expand *in vitro* putative PASCs from GH-secreting PAs (GHomas) and NFPAs post-surgical specimens (81), obtaining a similar success rate. In particular, 68% of the PAs ($n = 38$) were able to proliferate as spheroids when selected in stem-cell permissive medium (77). Importantly, in this study we demonstrated that PASC selection, performed either by growth in stem cell-permissive medium or by sorting for CD133 expression, gives rise to cell subpopulations endowed with comparable stem cell-like features; these results highlight the relevance of CD133 in PASCs, as also reported in CSCs from different malignant tumors (82). A similar approach was described by Zhao et al. who sorted $CD133^+$ and $NESTIN^+$ co-expressing cells from dispersed PA cells. These cells represented up to the 3% of total PA cells and were able to generate spheres for several passages (83). In other reports, stem cell-permissive medium allowed the selection of sphere-forming cells in cultures from all the 12 (71) or 14 (84) tumor analyzed including GH-, ACTH-, FSH/PRL-secreting PAs and NFPAs. Similar results were obtained in a mouse model ($Rb^{+/-}$ mice), which spontaneously develops PAs: the growth of explanted tumor cells in stem- permissive culture conditions held the isolation of sphere-forming putative PASCs (72). A stem-like cell subpopulation was also derived from three human GHomas and three NFPAs, growing dispersed cells using culture conditions developed for mesenchymal stem cell (MSC) cultures, although these PASCs showed peculiar characteristics as compared to the others studies (see below) (74).

A different approach was used in another study (73), in which the same methodology used to isolate adult PSCs from normal murine pituitaries was applied (85). Since one of the typical features of cells with a stem-like signature is the overexpression of ATP-binding cassette (ABC) multidrug transporters, which confers resistance against toxic stimuli (included those exerted by drugs), they analyze PA cultures for the ability to extrude, via these transporters, the fluorescent DNA-binding dye Hoechst 33342, appearing in the FACS analysis as a "side population" (SP). Interestingly, SP cells were isolated form all the 60 tested adenomas (GH- and ACTH-secreting, or NFPAs), with a calculated presence of putative PASCs ranging from 0.5 to 2% (mean 1.9%), with the occasional observation of a NFPA showing up to 17.2%. SP cells grew as spheroids *in vitro*, even though only short term proliferation was observed, and, to perform a more detailed characterization of stem properties, SP cells derived from the established murine corticotroph adenoma cell line AtT20 were analyzed (73).

Stem Cell Markers and Intracellular Pathways in Pituitary Adenoma Cells

Although tissue-specific transcription factors can be identified in normal stem cells from different tissues, normal stem cells are generally defined by the expression of a common subset of stemness-related factors (e.g., SOX2, NANOG, OCT4, NOTCH, CXCR4/CXCL12, CD44, etc.) which confers the peculiar properties of self-renewal and pluripotency, during both embryonic development and adult stage (13). Candidate PASCs also display expression of several markers used for stem cell identification by immunophenotyping. For example, SOX2 and NANOG are two pluripotency-associated transcription factors expressed by embryonic and adult stem cells, involved the

TABLE 1 | Isolation and functional characterization of pituitary adenoma stem cells.

	References	**Xu et al. (69)**	**Yunoue et al. (70)**	**Chen et al. (71)**	**Donangelo et al. (72)**	**Mertens et al. (73)**	**Orciani et al. (74, 75)**	**Megnis et al. (76)**	**Wurth et al. (77)**	**Peverelli et al. (78)**
Experimental model and procedure	PA type	GH-oma, NFPA	GH-/ACTH-/ TSH-/PRL-oma, NFPA	Unknown	Spontaneous PA from $Rb^{+/-}$ *mice*	GH-/ACTH-/PRL-oma, NFPA	GH-oma, NFPA	GH-/LH-oma, NFPA	GH-/ACTH-/ GH-PRL-oma, NFPA	NFPA
	Model	HUMAN	HUMAN	HUMAN	MOUSE	HUMAN	HUMAN	HUMAN	HUMAN	HUMAN
	Isolation approach	Floating spheres, serum-free medium	$CD133^{+}$cell-identification by FACS analysis	Serum-free medium-	Floating spheres, serum-free medium	SP, serum-free medium	Cell adhesion, MSC-medium	Cell adhesion, serum-containing medium	Serum-free medium and $CD133^{+}$ cell sorting	Serum-free medium
Cancer stem cell criteria	Spherogenesis (self-renewal)	Yes	n.d.	Yes	Yes	Yes	No	n.d.	Yes	Yes
	Multipotency (differentiation)	Yes (hormone-secreting cells)	n.d.	Yes (neural lineages)	Yes (hormone-secreting cells)	n.d.	Yes (mesenchymal lineages)	Yes (mesenchymal lineages)	Yes (hormone-secreting cells)	n.d.
	In vivo tumorigenesis (animal model)	Yes (M)	n.d.	Yes (M)	Yes (M)	No	n.d.	n.d.	Yes (Z)	Yes (Z)
	Other features	Cytotoxic drug resistance	-	Low proliferative activity *in vitro*	-	-	SSTR1-5 expression, sensitivity to anti-proliferative effects of SSTR agonists	SSTR1-5, D2R expression (low)	SSTR2, SSTR5, and D2R expression; sensitivity to anti-proliferative effects of D2R/SSTR chimeric agonist	SSTR2 and D2R expression, sensitivity to antiproliferative effects of SSTR2 and D2R agonists

PA, pituitary adenoma; M, mouse; Z, zebrafish embryo; SP, side population; MSC, mesenchymal stem cell; SSTR, somatostatin receptor, D2R, dopamine receptor2; serum-free medium, stem cell permissive medium with EGF and bFGF; n.d., not determined.

maintenance of stem cells in various adult tissues, and, in mouse, $Sox2^+$ cells are involved in pituitary regeneration (26). NOTCH signaling contributes to stem cell proliferation (86) and prevents progenitor cells from premature differentiation (87), while OCT4, essential during embryogenesis and to retain pluripotency in the adult tissues, is overexpressed in CSCs of various cancers conferring drug resistance (88). CD133 (prominin 1) is a membrane glycoprotein involved in retinal development, whose overexpression is related to aggressiveness of ovarian, colorectal, prostate, and lung cancer, and glioblastoma, representing a signature for putative CSCs in these neoplasms (89); the chemokine CXCL12 and its receptors CXCR4/CXCR7 are involved in self-renewal and migratory behavior of normal stem cells and CSCs (90, 91) acting via autocrine/paracrine mechanisms (52, 55). Importantly, SOX2, NESTIN, GFRα2, among others were established as markers to identify normal PSCs and progenitors (92). For example, SOX2 and OCT4 expression was detected in scattered cells within human normal pituitary samples, while more diffuse expression was observed for CXCR4 (77).

The evaluation of the expression pattern of above stem markers, summarized in **Figure 1**, represents the basis for the characterization of CSC isolated from PAs.

Stem Cell Markers in Histological Pituitary Adenoma Preparations

The first evidence of the presence of putative stem-like cells within PAs was obtained from histological preparations of human tissues. CD133 was analyzed in a series of 70 PAs, observing that 25.7 % of them showed positive immunolabeling, with higher frequency in NFPAs as compared to GHomas (15/45 in NFPAs vs. 3/25 in GHomas and prolactinomas) (70). After cell dispersion and cytofluorimetric analysis, CD133-positive cells were shown to represent an average of 2.9% of total adenoma cells in NFPAs ($n = 5$), 0.8% in GHomas ($n = 2$) and 7% in the single prolactinoma analyzed. However, CD133 expression levels were not correlated with tumor size, or post-operative recurrence rate. $CD133^+$ cells also co-expressed NESTIN and, although with high intertumor variability, CD34. However, no statistically significant correlation between CD34 and CD133 expression was observed. Wurth et al. identified, by immunofluorescence, the expression of SOX2, OCT4, NESTIN, and CD133, in seven human GHoma, and five NFPA samples, while NANOG and NOTCH1 were detected in 25 and 50%, respectively, of the samples analyzed; these markers restricted to subsets of cells diffuse within tumor mass, but, in GHomas, do not co-localized with GH-secreting cells (77). Subsequently, small cell subpopulations expressing CD133 and NESTIN were identified in 12 human prolactinomas by immunofluorescence analysis. Interestingly, CD133 and dopamine D2 receptor (D2R) expression, analyzed by FACS, segregate in different subpopulations (93). In another series of human PAs, NOTCH 1–4 receptor subtypes, and their ligand JAGGED1 were detected by RT-PCR, suggesting the presence of a constitutive autocrine/paracrine NOTCH system activation in a subset of tumor cells (94). In this study, NOTCH3 immunohistochemistry showed that NFPAs, prolactinomas, GHomas, and ACTH-secreting PAs (ACTHomas) express the protein in the cytoplasm and membrane of tumor cells. SOX2 expression was also detected by immunohistochemistry in about 50% of 16 PAs, with prevalent expression in GHomas and prolactinomas (60% of cases) than in NFPAs (only 20% of cases) (95). Transcriptomic analysis confirmed an increased expression of NOTCH3 in human NFPAs compared to normal pituitaries (96), as further demonstrated by other studies at both mRNA and protein levels (97–99).

In a large cohort of 65 PA specimens, *CXCR4* mRNA was detected in 92% of GHomas and 81% of NFPAs, whereas the ligand *CXCL12* was identified in 63 and 78% GHomas and NFPAs, respectively. These data were confirmed by immunohistochemistry and immunofluorescence, in GHomas and NFPAs showing higher level of expression than normal human pituitary samples (48). Flow cytometry and immunofluorescence analysis of 35 PAs (21 invasive and 14 non-invasive) also revealed that CXCR4 and CXCL12 expression was significantly higher in the invasive subset than that of the non-invasive PAs (100); conversely no correlation was observed for other markers such as CD44 and CD147. Interestingly, CXCR4 activation in the rat somato-prolactinoma GH4C1 cells enhances proliferation and hormone release (101), suggesting a pivotal role for this chemokine system in PA functioning.

Pituitary Adenoma Stem Cell Markers in Enriched *in vitro* Cultures

One of the first study reporting the phenotypical characterization of putative stem cells isolated by two PA cell cultures (one from a GHoma and one from a NFPA) was performed by Xu et al. (69). They obtained pituitary cell spheroids which express OCT4, NOTCH4, JAG2, CD133, and NESTIN. Subsequently, similar or partially overlapping results were obtained in many different studies in which PASCs were obtained with different experimental approaches. In detail, a second study showed that sphere colonies derived from PAs express CD133, NESTIN and other stem markers typical of neural cells: NCAM (neural cell adhesion molecule) and neuron-specific class II β-tubulin (71). Moreover, in agreement with previous studies, PASCs isolated as SP, besides NESTIN, revealed the expression of other stem cell-related markers, such as CD44, CXCR4, KIT, KLF4, and SOX2 (73). Importantly, EMT-related gene expression, such as *TWIST*, *ZEB1* and *2*, and *SNAI1* and *2* was up-regulated in candidate PASCs, as compared to non-SP cells, while the epithelial marker genes *CDH1* and *CLDN1* were down-regulated (73), indicating that EMT is active in PASCs. The highest relative levels of some EMT-associated genes (*ZEB2, SNAI2*, and *TWIST1*) occurred in the SP derived from invasive PAs, further highlighting the clinical implication of these regulatory pathways in PASCs (**Figure 2**). However, the EMT activation in PASCs will require further validation.

In agreement with these results, our group reported that PASC-derived pituispheres express SOX2, OCT4, NESTIN, CD133, NOTCH1, and CXCR4 in almost all the culture analyzed (93, 100, 87, 91, and 100%, respectively), while NANOG expression was confined to only half of the PAs (77). A partially divergent result was reported by Orciani et al. in a subset of six GHomas and six NFPAs, in which, together with the expression

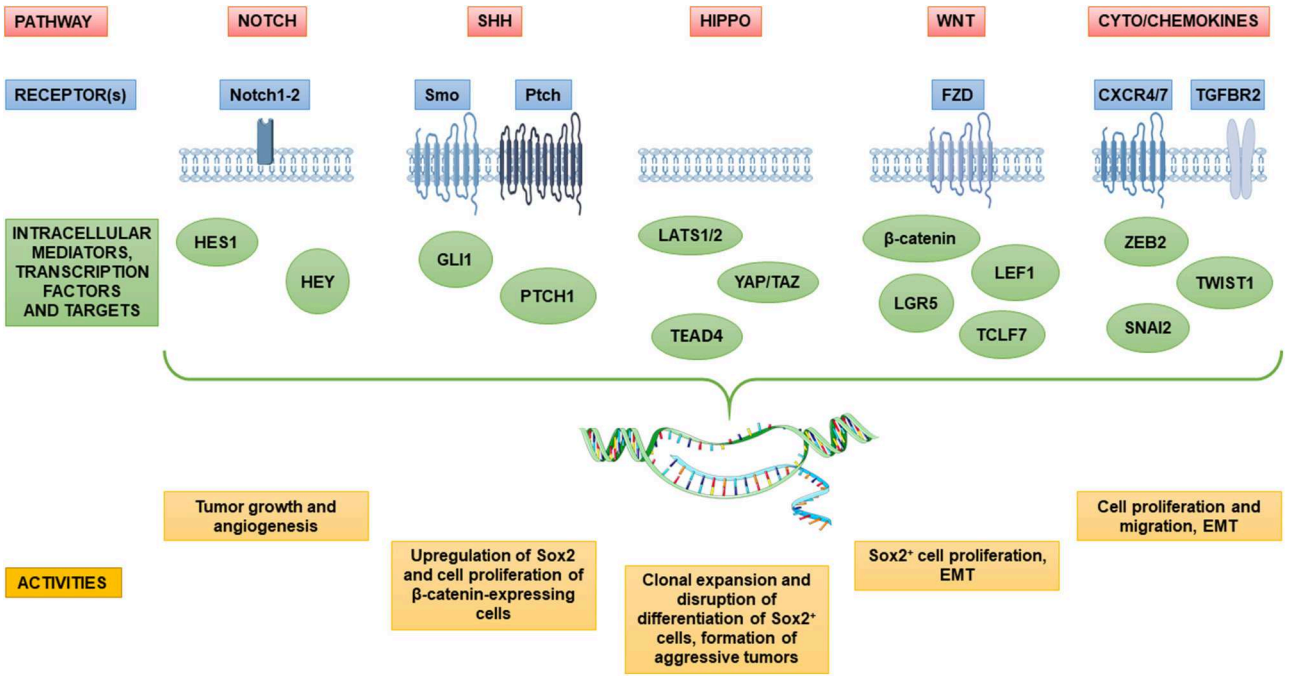

FIGURE 2 | Dysregulation of major stem cell pathways in candidate pituitary adenoma stem cells and their involvement in pituitary tumorigenesis. The diagram depicts key signaling pathways in pituitary adenoma implicated in sustaining the putative pituitary adenoma stem cell populations and its functions in tumor maintenance and progression. Several pathways including Notch, Sonic Hedgehog (SHh), Hippo and Wnt, which are tightly regulated in normal stem and precursor pituitary cells, are aberrantly regulated in tumors, likely in defined subsets of cancer stem cells within pituitary adenomas. Signaling pathways are often linked to epithelial–mesenchymal transition (EMT) in the gain of tumor stem cell properties, and migration ability. An overview of main receptors and transcriptional mediators upregulated in pituitary adenomas is represented as well as significant effects on the pathogenesis of pituitary adenoma.

of pluripotency factors (OCT4, NANOG, KLF4, CD133, and SOX2), the typical MSC surface markers were identified in PASCs (74). Indeed, these cells were isolated using MSC-permissive culture medium and, in appropriate culture conditions, they were able to differentiate into osteogenic, lipogenic, and chondrogenic cell lineages. Subsequently, using similar cultures from different tumors, these Authors reported that PASCs also express markers reminiscent of EMT (75). Similarly, in a different study, PASCs isolated from two GHomas, three prolactinomas, and two NFPAs displayed MSC features, as far as morphology, cell surface marker expression (CD73, CD90, CD105, CD44, and vimentin), and differentiating ability into osteocytes and adipocytes (76). These results, in line with reports on better characterized malignant tumor CSCs, i.e., glioblastoma (102), suggest the existence of different CSC subpopulations within PAs, characterized by different identities and, possibly, functions and origin within adult pituitary, although partially sharing pluripotency markers.

In another report (78), putative PASCs were isolated from 46 NFPAs and characterized for the expression of *SOX2, OCT4,* and *KLF4* mRNAs. SOX2 and NESTIN expression was also confirmed by immunohistochemistry/immunofluorescence experiments, in a subset of PASC-enriched cultures grown as spheroids. Moreover, indirect immunofluorescence analysis revealed the nuclear co-expression of the pituitary specific transcription factor PROP1 and SOX2 in about 2–10% of cells, while PROP1 was cytoplasmic in $SOX2^{-}$ cells, highlighting the presence, within the culture, of heterogeneous populations of stem ($PROP1^{+}/SOX2^{+}$/) and precursor ($PROP1^{+}/SOX2^{-}$) cells Finally, the analysis of cells from 22 PAs of different subtypes showed a preferential high expression of CD15, as compared to CD133. Moreover, by comparative analysis within each tumor cell culture, of $CD15^{high}$ and $CD15^{low}$ subpopulations, it was reported that $CD15^{high}$ cells are also associated to higher *SOX2* and *PAX7* expression, and were detected in higher percentage in recurrent PAs as compared to the matched primary tumor (84).

To date only one study formally described the *in vitro* isolation of murine PASC, spontaneously originated from pituitary intermediate lobe in $Rb^{+/-}$ mice. Isolated cells were grown as spheroids and expressed Sox2, Nestin and CD133, as well as Sca1, in line with what reported in normal PSCs (72). Nevertheless, this study is particularly important since it implies a common phenotype of PASCs, independent from the human or murine origin.

Stem Cell-Related Pathway Activity in Pituitary Adenoma Cell Cultures

Altogether, previous described literature supports the alteration of stem cell regulatory pathways in cell subpopulations within PAs. Among these, the dysregulation of NOTCH pathway was associated with PA, being gene expression of components of its signaling (e.g., *HES1*, *HEY*, *NOTCH1*, *NOTCH2*) upregulated in the SP from human PA (73). However, functional data

able to clarify its role in PASC tumorigenesis are very limited, and mainly obtained in established PA cell lines. To address this issue, Zubeldía-Brenner et al. (103) used the rat somatoprolactinoma cell line GH3 [previously showed be positive for Notch1-4 (94)] to determine whether the inhibition of Notch signaling would affect growth and angiogenesis of tumor xenografts in immune-compromised mice. For this purpose, they used a γ-secretase inhibitor (DAPT), which prevents cleavage of intracellular Notch domains and, consequently, modifies target specific transcription factors in the nucleus. Interestingly, the inhibition of Notch signaling led to tumor mass reduction and decreased neoangiogenesis. In order to elucidate whether putative PASCs with constitutive active NOTCH system could be targeted by inhibition of this pathway, a significant research work in tumor subtype-specific manner has still to be done. Nevertheless, these recent data may support the downregulation of NOTCH signaling as a potential therapeutic tool in aggressive or resistant PAs.

Other studies identified a relationship between the expression and/or activity of different specific stem cell markers in pituitary tumorigenesis. The activity of SONIC HEDGEHOG (SHH) pathway, crucial during embryogenesis and organ development, differs among PA subtypes, being downregulated in NFPAs as compared to normal pituitary tissues, while GLI1, one of the transcriptional effectors that mediate SHH signal transduction, is overexpressed in GHomas and ACTHomas (73, 104). Excess Shh signaling increases both the proliferation of $Sox2^+$ and $Sox9^+$ adult mouse PSCs and the expression of ACTH, GH and PRL in adult pituitary (105). SHH and its target gene *GLI1* were also shown to be overexpressed in GHomas, ACTHomas, and prolactinomas, further supporting that excess SHH signaling is involved in the development/maintenance of hormone-producing PAs. SHH administration to the HP75 human PA cells caused the upregulation of SOX2 and proliferation of β-catenin-expressing cells. Similarly, the addition of WNT3a in SOX2-expressing PA cells sustains cell proliferation. It was proposed that SOX2 mediates crosstalk between SHH and WNT/β-catenin pathways to promote the proliferation of PAs (106). In fact, aberrant WNT/β-catenin activity has been observed in pituitary tumors (107), and gene expression of components of the pathway (*LEF1, LGR5, TCF7L1*) was identified in PA SP cells (73).

The HIPPO kinase cascade is a crucial pathway that regulates cell growth and fate in numerous organs, and may be implicated in the regenerative response of PSCs (58). In mouse PSCs, this signaling activity, in association with its effectors Yes-associated protein (Yap) and transcriptional co-activator with PDZ binding motif (Taz), was detected in Sox2-expressing subpopulations. Moreover, overexpression of HIPPO pathway components (i.e., large tumor suppressor homolog 2, LATS2, and TEAD4), has been described in PA SP cells (73) as well as in poorly differentiated pituitary tumors (null-cell PAs, adamantinous and papillary craniopharyngiomas) which display enhanced YAP/TAZ activity (108). Importantly, the activation of HIPPO pathway, by silencing the kinase LATS1, inhibits promoter activity of *GH1* and *PRL* genes, correlating high YAP/TAZ activity with repression of pituitary differentiation (108). Using a genetic approach to perform gain- and loss-of-function experiments in mice, it was shown that *Yap/Taz* activation during development is essential for normal pituitary formation from $Sox2^+$ PSCs. However, postnatal upregulation of Yap/Taz activity causes uncontrolled clonal expansion of $Sox2^+$ pituitary cells, disruption of their differentiation, and formation of aggressive NFPAs, highlighting that this regulatory signaling cascade is a relevant component for both pituitary development and tumorigenesis (109).

Figure 2 summarizes the signaling pathways and transcriptional networks involved in PASC maintenance and tumorigenesis.

Assessment of Biological and Functional Activity in Putative Pituitary Adenoma Stem Cells

As highlighted before, CSCs are not a single population within a given tumor, but they set apart into subpopulations characterized by heterogeneous phenotypes (110, 111). Thus, beside marker expression, the characterization of these cells requires the establishment of biological and/or functional specific activities. In particular, by definition, CSCs should display: (i) self-renewal, mainly assessed by serial sphere-forming ability, (ii) long term proliferation *in vitro*, and (iii) capacity to differentiate into specific cell types related to the tissue of origin.

In vitro Self-Renewal Activity

In vitro spherogenesis is a commonly accepted index of the self-renewal ability of a cell population. Although all the studies addressing the role of PASCs as PA-initiating cells, used the ability to form pituispheres to identify these subpopulations, in order to validate the CSC phenotype, this ability should be a persistent feature of the isolated cells. This can be assessed measuring the generation of secondary (tertiary, or more) spheres after disaggregation of the initially generated spheroids. This ability was indeed described in most of the previously mentioned studies: Xu et al. reported that after up to 15 passages PASCs were still able to generate spheroids (69); Chen et al. observed that, after dispersion of PAs to single cells, primary spheres are generated after 2–3 weeks in culture and that daughter cells derived from the spheres formed secondary spheres within 2–4 weeks after reseeding (71); Peverelli et al. showed that cells from 46 PAs form primary spheres after about 2 weeks in stem cell-permissive medium, with sphere-forming efficiency (SFE), calculated in a subset of tumors, in about 2–5% of the plated cells. Cells derived from primary spheres and re-plated as single cell were able to generate secondary spheres with a SFE of about 4–8%, while no tertiary spheres were obtained (78). In our study, sphere formation occurred after 7–10 days in 14 out of 16 GHoma and NFPA cell cultures, but proliferation within spheroids lasted for several weeks (77). In a subset of samples in which it was calculated, SFE was in the range of 1.3–7%. Cells harvested from disaggregation of 7 days living spheroids were able to generate secondary spheres within 13 days culture in stem cell-permissive medium, with a slightly higher SFE in secondary spheres as compared to that of the primary spheroids derived from the same PA (77). Similar data were obtained in PA cultures derived from

the $Rb^{+/-}$ mice: spheres were generated starting after 3 days *in vitro* and continued to grow up to 10–12 days. After dissociation, although the number of viable cells declined, cells from primary spheres generated secondary spheres displaying similar growth rate, for up to 12 passages. As compared to non-stem population (identified by the lack of Sca1 expression), PASCs showed four-time higher propensity to generate spheroids (72).

Spheres also developed from SP cells isolated after dispersion of PA tissues, while the main population was devoid of such activity. However, SFE was very low, with only 2–4 per 20,000 cells seeded, which did not allow to have viable cells in a sufficient number to test spherogenesis in further passages (73).

Proliferation and Migration Abilities

Primary cultures of non-stem PA cells have a very low proliferative activity, with no more than 1–2 *in vitro* cell divisions (112, 113). Thus, sustained and long-lasting proliferation *in vitro* may represent a relevant index to identify cell subpopulations endowed with stem-like characteristics. However, only few studies analyzed this feature: Wurth et al. reported that cells derived from 11 PASC-enriched cultures proliferate up to 28 days increasing up to 400% the initial number, although for longer culturing time the proliferative potential declines. Conversely, non-stem PA cell cultures barely duplicated once, and started to degenerate from the second week *in vitro* (77). Peverelli et al. reported that NFPA PASC spheroids contain a high percentage of $Ki67^{+}$ cells, which lasted up to 4 weeks, while the differentiated cell counterpart retains a proliferative activity for no longer than 1 week (78). Similarly, stem-like cells derived from $Rb^{+/-}$ mice PAs, characterized for Sca1 expression, exhibit a proliferative advantage over $Sca1^{-}$ cells, evaluated up to 3 days in culture and reaching a 4.4-fold increase in growth rate (72). Orciani et al. reported a linear growth of mesenchymal PASCs for up to 12 days, with a doubling time of about 20 h for the first eight passages (74). However, at the 12th *in vitro* passage the doubling time reached 40 h, further confirming, that PASCs tend to lose the stem cell-like features, as observed in different *in vitro* models (77). Megnis et al. reported a continuous proliferation in all the 6 PASC-enriched cultures analyzed, up to 14 days (76). Other studies were not able to observe a sustained *in vitro* proliferation and did not analyze this parameter (73). The limited *in vitro* growth of PASCs, although much more prolonged as compared to non-stem PA cells, might either reflect intrinsic features or technical limitations in the cultivation of these cells. In this respect, the benign nature of PAs, in which the activation of senescence program limits tumor expansion, could determine the self-limiting growth of these cells *in vitro*. Moreover, it should be considered that culture conditions for PASCs are mainly adapted from CSC cultures of different tumors (79), and may be inappropriate or insufficient to support their continuous growth. Further studies are required to address this issue.

$CD133^{+}$ cells isolated from both GHomas and NFPAs also possess a higher migratory activity as compared to $CD133^{-}$ cells isolated from the same tumor (74). This observation provides a direct support to the correlation previously reported of a higher number of $CD133^{+}$ cells in invasive PAs as compared to those with a non-invasive clinical behavior (71).

Differentiation Ability

Stem cell are undifferentiated cells acting as a reservoir from which differentiated cells origin to replace cells during normal adult tissue turnover or after injuries. This feature is also present in CSCs which are the source of all the "differentiated" tumor cells forming the bulk of the tumor. In several tumors CSCs possess a marked *in vitro* differentiation ability. For example, glioblastoma CSCs, grown in the absence of growth factors and in the presence of fetal bovine serum (FBS), are able to differentiate into astrocyte- and/or neuron-like cells (114). Contrasting results were reported as far as PASC differentiation ability: in most studies this PA subpopulation does not express pituitary hormones (69, 70, 77), while, in others, the immunohistochemical detection of LH in $Sox2^{+}$ cells within spheroids was reported, although evidence of co-localization in the same cells was not provided (78). However, relevant to the CSC ability to originate all the cell populations composing the tumor, few studies actually showed that PASCs, isolated from hormone-producing tumors, can differentiate into hormone-releasing cells *in vitro*. Xu et al. showed, using specific ELISAs, that when shifted in differentiation medium (in which growth factors were replaced by FBS) for 2 weeks, PASCs derived from a GHoma released GH in response to GRF, and LH and FSH when exposed to LHRH (69). This was not observed in the same cells grown in stem cell permissive medium. However, both stem-like and differentiated cells released PRL in response to PRL-releasing peptide, and TSH in response to TRH. NFPA PASCs also released LH in basal conditions, as also demonstrated by Peverelli et al. (78), and, after differentiation, released both LH and FSH after LHRH treatment. Thus, PASC differentiation potential was demonstrated, but it was still unaddressed the lack of a tumor-specific differentiation, since LH, FSH, and TSH were also produced by GH-secreting PA culture. PASC ability of to differentiate into cells with an adult pituitary cell phenotype was tested evaluating GH production in 2 GHoma PASC cultures after differentiation induced by shifting the cells in FBS-containing medium for 10 days (77). In these experimental conditions GH immunoreactivity, undetectable in the cells kept in stem cell-permissive medium, was clearly evident. Interestingly in the same cells the expression of some stem cell markers (CD133, OCT4, NESTIN) was reduced after differentiation, while others were retained (i.e., CXCR4). These data clearly support the differentiation potential of these PASC cultures, in which the loss of stemness markers is associated to the induction of hormone production. However, when cells were grown for 30 days in stem-permissive medium, few GH-expressing cells were observed also in these conditions, although in a lower number than observed after differentiation (77). Thus, a low level of spontaneous differentiation may occur in the PASC subpopulation, in line with the loss of proliferative activity after prolonged *in vitro* culture (see above). Similarly, PASCs, isolated from tumors developed in $Rb^{+/-}$ mice and grown in the presence of growth factors, are completely hormone-negative, but after 9 days of culturing in differentiation medium

all six pituitary hormones were detected in the cultures (72). Also in this experimental setting, stem cell markers Sox2 and Sca1 were downregulated, although few Nestin$^+$ cells were observed in differentiated PASCs, sometime co-expressed with hormones (72).

Other studies reported the occurrence of heterologous type of differentiation. Differentiated PASCs were shown to express neural (β-III tubulin), astrocytic (glial fibrillary acidic protein, GFAP), and oligodendrocyte (2′,3′-cyclic nucleotide-3′-phosphodiesterase, CNPase) markers (69, 71).

On the other hand, when PASCs were selected according to MSC isolation procedures and characterized by the expression of mesenchymal markers, differentiation follows this lineage and not the pituitary-related phenotypes. In fact, shifting the cultures in defined media, cells acquire osteogenic [expression of alkaline phosphatase after 10 (74) or 21 (76) days], adipogenic (presence of cytoplasmic lipid vacuoles after 14/15 days) (74, 76), or chondrogenic (detection of safaranin-O staining after 30 days) (74) phenotypes.

These latter studies further highlight the possibility that different stem-like subpopulations may be present within PAs, which can be preferentially expanded *in vitro* according to the isolation procedure used. Thus, more in depth lineage studies are necessary to address the cellular origin of these subpopulations.

In vivo Tumorigenic Ability

The ability to regenerate in animal models the tumor from which stem cells were isolated is still the main feature which defines CSCs (115). Thus, the tumorigenic ability is a required feature also for PASCs. However, when benign, slow-growing tumors are studied, the development of animal model is a critical issues and, to date, contradictory results were reported.

Several of the previously analyzed studies did not address this issue in the characterization of PASCs (70, 73, 74, 76), while others used immunodeficient mouse models to perform xenograft experiments, as commonly performed for CSCs from malignant tumors (glioblastoma, breast cancer, mesothelioma, osteosarcoma) (114, 116–119).

Xu et al. succeeded in inducing tumor development after brain parenchyma injection of 10,000 cells from both the two cultures of PASCs they isolated. Xenografts were slow-growing, being detectable after 3 months from the inoculation, but still expanding after 6 months (69). Interestingly, the nature of the original tumor was retained since tumors originated from GHoma-isolated PASCs showed expression of GH (and PRL), which was not observed when PASCs derived from NFPA were injected. Moreover, PASCs recovered from tumor xenografts were expanded *in vitro* as spheroids and re-injected in mouse brains, retaining tumorigenicity. Similarly, a small tumor mass was observed 12 weeks after subcutaneous (s.c.) inoculation of 10,000 cells from spheroid cells isolated from a single PA (71). The resulting single tumor displayed low proliferation rate (as for Ki67 labeling), histology resembling PA structure, and expressed synatpophysin, but it was not further characterized. Similar results were obtained by the intracranial injection in nude mice of 10,000 CD133$^+$/nestin$^+$ PA cells, which gave rise to tumors, detectable after 6 weeks from the graft (83). In another study, one PASC culture from a null PA, sorted for CD15 expression, was pseudo-orthotopically injected in the brain of three mice (84). In agreement with the CSC theory, 50,000 CD15$^+$ cells generated tumors when injected in the mouse brain, while up to 500,000 CD15$^-$ cells were not tumorigenic. The explanted tumors showed high number of mitosis and nuclear atypia, and, in agreement with the histological subtype of the non-secreting original tumor, immunoreactivity for pituitary hormones was not detected. Comparing the original tumor with the xenograft, both specimens were negative for GFAP, alpha-smooth muscle actin, EMA, and TTF1, and, according to the neuroendocrine derivation, both expressed synaptophysin and CD56. Interestingly, desmin and myogenin were unexpectedly expressed only in the xenograft but not in the original PA, suggesting that CD15$^+$ PASCs possess a microenvironment-dependent multi-lineage potential (84).

Conversely, Mertens et al. failed to demonstrate tumorigenicity of PA cells *in vivo* by xeno-transplantation in immunodeficient mice, regardless their hormonal phenotype. In these experiments small tumor pieces were implanted s.c. or under the kidney capsule in different mouse models: SCID mice, or the more immunodeficient NOD–SCID and NOD-SCID IL2rγ^{null} (NSG) mice, which lack mature T, B, and natural killer cells. Tumor specimens apparently survived for 3–4 months after implantation but no growth was detected. Similar negative results were also obtained using xenografts of dissociated PA cells (73).

Also in our experience, we did not detect tumor development within 8 months from the injection of PA cells that fully accomplish the *in vitro* criteria for CSC definition (i.e., stem marker expression, undifferentiated phenotype and differentiation ability in hormone-producing cells, high proliferation potential, self-renewal); similar negative results were observed after grafting the cells either s.c. into the flank of animals in the presence of Matrigel (20,000 cells, from 2 PAs) or pseudo-orthotopically in the striatum (50,000 cells, from 4 PAs) (77). Thus, we used a different animal model: zebrafish embryos, which allow the evaluation of two relevant CSC features which define PASC tumorigenicity: invasive and angiogenic potential (120). PASCs from 5 PAs were xenografted into embryos of the Tg(*fli1:EGFP*)y1 zebrafish line that express EGFP in endothelial cells, thus allowing to detect whether neovessels are formed in proximity of the injected cells. PASCs were stained with a red fluorescent dye, to follow their migration activity. In this model, only a 500 cells/embryo are required and all the events occur within 2–3 days. Grafted PASCs were easily detected soon after the injection and, when recovered after 48 h, confirmed the human nature and CD133 expression. However, after 24 h injected PASCs start to migrate outside the tumor cell mass, invading the yolk and the caudal areas. Moreover, at odd with control embryos, grafted embryos showed the sprouting of new vessels from the subintestinal vein plexus, which were directed toward the tumor cells, demonstrating the induction of *in vivo* neo-angiogenesis. Similar results were also reported by Peverelli et al., who grafted sphere-derived cells from 2 NFPAs in zebrafish embryos. After 24 h, tumor cells from both tumors migrated outside the site of the injection confirming an invasive

ability. Moreover, also in this study injected spheroid-derived cells stimulated the formation of endothelial sprouts from the subintestinal vessels plexus in 1/3 animals (78).

Finally, tumorigenic ability was also reported for PASCs derived from $Rb^{+/-}$ mice, although this occurrence was somehow expected since they contain the specific genetic alteration responsible for the formation of these adenomas (72). In these experiments $Sca1^{+}$ and $Sca1^{-}$ cells were compared, demonstrating that the stem-like cell subpopulation was endowed with higher tumorigenic potential when injected in the striatum of NSG mice. Importantly, although injected cells were hormone-negative, after *in vivo* tumor development multiple hormones were detected in 91% of the 11 tumors analyzed. *Sca1* expression was abolished in almost all the tumors, whereas other stemness markers were retained (Nestin, CD133, Sox2) in subsets of cells, altogether with the astrocytic marker Gfap (72).

DRUG SENSITIVITY OF PITUITARY ADENOMA STEM CELLS

One of the most relevant information that can be obtained by identification of CSC from different tumors, is the possibility to analyze, with good translational potential, the sensitivity of this subpopulation to traditional or innovative pharmacological treatments. For example, studies on CSC from glioblastoma, breast cancer or osteosarcoma (119, 121–123), demonstrated a higher sensitivity of this subpopulation to metformin, as compared to "differentiated" tumor cells, determining the development of a drug repositioning approach (124, 125). In particular, while it is well-accepted that stem-like cells are particularly resistant to classical cytotoxic drugs due to the overexpression of drug-extruding pumps and DNA-repairing enzymes (110), in CSC cultures from different tumors the sensitivity to molecular-targeted drugs, in particular to tyrosine kinase inhibitors, is retained (114, 126).

In line with these premises, PASCs, isolated as SP, showed 1.5-fold up-regulation of the multidrug transporters ABCB1 and ABCG2 (73), thus suggesting their ability to extrude cytotoxic drugs. Accordingly, Xu et al. reported that PASCs from a GHoma were insensitive to both carboplatin and etoposide (69) and temozolomide resistance was described in another study (66). Interestingly, in a drug repositioning study, *in vitro* and *in vivo* treatment with disulfiram, a clinically approved drug for the treatment of alcoholism, sensitizes $CD133^{+}$/$nestin^{+}$ PASCs to temozolomide cytotoxicity, preventing drug-induced DNA damage repair by inhibiting O-6-methylguanine-DNA methyltransferase expression (83). Although CSC resistance to cytotoxic drugs is still a relevant issue in the treatment of all types of tumor, including aggressive PAs, PASCs might retain sensitivity to biological treatments. Thus, the sensitivity to commonly used drugs for PAs, somatostatin and dopamine agonists, and the expression of their receptors were tested in PASC subpopulations.

Both Wurth et al. and Peverelli et al. studies compared, in adenoma tissues and in PASC-enriched spheroids, the expression of somatostatin (SSTR) and dopamine (D2R, dopamine receptor type 2) receptors, which are targeted by currently used pharmacological treatments (i.e., SSTR2 and SSTR5, which are activated by lanreotide and octreotide, and D2R, activated by cabergoline) (77, 78). In the first study both SSTR2 and SSTR5 were expressed by the original GH-secreting tumors and within the analyzed sections, small groups of putative PASCs (immunopositive for NESTIN) also express SSTR2. Hence, it was suggested that these cells are not completely undifferentiated but could represent a progenitor cell subpopulation, expressing relevant regulatory neuropeptide receptors. This possibility was confirmed by *in vitro* studies on PASC spheroids which were mainly formed by cells co-expressing NESTIN, SSTR2 and SSTR5; moreover, also $OCT4^{+}$/$D2R^{+}$ double positive cells were detected in spheroids (77). Similar results were obtained by Peverelli et al., who reported the expression of mRNAs for *SSTR2* and *D2R*, in both histological preparations and spheroids from NFPAs, evaluated by RT-PCR. Unexpectedly, *SSTR5* mRNA was undetectable in spheroids although expressed in all tissues analyzed (78). The expression of all the five *SSTRs* was analyzed and compared with the original tumors also in PA MSCs derived from six GHomas and six NFPAs (75), although a different picture was reported. Mesenchymal PASCs derived from both PA subtypes express *SSTR1* at the highest level, while GHomas show higher expression of *SSTR1–4–5* than NFPAs, and lower level of for *SSTR2–3* was detected in both subtypes.

PASC expression of these pharmacologically relevant receptors prompted the analysis of their possible modulation to induce antiproliferative effects. In NFPAs, the role of SSTR2 and D2R activation in mediating antiproliferative signals was analyzed using two selective agonists: BIM23120 for SSTR2 and BIM53097 for D2R (78). The analysis was performed by BrdU incorporation assay either soon after cell dispersion (day 3) or after 30 days *in vitro*, when PASC-containing spheres were completely formed. Interestingly, a different responsivity among the cultures was observed, with about 33% of tested adenomas showing reduced proliferation in response to BIM23120, and 43% to BIM53097. Sensitive tumors showed a similar response in both culture conditions, indicating that drug sensitivity was already present within tumors and not acquired during the *in vitro* selection and expansion. On the average, although variability among the cultures was observed, as expected, BIM23120 (10 nM for 72 h) reduced DNA synthesis in the spheroids by 65%, while BIM53097 by 45% (78). Moreover, both drugs increased spheroid cell expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and decreased cyclin D3 content, as molecular correlate of the antiproliferative effects of these receptors, as already reported for SSTRs (127). Importantly, no difference in the frequency of sphere formation among drug sensitive and resistant adenomas was observed, although the former were larger in size (78). Wurth et al., in light of the co-expression of SSTR2, SSTR5, and D2R in the same spheroids, used the MTT reduction assay to test the antiproliferative effects mediated by these receptors using an innovative chimeric SSTR2/SSTR5/D2R agonist, named BIM23A760 (128). In all the cultures analyzed, BIM-23A760 (1 nM, for 24 h) inhibited growth of spheroid-derived PASCs, reaching a statistical significance in

TABLE 2 | Drug sensitivity of pituitary adenoma stem cells.

PASC origin	PA histotype	Drug	Target (s)	Effect	References
HUMAN	GH-oma	Carboplatin	DNA alkylation	Drug resistance	(69)
		Etoposide	Topoisomerase II	Drug resistance	
HUMAN	GH-oma	Temozolomide	DNA alkylation	Drug resistance	(66)
HUMAN	n.s.	Temozolomide	DNA alkylation	Drug resistance (MGMT-dependent)	(83)
		Disulfiram	ALDH	Cell sensitization to temozolomide	
HUMAN	NFPA	BIM23120	SSTR2	Anti-proliferative	(78)
		BIM53097	D2R	Anti-proliferative	
HUMAN	NFPA	BIM23A760	SSTR2,5-D2R	Anti-proliferative	(77)
HUMAN	GH-oma	Somatostatin	SSTR1-5	Anti-proliferative	(75)
		Octreotide	SSTR2,5	Anti-proliferative	
		Pasireotide	SSTR1,2,3,5	Anti-proliferative	
HUMAN	NFPA	Somatostatin	SSTR1-5	No effect	(75)
		Octreotide	SSTR2,5	No effect	
		Pasireotide	SSTR1,2,3,5	No effect	
MOUSE	AtT20 cells (ACTH-oma)	Plerixafor	CXCR4	Inhibition of EMT-associated motility and xenograft tumor growth	(73)

PASC, pituitary adenoma stem cell; PA, pituitary adenoma; MGMT, O-6-methylguanine-DNA methyltransferase; ALDH, aldehyde dehydrogenase; SSTR, somatostatin receptor; D2R, dopamine receptor 2; n.s., not specified.

6 out of 7 PAs (ranging from −14 to −30% of vehicle-treated controls), independently from the length of the period of growth *in vitro* (analogous results were obtained in cultures lasting from 7 to 30 days) (77). An indirect evidence of the antiproliferative activity of D2R in PASCs was provided in *D2R* knockout mice, which develop prolactinomas starting at 6–8 months of age, and whose tumors show higher colony forming activity and a 2.4-fold increase in $Sox2^+$ cells than the WT glands (73). An unexpected difference in SSTR agonist sensitivity between mesenchymal PASCs derived from GHomas and NFPAs, was also reported (75), using native somatostatin (1 μM), which activates all the five SSTRs, octreotide, able to bind SSTR2 and SSTR5, and pasireotide, which activate all SSTRs but SSTR4. In fact, using both XTT test and direct cell count, a statistically significant inhibition of proliferation was observed with all the agonists in PASCs derived from GHomas after 72 or 144 h of treatment (max inhibition about−40% for both octreotide and pasireotide), without affecting EMT or the induction of apoptosis. Conversely, no effects were detected in mesenchymal PASC from NFPAs, although SSTRs were expressed in these cells, and previous studies reported antiproliferative activity of somatostatin, octreotide, and pasireotide (112, 129, 130).

Finally, a different experimental model using PASCs isolated as SP from tumors originating from the murine ACTHoma cell line AtT20 (73) was used to demonstrate the role of the chemokine CXCL12 (131) in PASC proliferation. The inhibition of CXCR4, the CXCL12 receptor, using the clinical approved CXCR4 antagonist plerixafor, reduced EMT-associated PASC motility *in vitro*, and xenograft tumor growth *in vivo* (73).

All these data (summarized in **Table 2**) support the notion that in PASCs, although isolated from different PA subtypes, with different procedures, showing an undifferentiated phenotype, express most of the membrane receptor systems involved in pituitary cell stimulation (CXCR4) or inhibition (SSTRs, D2R) of cell proliferation are expressed and functioning. Thus, their modulation may represent a valuable pharmacological goal for this otherwise drug-resistant subpopulation.

CONCLUSIONS AND FUTURE PERSPECTIVES

From the literature we reviewed it is clear that, although consistent information is currently available concerning the actual existence of PASCs, many unsolved questions will need to be further explored.

Notwithstanding the highlighted differences among the published studies, the presence of stem-like cells in benign tumors seems to be a confirmed notion. In particular, the reported cell heterogeneity in PAs, a tumor type which by definition is considered monoclonal in origin, further reinforces this assumption. However, the divergent differentiation fate of PA cells during tumor development (retaining of stem-like phenotype, differentiation in hormone producing cells, acquisition of mesenchymal or neural features) rises the relevant issue of which factors actually control this transition, and the identification of possible stem cell niche(s) within the tumor mass where PASC can self-renew and act as reservoir for the bulk of tumor cells. To date, although some tumor areas were identified as putative PA niches, no formal demonstration has been provided. Importantly, although discrepancies among the papers that analyzed PASC features are clearly related to the procedures (different permissive media, side-populations, etc.) or the model (human PA of different subtypes, or mouse tumors) used, it is also evident that multiple stem-like cell populations may exist within PAs. Thus, it is very important to define the characteristics of these cells not only as far as the biological

features are concerned, but also to address whether different PASC subpopulations co-exist in the same tumor or different PAs develop from stem-like cells with distinct phenotypes. In this respect, another unsolved issue is the actual cell of origin (the real tumor stem cell) of PAs. While in the past most evidence was pointing out the role of FS cells, now it seems more likely that oncogenic transformation should occur in adult PSCs, or more differentiated pituitary progenitors. In this respect, novel technologies, including single cell RNA sequencing, may provide a significant contribution to address the question regarding the origin of different PASC populations within PAs, as recently performed in adult pituitaries (132). In this context, the complete characterization of the different PA subpopulations will allow the identification of the bases of the differential efficacy of cytotoxic and biological pharmacological treatments on PASCs. In fact, although cytotoxic drugs are scarcely effective on PASCs, as expected in putative CSCs, these cells express SSTRs and D2R and are responsive to the respective agonists. Considering that the clinically approved receptor agonists, such as octreotide, lanreotide, pasireotide, and cabergoline, display both anti-secretory and, although less defined, antiproliferative activities (133), it is conceivable that these dual effects may involve the modulation of receptors in different PA cell subsets: the former likely mainly acting on differentiated cells, and the latter on PASCs. Further studies trying to clarify this issue are most warranted.

Another relevant concern is the identification of mechanisms controlling PASC proliferation, self-renewal, and differentiation. Recently a role of miRNA in pituitary tumorigenesis was proposed (134, 135). miRNAs are small non-coding RNAs that post-transcriptionally modulate gene expression, and whose expression is altered in several human neoplasia. For example, relevant for the topic of this review, a different miRNA expression pattern was observed in NFPAs and normal pituitary (136) with a significant influence on stem cell-related pathways, such as NOTCH (137). The field of miRNA is rapidly evolving and its application to different PASC populations might provide important novel information concerning the pathogenic mechanisms activated in these cells to originate PAs.

Finally, a crucial issue for PASC research is the demonstration of their tumorigenic potential, a required feature to define putative CSCs. The development of animal models using human PA cells is also extremely relevant to provide pharmacological preclinical clues to be translated in clinical studies. As discussed in the previous paragraph, this topic is very puzzling using traditional mouse models, and novel approaches, such as zebrafish embryo, can address only few aspects of this matter (i.e., neoangiogenesis and cell migration). Recently, development of 3D organoid systems (63), derived from Sox2-expressing normal mouse pituitary cells allowed to maintain long-term growth and stem-like phenotype during the expansive culture but also to the ability to differentiate into hormone-producing subpopulations. The application of this methodology to PAs may contribute to definition of the actual tumor-initiating subpopulation, the cellular and molecular pathways underlying tumor growth and the possible acquisition of invasive features, overcoming limitations observed using animal models.

AUTHOR CONTRIBUTIONS

All authors contributed to the literature search and selection, the critical analysis of the data, and to writing the manuscript.

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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 Endocrinology

MINI REVIEW
published: 25 February 2020
doi: 10.3389/fendo.2020.00079

Cancer Stem Cells in Pheochromocytoma and Paraganglioma

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OPEN ACCESS

Edited by:
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Reviewed by:
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Michaela Luconi, University of Florence, Italy

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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *22 November 2019*
Accepted: *06 February 2020*
Published: *25 February 2020*

Citation:
Scriba LD, Bornstein SR, Santambrogio A, Mueller G, Huebner A, Hauer J, Schedl A, Wielockx B, Eisenhofer G, Andoniadou CL and Steenblock C (2020) Cancer Stem Cells in Pheochromocytoma and Paraganglioma. Front. Endocrinol. 11:79. doi: 10.3389/fendo.2020.00079

Pheochromocytoma (PCC) and paraganglioma (PGL) are rare neuroendocrine tumors associated with high cardiovascular morbidity and variable risk of malignancy. The current therapy of choice is surgical resection. Nevertheless, PCCs/PGLs are associated with a lifelong risk of tumor persistence or recurrence. A high rate of germline or somatic mutations in numerous genes has been found in these tumors. For some, the tumorigenic processes are initiated during embryogenesis. Such tumors carry gene mutations leading to pseudohypoxic phenotypes and show more immature characteristics than other chromaffin cell tumors; they are also often multifocal or metastatic and occur at an early age, often during childhood. Cancer stem cells (CSCs) are cells with an inherent ability of self-renewal, de-differentiation, and capacity to initiate and maintain malignant tumor growth. Targeting CSCs to inhibit cancer progression has become an attractive anti-cancer therapeutic strategy. Despite progress for this strategy for solid tumors such as neuroblastoma, brain, breast, and colon cancers, no substantial advance has been made employing similar strategies in PCCs/PGLs. In the current review, we discuss findings related to the identification of normal chromaffin stem cells and CSCs, pathways involved in regulating the development of CSCs, and the importance of the stem cell niche in development and maintenance of CSCs in PCCs/PGLs. Additionally, we examine the development and feasibility of novel CSC-targeted therapeutic strategies aimed at eradicating especially recurrent and metastatic tumors.

Keywords: cancer stem cells, adrenal, pheochromocytoma, paraganglioma, hypoxia

INTRODUCTION

The adrenal gland is composed of two main tissue types that establish a bidirectional connection; the catecholamine-producing chromaffin cells in the medulla and the primarily steroid-producing cells in the cortex. The inner medulla is derived from neuro-ectodermal cells of neural crest origin while the outer cortex is derived from the intermediate mesoderm. Close interactions between the

two components are necessary for differentiation and morphogenesis of the gland (1, 2). Although highly heterogeneous, pheochromocytomas (PCCs) and paragangliomas (PGLs) are generally slow growing neural crest-derived tumors comprised of chromaffin cells arising at intra- and extra-adrenal locations, respectively (3). The tumors show a very high rate of germline or somatic mutations in a large number of genes (4, 5). When occurring at a young age, the tumors are often multifocal, suggesting development during embryogenesis from a common progenitor during neural crest migration to different sites of tumorigenesis (6).

STEM CELLS IN THE NORMAL ADRENAL MEDULLA

The neural crest represents a migratory population of multipotent cells transiently present during embryogenesis, which gives rise to a diverse variety of cell types and tissues (7–9). At 11.5 days post coitum (dpc) in the mouse, early neural crest cells migrate near the dorsal aorta, and form the suprarenal ganglion. These progenitors will give rise to both chromaffin cells and neurons, which innervate the adrenal medulla. From 11.5 to 15.5 dpc, late neural crest cells migrate toward the dorsal root ganglion, where they acquire a Schwann cell precursor (SCP) fate and express the transcription factor SOX10, which is reported to be expressed in human, bovine and murine chromaffin progenitors and stem cells (10–12). SOX10-positive SCPs migrate along the nerves toward the medulla primordium, and give rise to over 50% of the adrenomedullary chromaffin cells (13). The majority of chromaffin cells of the Zuckerkandl organ, which is the largest extra-adrenal chromaffin body in mammals, are also generated from SCPs (14). Until recently, it was believed that sympathoblasts and chromaffin cells originate from a common progenitor (15), but the recent studies of SCPs show an earlier segregation of the two lineages (14). SCPs have lately attracted considerable attention due to numerous reports highlighting their role as multipotent progenitors generating various fates in the body (16–20). In addition to giving rise to chromaffin and mature Schwann cells, SCPs propagate to mesenchymal stem cells (21–23), endoneural fibroblasts (24), and melanocytes (25), which use the peripheral nerves to reach their specific locations in the developing embryo (18).

The postnatal adrenal medulla is composed of two main differentiated cell types: catecholamine-producing chromaffin cells, which represent the functional unit of the gland, and neurons stimulating the catecholamine production. A third cell type of the adrenal medulla has been identified as "sustentacular" or "support" cells. These cells are of glial nature and express S100B, GFAP, and Vimentin (26). Sustentacular cells are found in proximity to both chromaffin cells and nerve terminations, but their function has not been clarified yet. Sustentacular cell markers are expressed by Nestin-positive cells, proposed to be stress-responsive progenitors of the adrenal medulla (11). The cytoskeletal type VI intermediate filament Nestin was initially identified as a marker of neural stem and progenitor cells (27, 28), but was later shown to be expressed in a variety of tissues and other stem or progenitor cells (29). Nestin appears to be linked to essential stem cell functions including self-renewal/proliferation, differentiation and migration (29), and is expressed in progenitors of both the adrenal cortex and medulla (30, 31). Furthermore, Nestin seems to correlate with malignancy in several different cancer types (32). In the adrenal medulla, Nestin-positive cells overlap with CD133-positive cells and can differentiate into chromaffin cells and cells of the neural lineages (11). CD133 is expressed by progenitor/stem cells in several organs including the brain under healthy or cancerous conditions [for review see (33)], and expression of CD133 has also been demonstrated in primary cultures of human and bovine adrenal medullary cells grown under stem cell promoting conditions (10, 12).

TUMORIGENESIS OF PCCS AND PGLS

Around 30–40% of PCCs/PGLs are hereditary due to mutations in close to 20 currently identified PCC/PGL susceptibility genes, whereas another 40–50% show somatic mutations in the same as well as other genes (34, 35). At the time of PCC/PGL diagnosis, the mean age of patients is 40–50 years, though 10–20% of all cases are diagnosed in children (36). PCCs/PGLs can be divided into two main clusters depending on their underlying mutations in any of the susceptibility genes: the pseudohypoxia-associated cluster 1 and the kinase signaling-associated cluster 2 (4, 34). A third WNT signaling-associated cluster due to translocation of *MAML3* has also recently been described (35), but remains poorly characterized. Genes most commonly contributing to cluster 1 PCCs/PGLs are those encoding the four subunits of the succinate dehydrogenase (SDH) enzyme, namely *SDHA*, *SDHB*, *SDHC*, and *SDHD*, and the SDH assembly co-factor *SDHAF2*. Other genes associated with a pseudohypoxic signature include *VHL*, *EPAS1*, *FH*, *MDH2*, and *EGLN1/2*. Chromaffin cell tumors arising due to mutations affecting SDH, but particularly SDHB, are predominantly aggressive and often malignant. These tumors and those due to *VHL* mutations often occur in childhood suggesting development during embryogenesis from a common stem cell/progenitor. According to the classical "two-hit" model, two mutations are a prerequisite for tumorigenesis resulting from loss of function mutations. In addition to the original germline/somatic mutation, tumorigenesis requires a second somatic mutation of the same gene (37). However, compared to other tumors PCCs/PGLs exhibit a low somatic mutation rate (35) suggesting that at least in pediatric tumors a single mutation is sufficient for tumorigenesis. Cluster 2 tumors include mutations in the *RET*, *NF1*, *TMEM127*, and *MAX* genes and are characterized by activated PI3K/AKT/mTOR and RAS/RAF/ERK downstream kinase and protein translation signaling pathways (38). These tumors almost always originate in the adrenals, and clinically they do not display a particularly aggressive behavior. Furthermore, they have more mature catecholamine secretory pathways and phenotypic features, and they tend to develop later in life than tumors due to cluster 1 mutations (6, 39).

Normal stem cells are regulated by extrinsic cytokines as well as by intrinsic genetic programs within their niche (40).

This niche must be pliable to coordinate both homeostasis and repair; however, such flexibility can be distorted by chronic diseases and cancer. During embryonic development, especially before vascularization, cells exist in a relatively oxygen-poor environment. Consequently, oxygen sensing pathways play crucial roles in ensuring appropriate embryonic morphological development and survival (41). Similarly, intratumoral hypoxia provides a microenvironment that shields CSCs and stimulates their proliferation (42). Under changing oxygen levels hypoxia-inducible transcription factors (HIFs) activate genes that promote tolerance of hypoxia by decreasing the cellular requirements for oxygen and by increasing the supply of oxygen (43–45). This is potentially mediated by two HIF isoforms, HIF1α and HIF2α differentially coordinating migration, survival and differentiation of neural crest cells (46, 47).

The common denominator for the pseudohypoxic phenotype of all cluster 1 tumors involves HIF stabilization. It appears that stabilization of HIF2α rather than HIF1α is responsible for tumor development and the distinct phenotypic features of cluster 1 chromaffin cell tumors (47). Stabilization of HIF2α also provides the unifying mechanism responsible for the pseudohypoxic phenotypes of all cluster 1 PCCs/PGLs (48). Mutations in the *EPAS1* gene encoding HIF2α are almost always somatic, but still often involve a syndromic presentation including polycythemia (elevated volume of red blood cells in the blood) and somatostatinomas (49, 50). Although lacking the central pseudohypoxic footprint, the cluster 2 tumors relies on a glycolytic and glutaminolytic switch, necessary for cell proliferation and survival, as well as for chromatin remodeling. This means that even though genetically there is a high heterogeneity in PCCs/PGLs, the molecular pathways defining the three clusters are interrelated and all participate in developmental processes (51).

Especially in cluster 1 tumors that develop early in life, mutated SCPs might be one of the initiating tumorigenic cell types since recent data on SCPs reveal that they can give rise to both adrenal and extra-adrenal chromaffin cells. Furthermore, PCCs and PGLs share diagnostic markers. Other tumor-initiating cell types could be chromaffin cells, sympathetic-like chromaffin cells or sympathoblasts (**Figure 1**).

Even if the chromaffin cells in the adrenal medulla and extra-adrenal tissues, like the Zuckerkandl organ, have the same origin, they are quite different at later developmental steps (14). This suggests that the earlier the mutation happens, the higher the risk for developing multifocal tumors. When the mutation arises later, the progenitor cell is already committed to giving rise to either intra- or extra-adrenal chromaffin tissue. This might also explain the differences in metastatic potential between PCCs and PGLs as about 10–20% of PCCs are metastatic, whereas up to 50% of PGLs are metastatic (52).

The high prevalence of multifocal tumors at a young age may indicate secondary somatic mutations before the settlement of migrating SCPs at different locations. In support of this concept, PGLs carrying somatic *EPAS1* (HIF2α) mutations are associated with mosaicism and identical mutations in multiple tumor sites, consistent with the timing of tumor development as a result of postzygotic mutations during early embryogenesis (53, 54). During embryonic development, a large proportion of sympatho-adrenal progenitors undergo programmed cell death. This is also in keeping with observations that paraganglial chromaffin tissue, and particularly the Zuckerkandl organ, are well developed in the late-stage human fetus and regress after birth with only vestigial clusters of cells persisting into adulthood (55). These extra-adrenal clusters of chromaffin cells may reflect the remnants of the hypoxia-sensing peripheral catecholamine system, which is more important during fetal development than in adulthood. This is different from the neural crest-derived carotid body that remains important throughout adulthood for oxygen sensing. In a physiological state, the carotid body responds to chronic hypoxia with hyperplasia and hypertrophy of its neural and vascular

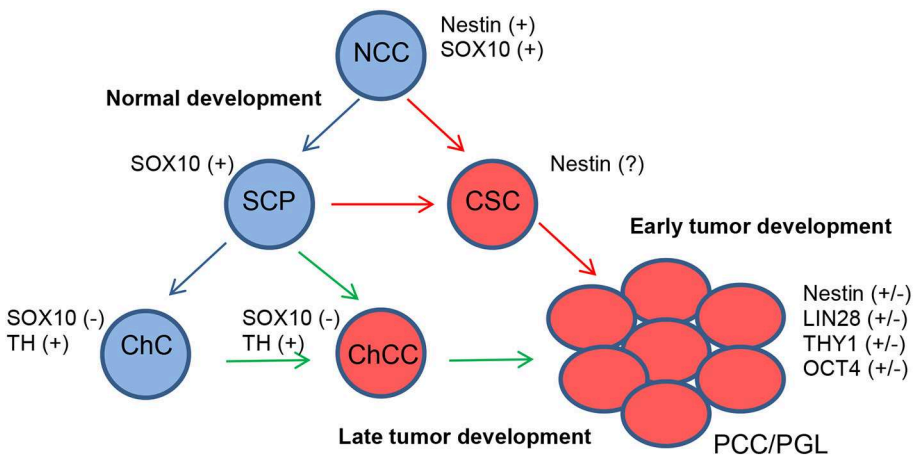

FIGURE 1 | Proposed model for the development of PCCs/PGLs. Under normal conditions, neural crest cells (NCCs) differentiate into SCPs and finally chromaffin cells (ChCs) (blue arrows). In PCCs/PGLs, developing at an early age, somatic mutations in SCPs give rise to CSCs from where tumors develop (red arrows). Somatic mutations happening in ChCs also give rise to PCCs/PGLs via chromaffin cancer cells (ChCCs) but with a later appearance (green arrows).

tissue components. In order to do this neural crest-derived progenitors in the carotid body retaining mesoectodermal differentiation potential are reactivated in a HIF-dependent manner (56–58).

CANCER STEM CELL MARKERS FOUND IN PCCS AND PGLS

CSCs are self-renewing and due to their stem-like properties they may be more resistant to chemotherapy and radiation (59). As these cells are potentially at the basis of tumor formation and thereby a potential target for future therapies, a lot of attention has been focused on their existence and impact in several other cancers, especially in endocrine and neural tissues. Even though many of the PCC/PGL mutations are related to stem cell markers (51), there are few studies focusing on CSCs in PCCs/PGLs. Increasing evidence suggests that CSCs are reliant on low oxygen conditions, and therefore on HIF1α and HIF2α to maintain their stem cell features (47, 60). Recently, it was shown that only in CSCs the pluripotency-promoting transcription factors NANOG and SOX2 cooperate with MYC to regulate HIF2α, which leads to a decrease in P53 expression and reduces the levels of reactive oxygen species in CSCs thereby promoting stemness (61). This is supported by other studies showing that primarily HIF2α is activated in CSCs (62).

Often the results on the expression of stem cell markers in PCCs/PGLs are contradictory, likely reflecting tumor heterogeneity (**Table 1**). Though the molecular pathways in the different tumors might be related, the amount of the different cell types in the tumors and their levels of differentiation, remain variable, possibly due to their microenvironment (51). For example, the expression of OCT4 is inconsistent as a study by Looijenga et al. concluded that PCCs/PGLs are negative for OCT4 (63), yet a subsequent study by Alexander et al. showed strong and diffuse cytoplasmic staining of OCT4 in PCCs and metastases (64). Oudijk et al. analyzed the expression of relevant CSC markers in tissue microarrays of a large number of PCCs and PGLs and showed that frequently CSC marker expression was associated with cluster 1 *SDHx*-mutated tumors (65), though expression of CSC markers has also been observed in PGLs from patients without *SDHx* mutations (66). Contrary to the findings within other adrenal tumors, OCT4, CD133, and NANOG expression was not detected in any of the samples from Oudjik et al., however expression of other stem cell markers such as DLK1/PREF1, NGFR, LIN28, SOX2, and THY1 was observed in 12-40% of cases, whereas the expression of Nestin, SOX17 and CD117 was identified less frequently in 2–3% of cases (65). In contrast, a case study of a PCC in pregnancy showed high expression of Nestin (67), which, as CD133, has also been detected in PGLs (66). As Nestin marks sustentacular cells, it raises questions regarding their role in PCCs/PGLs. To

TABLE 1 | CSC markers in PCCs/PGLs.

Marker	Subcellular location	Function	Expression in PCCs/PGLs (percentage of positive tumors)	References
OCT4	Nucleus and cytoplasm	Transcription factor Important for maintaining pluripotency, forms a complex with SOX2, regulates NANOG	+(0–100%)	(63–65)
NANOG	Nucleus	Transcription factor Important for maintaining pluripotency	–	(65)
SOX2	Nucleus	Transcription factor Expressed in the developing CNS Important for maintaining pluripotency, forms a complex with OCT4	+(12%)	(65)
SOX17	Nucleus	Transcription factor Regulates embryonic development	+(2%)	(65)
Nestin	Cytoplasm	Type VI intermediate filament Marker of neural stem/progenitor cells	+(3%)	(65–67)
CD90 (THY1)	Cell membrane	N-glycosylated, glycophosphatidylinositol anchored cell surface protein Marker of various stem cells	+(40%)	(65, 66)
CD117 (C-kit)	Cell membrane	Tyrosine-protein kinase acting as a receptor for c-kit ligand. Involved in growth and development of the cerebral cortex	+(3–14%)	(65, 66, 68, 69)
CD133 (Prominin-1)	Cell membrane	Cell surface glycoprotein Plays a role in cell differentiation, proliferation and apoptosis	±	(65, 66)
CD271 (NGFR)	Cell membrane	Nerve growth factor receptor Can mediate cell survival as well as cell death of neural cells	+(19%)	(65)
LIN28	Nucleus, endoplasmic reticulum, cytoplasm	RNA binding protein enhancing translation of IGF-2 Regulates self-renewal of stem cells	+(24%)	(65)
DLK1 (PREF1)	Cell membrane	Protein Delta Homolog 1, transmembrane protein belonging to the EGF-like homeotic protein family Plays a critical role in differentiation processes	+(19%)	(65, 66)

date, the precise role of this cell type in the normal adrenal medulla and in PCCs/PGLs has not been clarified. Several studies demonstrated differences in the number of sustentacular cells in primary and metastatic PCCs (70, 71), and a case of a distinctive neoplasm thought to have originated from S100-positive sustentacular cells has previously been reported (72). These observations support the notion that sustentacular cells may have a role in tumor formation or metastasis of PCCs/PGLs. However, these results also show the paucity of studies analyzing the expression of CSC markers in all clusters of PCCs/PGLs.

CSC TARGETED THERAPIES

When possible, surgery is always the therapy of choice for non-metastatic PCC/PGL. With metastatic disease, a primary tumor resection is recommended followed by radiotherapy or classical chemotherapy [reviewed in (73)]. In the case of progression or if classical therapies are not possible or tolerated by the patient, targeted therapies might be considered. As most PCCs/PGLs show a high expression of somatostatin receptor subtype 2, radiolabeled somatostatin analogs have been investigated as a treatment option (74). Other targeted therapies with for example different tyrosine receptor kinase inhibitors are under investigation and show promising results (73). As PCCs/PGLs occasionally express CD117 (**Table 1**), the tyrosine kinase inhibitor imatinib has for example been tested on *in vitro* cultures of PGL cell cultures, where it was shown to inhibit growth of both *SDHx*-unrelated and related tumor cells. Furthermore, it prevented xenograft formation in mice transplanted with patient-derived cells (66).

CSCs are known to be more resistant to conventional chemo- and radio-therapy compared to non-CSC populations. Pre-clinical and clinical trials in multiple tumor types have targeted CSCs via surface markers, inhibition of developmental stem cell pathways, or ablation of CSC niches [reviewed in (75)]. In neuroblastoma (NB) a common link between signaling cascades involved in tumorigenesis is hypoxia, and in hypoxic regions of NB, HIF1, and HIF2 have been shown to be expressed in cells with stem-like features (76). NB is the most common extracranial pediatric solid tumor, which often arises in infants and children up to 5 years (77). It originates from embryonic neural crest cells during early embryogenesis and mutated SCPs are likely also in these tumors the initiator cells (14). In contrast to PCCs/PGLs, the expression of stem cell markers in NB have been more studied (78). The existence of CSCs in NB has been associated with malignancy, resistance to chemotherapy and recurrence (79), as in chemo- and/or radiation-resistant NB cancer stem-like cells an increased expression of the stem cell markers CD133, SOX2, ALDH1, Nestin, OCT4, and NANOG was observed (80). Additionally, populations of cells positive for Nestin, CXCR4, and OCT4 were increased in late-stage NB (81). It has been suggested that differentiation of such NB CSCs may render them more sensitive to therapeutic intervention (78). One way to induce cancer differentiation in NB is to use retinoic acid (82), and actually combined retinoic acid treatment and proteasome inhibition demonstrated the ability to inhibit tumor-sphere formation and apoptosis in NB CSCs. Also the expression of Nestin, SOX2, and OCT4 was reduced with this treatment (83). Other combination therapies for NB CSCs showed promising results as well [reviewed in (78)]. Because of the similarity in origin and CSC marker expression between NB and PCCs/PGLs, these therapies might also be an option for the treatment of PCCs/PGLs.

In glioblastoma, the epidermal growth factor receptor (EGFR) antagonist nimotuzumab targeting CD133 has been shown to radiosensitize subpopulations of cells (84). Furthermore, anti-EGFR therapy in combination with conventional therapy has shown promising results in patients with epithelial cancers (85–87). Furthermore, in a cohort of advanced basal cell carcinoma patients, Von Hoff *et al.* demonstrated a favorable response to treatment with a small-molecule inhibitor of the hedgehog pathway (88). Other studies have investigated the NOTCH and WNT signaling pathways in multiple solid tumor patient populations (89). Stem cell specific niche constituents and their cognate receptors such as fibronectin and the fibronectin receptor in acute myeloid leukemia and breast cancer, respectively, have also been targeted and demonstrated strong anti-tumorigenic activity (90, 91). Since many of these targets, as e.g., NOTCH1 and CD133, are also expressed in metastatic PCCs/PGLs (66), the CSC-targeted therapies mentioned above might also be an opportunity for the treatment of these tumor types.

CONCLUSION

While the existence of CSCs has been observed not only in PCCs/PGLs but also in several other endocrine and neural tumor types, the precise role of these cell types in tumor formation and homeostasis remains largely unknown. PCCs/PGLs include a subset of tumors in a pseudohypoxic state. The signaling and tissue microenvironments described in this state seem to encourage cell proliferation and survival and may even support persistence of cells expressing embryonic stem cell markers. Expression of several stem cell markers appears to be associated with PCC/PGL mutations, although the precise nature of this possible relationship needs to be further elucidated. Additionally, sustentacular cells may play a role in tumor development or invasiveness. However, there is still a need for thorough characterization of these cells in tumor formation. Finally, investigations into the homeostatic role of adult stem cells within the normal adrenal medulla in comparison to the tumor state may help understand the contribution of CSCs to PCCs/PGLs, which might lead to new therapies targeting CSCs in recurrent and metastatic PCCs/PGLs. Until now, CSC targeted therapies have not been employed for the treatment of PCCs/PGLs. However, due to similarity in the expression of CSC markers known from other tumor types, where CSC targeted therapies have shown promising results,

these therapies might be an option for PCCs/PGLs as well. In conclusion, characterization of CSCs in PCCs/PGLs and the regimen of CSC targeted therapies of these tumors require further research.

AUTHOR CONTRIBUTIONS

LS and CS wrote the first draft of the manuscript. ASa, CA, GE, and CS wrote sections of the manuscript. LS, SB, ASa, ASc, GM, AH, JH, BW, GE, CA, and CS contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) within the CRC/Transregio 205/1: The Adrenal: Central Relay in Health and Disease and the IRTG2251: Immunological and Cellular Strategies in Metabolic Disease.

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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 Endocrinology

REVIEW
published: 25 August 2020
doi: 10.3389/fendo.2020.00566

Cancer Stem Cells in Thyroid Tumors: From the Origin to Metastasis

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Thyroid tumors are extremely heterogeneous varying from almost benign tumors with good prognosis as papillary or follicular tumors, to the undifferentiated ones with severe prognosis. Recently, several models of thyroid carcinogenesis have been described, mostly hypothesizing a major role of the thyroid cancer stem cell (TCSC) population in both cancer initiation and metastasis formation. However, the cellular origin of TCSC is still incompletely understood. Here, we review the principal epigenetic mechanisms relevant to TCSC origin and maintenance in both well-differentiated and anaplastic thyroid tumors. Specifically, we describe the alterations in DNA methylation, histone modifiers, and microRNAs (miRNAs) involved in TCSC survival, focusing on the potential of targeting aberrant epigenetic modifications for developing novel therapeutic approaches. Moreover, we discuss the bidirectional relationship between TCSCs and immune cells. The cells of innate and adaptive response can promote the TCSC-driven tumorigenesis, and conversely, TCSCs may favor the expansion of immune cells with protumorigenic functions. Finally, we evaluate the role of the tumor microenvironment and the complex cross-talk of chemokines, hormones, and cytokines in regulating thyroid tumor initiation, progression, and therapy refractoriness. The re-education of the stromal cells can be an effective strategy to fight thyroid cancer. Dissecting the genetic and epigenetic landscape of TCSCs and their interactions with tumor microenvironment cells is urgently needed to select more appropriate treatment and improve the outcome of patients affected by advanced differentiated and undifferentiated thyroid cancers.

Keywords: cancer stem cells, thyroid tumors, epigenetic alterations, microenvironment, immune system

OPEN ACCESS

Edited by:
Natalia Simona Pellegata, Helmholtz Zentrum München, Germany

Reviewed by:
Giovanni Vitale, University of Milan, Italy
Marcello Maggiolini, University of Calabria, Italy

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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *29 May 2020*
Accepted: *10 July 2020*
Published: *25 August 2020*

Citation:
Veschi V, Verona F, Lo Iacono M, D'Accardo C, Porcelli G, Turdo A, Gaggianesi M, Forte S, Giuffrida D, Memeo L and Todaro M (2020) Cancer Stem Cells in Thyroid Tumors: From the Origin to Metastasis. Front. Endocrinol. 11:566. doi: 10.3389/fendo.2020.00566

THYROID TUMORS AND CANCER STEM CELLS

Thyroid cancers (TCs) are highly heterogeneous and represent the most frequent tumors among the endocrine neoplasms (1, 2). In the past years, TC incidence increased worldwide, and the gender disparity became more pronounced, especially considering women at age 40–49 with a female/male ratio of about three times higher (3). The molecular basis of these gender differences in TC is still unclear. However, it has been postulated that female steroid hormones play a critical role in TC development mediated by the differential expression of the nuclear α- and β-estrogen receptors in various TC histological subtypes (4). Moreover, estrogens increase adherence, invasion, and migration capability of thyroid cancer cell lines (5). Recently, it has been demonstrated that estradiol regulates a higher production of reactive oxygen species (ROS) in adult femal rats

compared with male counterparts (6, 7). Overall, the risk of thyroid proliferative diseases is increased during pregnancy, while the specific risk for TC is decreased after menopause.

According to their histopathological features, it is possible to distinguish four subtypes of thyroid carcinoma: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), anaplastic thyroid carcinoma (ATC), and medullary thyroid carcinoma (MTC). PTC, FTC, and ATC derive from malignant transformation of follicular cells, while MTC derives from calcitonin-producing parafollicular C cells. PTCs and FTCs represent the majority of differentiated TCs with good prognosis, accounting for 80–85 and 10–15% of all TCs, respectively. On the contrary, ATC is a rare and undifferentiated TC (UTC), characterized by an aggressive phenotype and poor prognosis. Although current therapeutic strategies including surgery, radioiodine therapy, and chemotherapy are able to eradicate the majority of primary TCs, the management of advanced and undifferentiated TCs is still a clinical hurdle. The existence of cancer stem cell (CSC) population explains the aggressiveness of TCs and their resistance to the clinical treatments. Scientific advances in stem cell biology have paved the way to a better understanding of the molecular mechanisms driving tumorigenesis in many types of cancers, including TCs (8) (**Figure 1**). CSCs are a small subset of cancer cells within tumors that exhibit exclusive self-renewal ability, clonogenic, and metastatic potential. They show a key role during the initiation, progression, drug resistance, and cancer recurrence or metastasis (9, 10). The isolation and characterization of thyroid cancer stem cell (TCSC) population in different thyroid tumors improved the knowledge about TC initiation. Nevertheless, there are still many questions to elucidate: (i) how TCSCs influence the initiation, progression, and metastasis within the four subtypes of TCs; (ii) how the tumor microenvironment (TME) affects TCSCs; (iii) which are the interactions between TCSCs and tumor bulk population; and (iv) which is the broad genetic/epigenetic landscape of TCs. Here, we provide an overview of the principal markers and pathways sustaining TCSC survival, their epigenetic alterations and interactions with immune cells, and TME cell components. We summarize the potential and innovative therapeutic approaches targeting TCSCs. Nowadays, dissecting the role of TCSCs in TC initiation, progression, and invasiveness may lead to the development of more effective therapies in advanced TCs.

Thyroid Cancer Model of Origin

Different carcinogenesis models have been proposed to describe TC origin. According to the multistep carcinogenesis model, ATC cells derive from FTC or PTC cells following a dedifferentiation process and the accumulation of different mutations, particularly the inactivating mutations of *TP53* and *CTNNB1* (11, 12). This model is supported by scientific evidence, such as the presence of *TP53* and *BRAF* mutations in differentiated and undifferentiated carcinomas, including ATCs (13), but it cannot explain the presence of specific *RET/PTC* rearrangements and *PAX8/PPARγ* gene fusion that may occur in ATCs (14). Moreover, the slow cell cycle of follicular thyroid cells reduces the potential accumulation of mutations, which sustain cancer progression (15). Thus, according to the multistep carcinogenesis model, TCSCs can derive from thyrocytes upon genetic alterations and the epithelial–mesenchymal transition (EMT) process, which induces in TCSCs a more aggressive phenotype able to give rise to metastasis (16). During EMT, TCSCs lose polarity and adhesion, thus acquiring an invasive phenotype through Snail upregulation, which impairs E-cadherin expression (17). The fetal cell carcinogenesis model foresees that thyroid cancer cells derive from thyroid fetal cells upon the acquisition of specific mutations. The term thyroid fetal cells refers to TCSCs or thyroid precursor cells such as thyroblasts or prothyrocytes (18). TCs present a different cell of origin as well as a different mutational profile based on their distinct histopathological features. Specifically, ATC originates from fetal TCSCs expressing onco-fetal fibronectin without the expression of any differentiation markers, while PTC originates from thyroblasts expressing onco-fetal fibronectin and differentiation markers as thyroglobulin (Tg). Finally, FTC originates from the prothyrocytes, representing a more differentiated thyroid cell (19). Interestingly, to date, many evidence support the CSC model also for TC origin and initiation (20). CSCs represent a small population in the tumor bulk. They are placed at the apex of the hierarchical pyramid that includes also progenitors and differentiated cells. CSCs are able to drive cancer initiation and progression by acquiring genetic mutations and epigenetic alterations (21). Nowadays, the dynamic CSC model superseded the CSC model. Indeed, CSC phenotype has been considered plastic and dynamic, able to interchange between a CSC state and a non-CSC state, spontaneously or in response to microenvironment stimuli (EMT or IL-6) in various cancer types (22–24). An extensive description of the thyroid carcinogenesis models can be found in (25). Given that all the above-described models do not completely explain the phenotypic and genetic heterogeneity of tumor bulk, it has been recently postulated the genetic mutation model to elucidate the molecular mechanisms underlying the distinct TC histopathology and behavior. Specifically, according to this model, a variety of genetic mutations may occur in the same CSC by leading to different tumor phenotypes. This can be termed as the CSC genetic mutation model in TC (**Figure 1**).

Normal vs. Thyroid Cancer Stem Cells

To date the origin of TCSC population is still incompletely understood. Specifically, whether TCSCs originate from precursors or mature cells and whether TC cells are the result of genetic mutations or epigenetic alterations occurring in thyroid stem cells (TSCs) are issues to be clarified. Nevertheless, the existence of TCSCs is confirmed by *in vitro* thyrosphere generation and by the development of *in vivo* mouse models. To date, several markers have been proposed to identify TCSCs, such as CD133, CD44, and aldehyde dehydrogenase gene (ALDH) (20, 26). Several studies highlighted that TCSCs present specific features that distinguish them from normal TSCs. Both CSCs and stem cells (SCs) undergo symmetric division, but their clonogenic and differentiative potential is different. Giani et al. observed that thyrospheres generated from PTC-derived CSCs (PTC-CSCs) were larger and irregular compared with

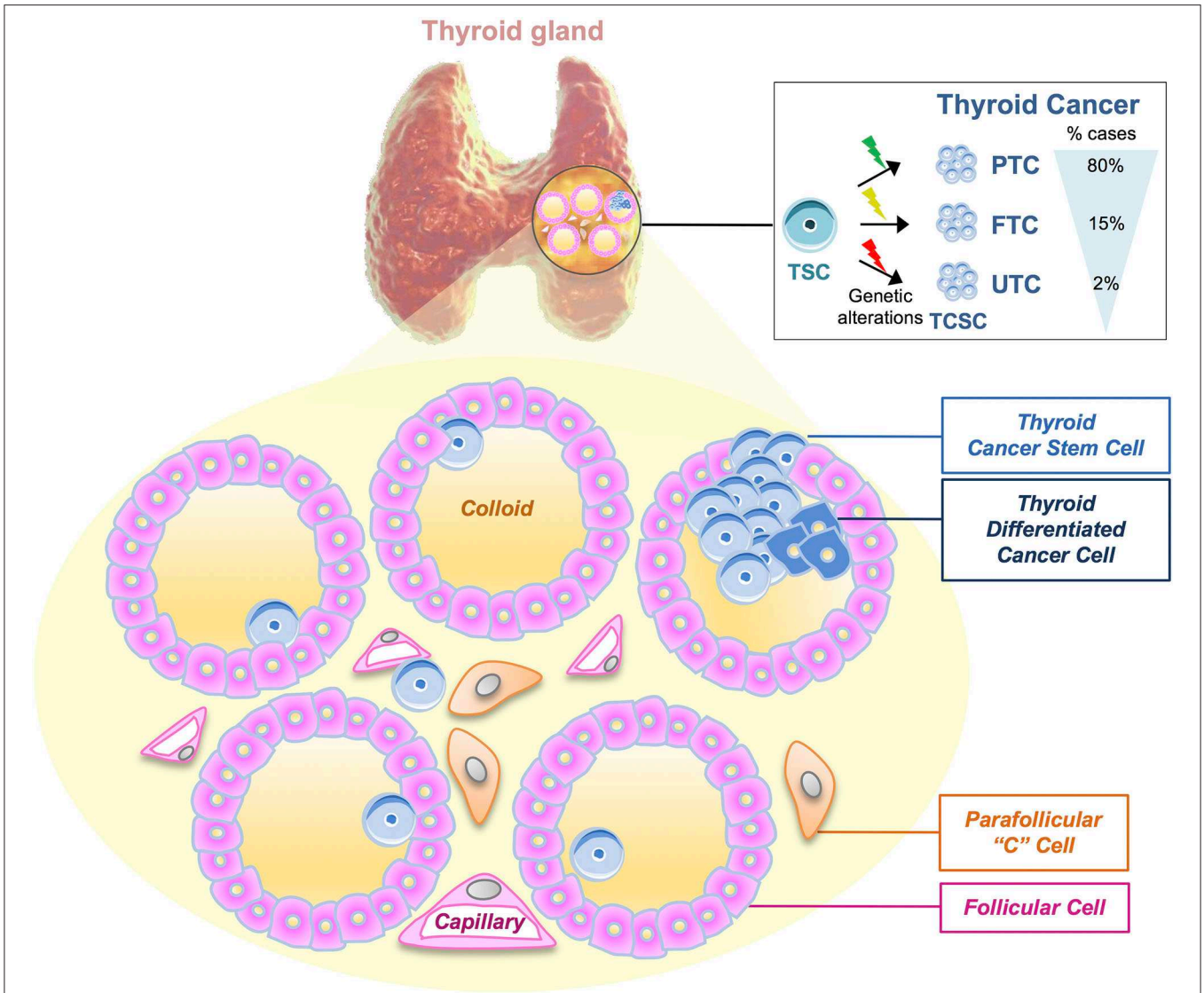

FIGURE 1 | Thyroid tumors and thyroid cancer stem cell genetic model. Thyroid cancers (TCs) are heterogeneous diseases varying from well-differentiated [papillary thyroid carcinomas (PTCs), follicular thyroid carcinomas (FTCs)] to undifferentiated tumors (UTCs). According to the most recent thyroid cancer stem cell model, the cell of origin of TC may be represented by the same cancer stem cell, which is subjected to different types of genetic mutations, leading to the development of PTC (80% of cases), FTC (15%), and UTC (2%), respectively. The model shown represents the microscopic structure of thyroid follicles, in which walls of follicular cells surround the follicle lumen filled with colloid, the center of thyroid hormone production. Moreover, following this model, thyroid cancer stem cells (TCSCs) along with thyroid differentiated cancer cells may populate the thyroid follicle. Parafollicular "C" cells are the cells of origin of medullary thyroid carcinoma (MTC).

normal TSCs. The clonogenic potential and expression levels of stemness markers (Oct-4, Sox-2, ABCG2) and EMT markers, as vimentin, were higher in PTC-CSCs compared with normal TSCs. Moreover, TCSCs showed lower expression levels of differentiation markers such as Pax-8 and TTF-1, and their differentiation efficiency was poorer than normal TSCs (27). Malguarnera et al. dissected the differences between CSCs derived from PTC and thyroid normal stem/progenitor cells. Both cellular types are able to generate thyrospheres in culture. However, only thyrospheres derived from normal thyroid stem progenitor cells could differentiate when plated in adhesion in presence of thyroid-stimulating hormone (TSH). Stemness markers as CD133, CD44, Oct-4, Sox-2, and Nanog were revealed in both normal and cancer thyrospheres; conversely, thyroid differentiation markers [thyroperoxidase (TPO), thyroglobulin (Tg), thyroid-stimulating hormone receptor (TSH-R)] were detected at low levels in both cellular types. In addition, the authors showed that insulin receptors (IR-A and IR-B), insulin growth factors (IGF-I and IGF II), and the IGF receptor (IGF–IR) were expressed at higher levels in CSCs compared to the differentiated cells. These findings confirm that insulin resistance is related to an enhanced susceptibility to develop TC, and

therefore, insulin and/or IGFs should be considered as novel potential targets for TC treatment (28).

Markers Identifying TCSCs and Pathways Sustaining Their Maintenance

Many studies have been carried out to identify specific biomarkers of TCSCs in the four histopathological TC variants, but their combination and/or association needs to be further investigated. Friedman et al. demonstrated that cell lines derived from ATCs are $CD133^+$, and when transplanted in immunodeficient non-obese diabetic (NOD)/severe combined Immunodeficiency (SCID) mice, they are able to develop tumor (29). Todaro and coworkers were the first to isolate TCSCs from primary thyroid tumors using ALDH activity. The authors highlighted the ability of TCSCs to form thyrospheres and recapitulate the parental tumor behavior when transplanted in murine thyroid gland. They found that a higher activity of ALDH in ATCs compared with FTCs and PTCs is correlated to the migration ability of TCSCs. $ALDH^+$ TCSCs derived from ATC showed an enhanced migratory ability compared to $ALDH^+$ cells derived from FTC and PTC. This phenotype is associated with an increase in c-MET and AKT activation. Silencing of these genes completely blocks the $ALDH^+$ TCSCs metastatic capacity. Thus, c-MET and AKT have been proposed as potential therapeutic targets (20). Controversial data have been shown on cells derived from ATCs expressing stem cell markers, as Nanog and POU class 5 homeobox 1 (POU5F1) but lacking the expression of CD133 (30). Ahn et al. demonstrated that TCSCs present in PTCs express high levels of CD44, but no expression was revealed for CD24. The frequency of $CD44^+CD24^-$ CSC population was higher in recurrent PTCs than primary PTCs. POU5F1 was almost exclusively expressed by $CD44^+CD24^-$ cells compared with $CD44^+CD24^+$ cells (31). Shimamura et al. performed a comprehensive analysis of multiple markers (CD13, CD15, CD24, CD44, CD90, CD117, CD133, CD166, CD326, and ALDH activity) on eight cell lines derived from TCs and evaluated their ability to generate thyrospheres *in vitro* and tumor *in vivo*. This study identified ALDH activity and CD326 expression as reliable candidates to mark TCSCs (32).

Distinct signaling pathways are implicated in tumor initiation and progression by sustaining TCSC survival. In FTCs, the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways has been extensively described. In PTCs, the genetic alterations activate only MAPK pathway, while in ATCs, the MAPK, PI3K, and β- catenin pathways are activated. It has been widely demonstrated that *BRAF* and *RAS* mutations, *RET/PTC* rearrangements, and also *ALK* mutations activate MAPK pathway, which has a key role in thyroid tumorigenesis (33). The pathogenesis of TC, including angiogenesis, invasion, and metastasis process, is dictated by aberrant MAPK signaling pathway, altered production of chemokines, growth factors, matrix-metalloproteinases (MMPs), hypoxia-inducible factor 1α (HIF-1 α), and tissue inhibitor of metalloproteinases-1 (TIMP-1). In particular, in $BRAF^{V600E}$ PTC, TIMP-1 is upregulated by nuclear factor kappa B (NF-κB) activation, leading to invasiveness, inhibition of apoptosis, increase in proliferation rate, and resistance to chemotherapy (34). Despite its role as MMP inhibitor, TIMP-1 exerts also its function in promoting tumor proliferation and regulating metastatic potential through hepatocyte growth factor (HGF) induced by HIF-1α. Thus, TIMP-1 could be used as a predictive biomarker and therapeutic target in advanced PTCs (35). PI3K-AKT pathway plays an important role in tumor progression and vascular intravasation in FTCs. In particular, elevated levels of AKT1 and its nuclear localization promote the invasiveness and the metastatic potential of FTC (36). This finding is supported by the presence of *AKT1* mutations in metastatic TCs (37).

Recently, it has been demonstrated that NF-κB regulates the proliferative and antiapoptotic signaling pathways in TCSCs. In ATC, the administration of NF-κB inhibitors in combination with radio- or chemotherapy exerts antiproliferative effects and induces massive apoptosis (38). Moreover, NF-κB is also upregulated by the MAPK pathway, suggesting a direct coupling with the presence of $BRAF^{V600E}$ mutations as described above. In poorly differentiated TCs and ATCs, WNT-β-catenin signaling pathway is upregulated. This pathway has a role in the proliferation and differentiation of SCs, and its aberrant activation is a consequence of the activation of PI3K-AKT pathway by glycogen synthase kinase 3β (GSK3β) (39). Aberrant activation of the all above described signaling pathways represents the first step of TC tumorigenesis and can be considered as a target for novel therapeutic approaches.

EPIGENETIC ALTERATIONS SUSTAINING TCSCs ORIGIN AND MAINTENAINCE

Epigenetic regulation of gene expression and chromatin compactness is a reversible and dynamic process crucial in developmental biology and in many diseases, including cancer (40). The impaired differentiation of normal stem cells into tissue subtypes and their acquisition of CSC capabilities as self-renewal, chemoresistance, and metastatic potential are finely governed by epigenetic modulators (9). Epigenetic alterations fundamental in the tumorigenesis of many cancer types, including TC, are mainly related to the following mechanisms and/or modulators summarized in **Figure 2**: (1) DNA methylation, inducing the silencing of many tumor suppressor gene transcription through the DNA methyltransferases (DNMTs); (2) histone modifiers, which regulate gene expression by adding or removing mostly acetyl or methyl groups to the histone tails; (3) Chromatin remodelers that control the structural organization of chromatin by modulating its degree of condensation; (4) non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lnRNAs), which may exert their role in both inhibiting or aberrantly inducing gene transcription. In thyroid tumors many epigenetic modifiers are altered, but further studies are needed to better elucidate how the complex regulation of histone modifying enzymes and chromatin remodelers could interfere with or facilitate the tumor growth of the CSC subset. Here, we highlight the key points related to the most relevant epigenetic mechanisms altered in the different subtypes of

TCs: (i) Thyroid-specific differentiation and tumor suppressor genes aberrantly methylated in their promoter regions along with altered expression of miRNAs represent the most frequent epigenetic features in well-differentiated thyroid tumors, as PTC and FTC, while the amount of reports regarding the histone modifications are very limited. (ii) The most common altered epigenetic mechanisms that contribute to well-differentiated TC initiation and progression, lead to the activation of MAPK and PI3K/AKT survival pathways, which sustain TCSCs origin and maintenance. (iii) In UTCs in addition to MAPK and PI3K/AKT pathways, other signaling pathways, as Wnt/β-catenin and Notch, crucial for the survival of TCSCs are epigenetically compromised. Unlike the well-differentiated thyroid tumors, the DNA methylation profiling in the undifferentiated subtypes reveals more often the aberrant promoter hypomethylation compared with hypermethylation.

DNA Methylation in Thyroid Tumors

Global DNA methylation studies utilizing next generation sequencing (NGS) platforms (41–43) have identified different DNA methylation patterns associated with TC histological variants and their distinct mutational status. Specifically, the hypermethylation has been associated with the well-differentiated TCs while the hypomethylation with the undifferentiated ones. DNA methylation status has been correlated with *BRAF* mutation, tumor progression, and aggressive behavior in PTCs (44). Thus, genome-wide approach shed new lights on the methylome profiles of TC subtypes; nevertheless, novel potential biomarkers for early diagnosis, prognosis, and therapy resistance have not been identified yet. The majority of the thyroid-specific tumor suppressor genes, which have been found aberrantly methylated in their promoter regions, exert their role as antagonists or modulators of PI3K pathway, RAS signaling, and EMT process. Of note, *PTEN* (phosphatase and tensin homolog) acts as an inhibitor of PI3K pathway (45); RAS association domain family 1A *(RASSF1A*) (46) and *RASAL* (RAS protein activator like-1) (47) are both involved in RAS signaling, cell cycle regulation, and mitotic progression; *TIMP3* (tissue inhibitor of metalloproteinase 3), is an inhibitor of metastasis, angiogenesis, and invasion; and others are known as tumor suppressors as the death associated protein kinase *(DAPK)* and the retinoic acid receptor beta2 *(RARβ2)* (44). Silencing of E-cadherin (*CDH1*) by its promoter methylation has been hypothesized as a potential mechanism of enhanced EMT in FTC cells, whereas in PTC cells, *CDH1* expression levels are maintained (48, 49). Moreover, thyroid differentiation specific genes, as thyroid transcription factor-1 (*TTF1*) and sodium-iodide symporter (*NIS),* are also hypermethylated particularly in undifferentiated tumors (50). High expression levels of DNMT1 have been associated with silencing of *NIS* (51). The role of KISS1/KISS1R signaling is controversial in various types of cancers, as it has been associated with both roles in metastatic promotion and suppression (52–54). Savvidis et al. found increased levels of KISS1 in extrathyroidal tissues of advanced differentiated TCs while an inverse correlation between KISS1R and tumor size (55). Interestingly, it has been demonstrated that *KISS1R* promoter is hypermethylated particularly in FTCs (42). Therefore, further studies are needed to define the methylome profile in TCSC population in an attempt to find specific targets in this cell compartment. However, the fact that components of the principal pathways involved in TCSC survival are silenced by hypermethylation strongly suggests that this could be a mechanism specific also of this subset.

Histone Modifications and miRNAs Role in Thyroid Tumors

Aberrant alterations in the histone modifier epigenetic enzymes as well as in the chromatin remodeling complexes have not been completely studied in TCs and in the subset of TCSCs. Nowadays, there are few reports about the histone modifications in TCs. Histone deacetylase (HDAC) enzymes modulate the levels of histone tail residues acetylated. It is now widely accepted that treatment with HDAC inhibitors is very effective in TCs subtypes, as they impair cell growth and induce apoptosis of TC cells while increasing the radioiodine uptake. Of note, the global acetylation levels in TCs are higher than in healthy tissues (56). Moreover, UTCs express lower levels of acetylated H3 on lysine 18 compared with well-differentiated TCs, suggesting that the deacetylation is a step required for the acquisition of a more aggressive phenotype. Interestingly, in cells with reduced levels of *TTF1* because of hypermethylation, levels of acetyl H3K9 are reduced and dimethyl H3K9 are increased. BRD4 is a bromodomain protein, which binds the acetylated histones facilitating the recruitment of transcription factors and ultimately the transcription. The role of BRD4 as a potential oncogene in TCs has been recently elucidated. Levels of BRD4 are higher in ATCs compared to healthy tissues. The BRD4 inhibition, by JQ1, which also inhibits c-Myc and induce *TTF1*, or by AZD5153, impairs TC cell growth, suggesting that it is critical for thyroid proliferation (57, 58). KDM1A is a H3K9 demethylase frequently overexpressed in PTCs and required for migratory and invasive capabilities of PTC cells (59). TIMP1 has been described as a KDM1A target. KDM1A epigenetically silences TIMP1 and subsequently activates MMP9 promoting metastasis and migration (59). Finally, the enzymatically active component of the PRC complex, EZH2, involved in the regulation of embryonic development and responsible for the trimethylation of lysine 27 on H3 (H3K27me3), leading to silencing of gene transcription, is overexpressed in ATCs (60). Future studies are needed to better characterize the epigenetic modifications and their use as potential therapeutic targets in TCs.

miRNAs are short molecules of 19–23 nucleotides involved in blocking transcription or degradation of messenger RNA (mRNA), which have been associated with critical roles in TC initiation and progression because they can downregulate tumor suppressor genes or upregulate oncogene transcription. Their expression is also specific for each TC subtype. An extensive overview about the principal aberrant epigenetic events and, in particular, the most differentially up- or downregulated miRNAs involved in both well-differentiated or undifferentiated TCs is reported in (61, 62).

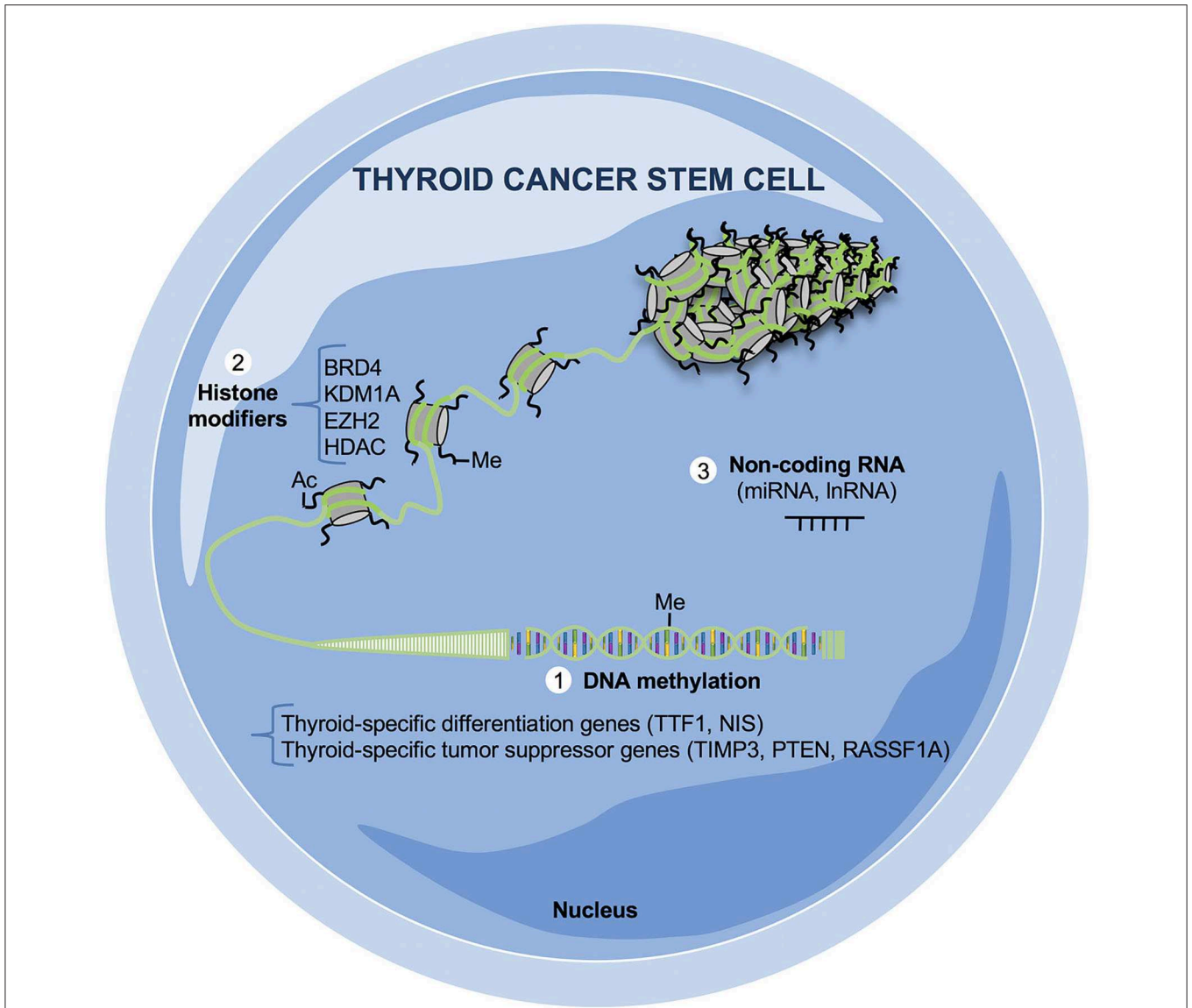

FIGURE 2 | Epigenetic mechanisms relevant in thyroid cancer stem cells (TCSCs). Altered epigenetic mechanisms crucial for the survival of TC cells and, in particular for TCSCs, have not completely elucidated yet. The majority of aberrant epigenetic events in TCs are based on (1) DNA methylation: the promoters of thyroid-specific differentiation as transcriptional thyroid factor 1 (*TTF1*) and sodium iodide symporter (*NIS*) and of tumor suppressor genes as tissue inhibitor of metalloproteinase-3 (*TIMP3*), phosphatase and tensin homolog (*PTEN*), Ras association domain family 1 isoform A (*RASSF1A)* are silenced by hypermethylation; (2) histone modifications: several histone-modifying enzymes are aberrantly expressed in TCs as bromodomain-containing protein 4 (BRD4), lysine demethylase 1A (KDM1A), enhancer of zeste homolog 2 (EZH2), and histone deacetylase (HDAC); (3) non-coding RNA (miRNAs, lnRNAs): many non-coding RNAs are up- or downregulated in both well-differentiated and undifferentiated TCs, influencing the transcription and the posttrancriptional regulation of several oncogenes and/or tumor suppressor genes.

Epigenetic Therapy in Thyroid Tumors

Epigenetic drugs can be used to revert the chemoresistance of TCs. Given that epigenetic modifications are reversible, these may be approached differently than genetic mutations, which are irreversible. HDAC and DNMT inhibitors represent the first Food and Drugs (FDA)-approved epigenetic drugs and are currently used in several clinical trials showing promising results for TC treatment (63). Recently, many *in vitro* and *in vivo* studies have reported that treatment with HDAC inhibitors alone or in combination with chemotherapy or other biological agent induces apoptosis and cell cycle arrest, impairing the growth while incrementing the radioiodine uptake of TC cells (64). Moreover, HDAC inhibitors, as Trichostatin A (TSA), and Valproic acid (VA) combined with DNMT inhibitors as azacitidine, exerts an antitumoral effect by reducing MMP2 and MMP9 levels in PTC and FTC. Unfortunately, some clinical limitations have been associated with the use of the epigenetic inhibitors in many solid tumors, including TC, as remethylation, the lack of specificity and general toxicity. Although the use of DNMT inhibitors was expected to revert the silencing of

many tumor suppressor genes involved in TC tumorigenesis, it has been demonstrated that they also promote oncogenes and prometastatic genes transcriptional activation through a global change in gene expression. Currently, only two clinical studies verified the efficacy of demethylating agents in patients affected by recurrent and/or metastatic differentiated TCs, with no partial or complete responses and severe side effects (www.clinicaltrials.gov, NCT00085293 and NCT00004062) (65). Nevertheless, these inhibitors may be potentially translated in clinical settings in combination with HDAC inhibitors and/or other targeted therapies such as mammalian target of rapamycin (mTOR) inhibitors (66). Notably, HDAC inhibitors, although generally well-tolerated, induced cardiac arrhythmias in patients with heart disease. The final data from the ongoing clinical trials are needed to understand their efficacy in treating TC refractory to current therapies (67, 68). The hurdles in the administration of these epigenetic drugs will be overcome with the use of highly specific inhibitors (for instance HDAC inhibitors specific for a single class or a single HDAC) and/ or nanoparticle (liposomes, nanogels, polymeric nanoparticles) to reduce off-target effects and improve the delivery system (69). Overall, so far, the successful preclinical results obtained in TC cells treated with the epigenetic drugs have not been recapitulated in clinical settings mostly due to the epigenetic landscape heterogeneity of thyroid tumors, the influence of immune cells, and the lack of a complete knowledge about the other TC histological subtypes other than PTCs. A better understanding of the epigenetic changes in TCSCs is crucial to design more effective molecular therapies (70).

THE CROSS-TALK BETWEEN IMMUNE SYSTEM AND CSCs IN THYROID CANCER

The first barrier to tumorigenesis is the immune surveillance. CSCs represent a heterogeneous subpopulation able to modulate the host immune response and ultimately escape the attacks mediated by the immune cells. The experts define this phenomenon as cancer immunoediting, a process that includes three different phases: the elimination, the equilibrium, and the escape. The elimination phase consists in the ability of innate and adaptive immune cells to recognize and destroy CSCs (71). In the equilibrium phase, the tumor is not eliminated but contained. In this phase, the immune system eliminates the immunogenic cancer cell clones. In the escape phase, the quiescent state and the low immunogenicity, typical of CSCs, allow them to remain in their niches without being recognized from host immune system. In particular, CSCs can escape immune destruction by reducing their expression of major histocompatibility complex I (MHC I) and by completely eliminating the expression of MHC II and co-stimulatory molecules.

The CSCs can also minimize the host immune response recruiting immunosuppressive cells by secretion of growth factors and interleukins, such as transforming growth factor beta (TGF-β), interleukin (IL)-6, IL-10, and prostaglandin E_2 (PGE2) (72). Immune cells with pro- and antitumorigenic roles have been detected in thyroid TME (73). Of note, specific classes of immune cells can be related to TC patient outcome. Unlike the infiltration of natural killer (NK) cells, presence of T regulatory lymphocytes in PTC is positively associated with advanced disease (74). Currently, in TC TME, the role of tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and tumor-associated mast cells (TAMCs) has been extensively described, while few studies have been reported regarding the presence of NKT cells, γδ T cells, Th9, and Th17 in different TCs (75–77).

In the following paragraphs, we will review in details the bidirectional cross-talk between the TCSCs and immune cells (**Figure 3**).

Immune Cells and Their Protumorigenic Role

The innate and adaptive immune cells are in charge of the defense against foreign agents. Among the immune cells, NK cells are the first to recognize and kill CSCs, with the resulting apoptotic cell fragments being eliminated by macrophages. Tumor neoantigens are processed and presented to the T cells by dendritic cells (DCs). The release of cytokines by DCs and other immune cells induce the activation of T and B cells promoting an inflammatory environment that further stimulates the innate immunity and supports the expansion of T cells and the production of antibodies toward antigens expressed on CSCs. In TME, immune cells exhibit not only an antitumorigenic role, but they can also induce a protumorigenic and anti-inflammatory condition contributing to CSC subpopulation survival (71). In TC TME, TAMs can exert different functions by dynamically interchanging between M1 (proinflammatory) and M2 (anti-inflammatory) phenotypes (78). TAMs polarize into M1 phenotype in presence of interferon gamma (IFNγ) and lipopolysaccharide (LPS), while acquiring an M2 phenotype for IL-4 and IL-13 action. Typically, TAMs with M2 phenotype play an important role in promoting tumor angiogenesis and progression (79) and are correlated with poor prognosis in several tumors, including TCs (80). In the different subtypes of TCs, the frequency of TAMs is variable and has been correlated with various prognoses. In ATCs, TAMs represent 50% of all immune cells and correlate with poor prognosis (81). In PTCs, the presence of TAMs is associated with large size tumors, presence of lymph node metastasis, and decreased survival (80, 82, 83). In poorly differentiated TCs, TAMS are correlated with negative outcomes as capsular invasion and extrathyroid tumor extension (80). The interaction between TAMs and TCSCs can be directly and indirectly mediated by the release of many cytokines and chemokines. It has been demonstrated that TAM-secreted CXCL8 and CXCL16 can promote the metastatic process in PTCs. Blocking CXCL8 and CXCL16 signaling reduced the invasiveness of PTC cells (83, 84), suggesting their targeting as a possible therapeutic strategy in PTCs. In PTCs, TGF-β produced by TAMs is overexpressed and correlates with the presence of $CD68^+$ TAM infiltration and with tumor invasion (85). In BRAF-induced PTC mouse model, Ryder et al. observed an increased of expression of two TAM chemoattractants: colony stimulating factor-1 (Csf-1) and CCL-2. The increase in Csf-1 is correlated with a high number of infiltrating TAMs expressing Csf-1r and CCR2 and with PTC

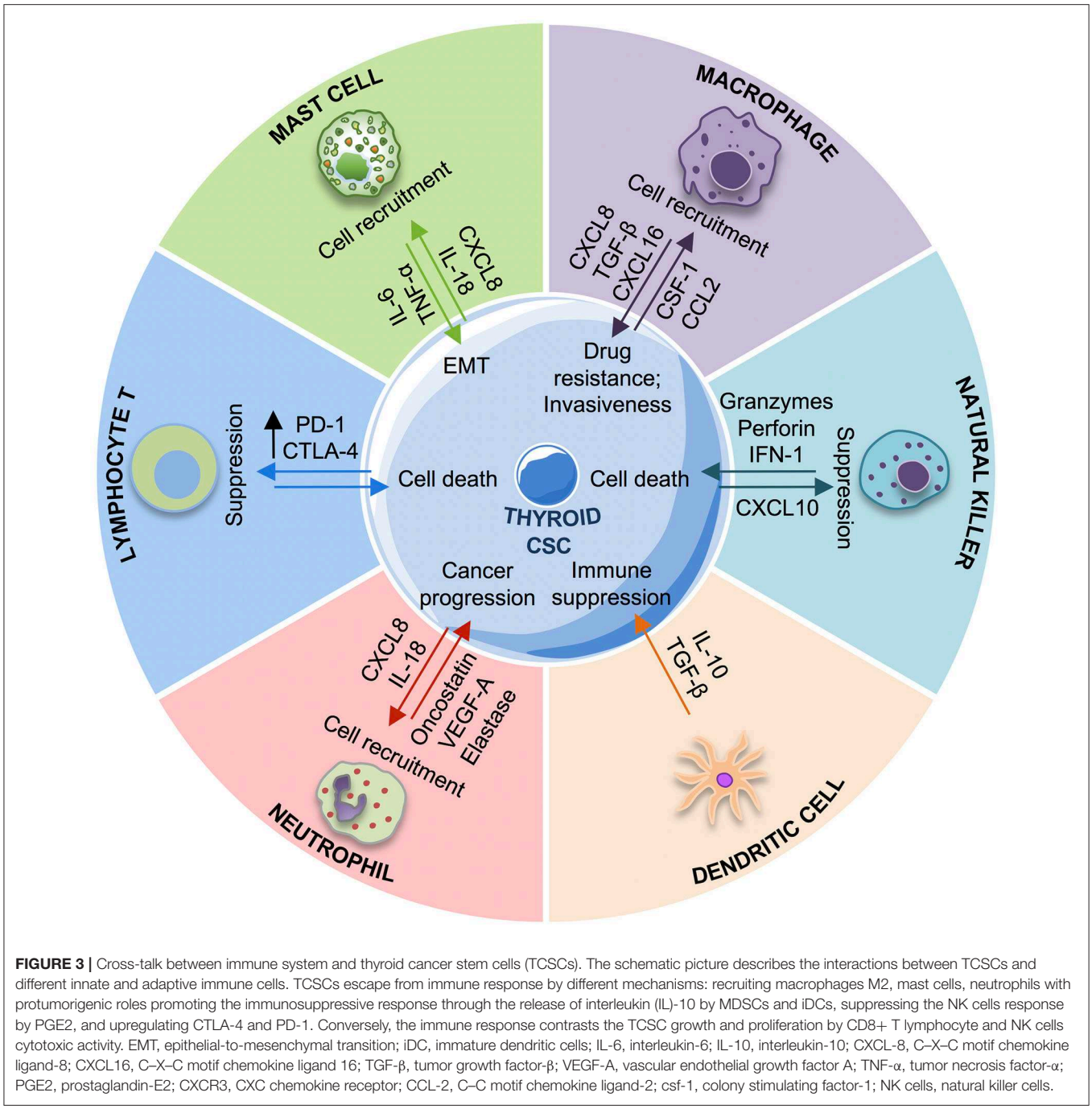

FIGURE 3 | Cross-talk between immune system and thyroid cancer stem cells (TCSCs). The schematic picture describes the interactions between TCSCs and different innate and adaptive immune cells. TCSCs escape from immune response by different mechanisms: recruiting macrophages M2, mast cells, neutrophils with protumorigenic roles promoting the immunosuppressive response through the release of interleukin (IL)-10 by MDSCs and iDCs, suppressing the NK cells response by PGE2, and upregulating CTLA-4 and PD-1. Conversely, the immune response contrasts the TCSC growth and proliferation by CD8+ T lymphocyte and NK cells cytotoxic activity. EMT, epithelial-to-mesenchymal transition; iDC, immature dendritic cells; IL-6, interleukin-6; IL-10, interleukin-10; CXCL-8, C–X–C motif chemokine ligand-8; CXCL16, C–X–C motif chemokine ligand 16; TGF-β, tumor growth factor-β; VEGF-A, vascular endothelial growth factor A; TNF-α, tumor necrosis factor-α; PGE2, prostaglandin-E2; CXCR3, CXC chemokine receptor; CCL-2, C–C motif chemokine ligand-2; csf-1, colony stimulating factor-1; NK cells, natural killer cells.

progression (86). A high number myeloid derived suppressor cells (MDSCs), considered as precursors of monocytes and neutrophils, has been reported in cancer patients as index of poor prognosis. In differentiated TCs, the presence of MDSCs has been correlated with tumor aggressiveness (87). MDSCs can be identified as M-MDSCs or PMN-MDSCs, with a phenotype similar to monocytes or neutrophils, respectively. Both cell types are activated in the TC microenvironment by CCL2, CCL5, and CSF-1 and act as inhibitors of the antitumor response by releasing IL-10 (88). Many studies have suggested that chemotherapy or other treatments such as tyrosine kinase and nitric oxide inhibitors improve TC patient prognosis by inducing MDSC differentiation or by inhibiting their function (89). Unlike other immune cells, DCs are not usually present in TCs, except for PTCs bearing $BRAF^{V600E}$ mutation. In PTCs, DCs are immature cells ($CD1a^{+}$ and $S100^{+}$) able to secrete cytokines as IL-10 and TGF-β, thereby promoting immune suppressive response (90). Similarly to DCs, Tregs are also elevated in PTCs and contribute to immune suppressive TME, favoring tumor cell survival. The mechanisms underlying this function have not been completely

clarified; nonetheless, it has been hypothesized that Tregs induce an anergy state in T effector cells, which become unable to recognize and attack the CSCs (74).

Mast cells are the first immune cells recruited during the inflammatory process, and they can also exert a protumorigenic role (91). The presence of mast cells has been associated with extrathyroidal tumor extension in 95% of PTCs and with invasiveness in poorly differentiated TCs and ATCs (77). Tumor-associated mast cells are more abundant in thyroid follicles of patients affected by PTCs compared with patients with thyroid adenoma (92). By releasing IL-6, TNF-α, and CXCL8/IL-8, mast cells can promote the EMT process in thyroid cells derived from all TC subtypes (FTC, ATC, and PTC). To date, the role of neutrophils in cancer is controversial. An elevated number of neutrophils in cancer patients have been correlated with better clinical outcomes (93–95) or with tumor progression (96, 97). TCSCs recruit the neutrophils by releasing CXCL-8/IL8 and upregulate their proinflammatory roles, thus promoting tumor progression. Galdiero et al. in TCs showed a correlation between the density of neutrophils and the tumor size, suggesting that these immune cells are involved in the tumorigenesis process. It has been demonstrated that TANS favor the tumor proliferation and invasion by the secretion of cytokines as oncostatin M and vascular endothelial growth factor A (VEGF-A) and by elastase action (98). In TC patients, it has been observed that a higher neutrophil/lymphocyte ratio correspond to a larger tumor size and high risk of recurrence (76). NK cells are responsible for the earliest stages of immune defense against microbe infections or the expansion of stressed or transformed cells. NK cells possess a cytotoxic activity, and their activation depend on two factors: they recognize the stimulatory receptors in the target cells and simultaneously their inhibitor receptors fail to link MHC I molecules on foreign cells. It is possible to distinguish two populations of NK cells, with different localization and functions: the $CD56^{bright}/CD16^{dim}$ cells, which predominate in the circulation and the $CD56^{dim}/CD16^{bright}$ cells, which are more cytotoxic and present in the majority of tissues (99). In the tumor, the NK cells $CD56^{bright}$ have a key role to eliminate the nascent tumor cells, identified as stressed and abnormal cells, with a low expression or lack of expression of MHC I class. In TCs, the role of NK cells needs further investigations. In PTC patients, the presence of NK cells is negatively correlated with disease stage (100). In fact, some studies have demonstrated that ATC cell lines are susceptible to NK-mediated immunotherapy (101, 102). However, tumor immunosuppression by NK targeting may also represent an obstacle to the activation of their cytotoxic function (103, 104).

TCSCs and Evasion Mechanisms

CSCs can attenuate the immune surveillance contributing to the tumor development. CSC interaction with immune cells consists in the secretion of immunosuppressive factors or the recruitment of immunosuppressive cells. In particular, CSCs downregulate some key elements for the antigen processing and presentation, contributing to their immune privileged status, thus leading to the inactivation of T cells (105). CSCs can escape NK cell action by different mechanisms: (i) increased expression of ligands that bind the inhibitor NK receptors; (ii) recruitment of T regs, which can promote the immune evasive state; (iii) NK and T-cells-mediated IL-22 secretion, which promotes CSC phenotype (72). The activation of a T-cell-mediated immune response needs not only the presence of MHC molecules but also of co-stimulatory molecules as CD80 and CD86. Tumor cells downregulate CD80/CD86 expression while upregulates the expression of PD-L1 (B7-H1), which binds to PD-1 on T cells promoting their anergy state (106). Thus, targeting PD-L1 represents a promising immunotherapy approach in many cancers and in advanced differentiated TCs and ATCs (107).

Immunotherapy and TCSCs

CSCs are resistant to different chemotherapeutic agents and radiotherapy due to high expression levels of drug efflux pumps, efficient DNA repair mechanisms, and the support of TME. Immunotherapy approach has been developed as a novel frontier also in targeting CSCs (108). Cancer immunotherapy consists in the administration of recombinant antibodies toward immune molecules defined as immune checkpoint (PD-1, CTLA-4), along with recombinant cytokines, oncolytic viruses, cancer vaccines, or engineered T cells (109). Given that immune cells play a key role in supporting the initiation and progression of TC, the identification of specific immune targets could improve the efficacy of TC therapy. Different immunotherapy strategies have been implemented for the treatment of TC: (i) failure of TAM recruitment, (ii) TAM polarization in M1 phenotype, (iii) identification of tumor antigens for the development of cancer vaccines, and (iv) inhibition of immune checkpoints (110). TAMs are highly represented in ATC TME, as compared to advanced differentiated TCs. High levels of CCL-2 and CSF-1, two TAMs chemoattractants, have been found in human TC tissues (82). In this context, blocking and targeting CCL-2/CCR-2 and CSF-1/CSF1R inhibits the recruitment of TAM with M2 phenotype and the repolarization of these cells into the anti-tumor M1 phenotype (86, 111). To date, clinical trials are ongoing to test the efficacy of the CSF-1R antibody (LY3022855) and CSF-1R inhibitor (PLX3397) (NCT01346358 and NCT01525601, respectively). TAMs represent the half of TME cells in ATC; in this way, the block of CSF-1 and CCL-2 represent a potential target therapy. TC cells express a specific inhibitory receptor membrane, CD47 that binds SIRPα, a ligand expressed on TAMs. The interaction receptor–ligand leads to inhibition of phagocytosis by TAMs and affects the tumor antigen presentation by DCs by supporting TC progression. The block of SIRPα/CD47 interaction in mouse model leads to tumor growth impairment and regression. Use of a specific antibody represents a way through which this pathological pathway re-educated in TAMs (112). Specifically, *in vitro* study using the anti-CD47 on TC cell lines shows the induction of apoptosis (113). Another potential strategy could identify TC-specific antigens to develop a successful vaccine for T cells and dendritic cells in the context of TME. Some studies suggest that potential TC antigens, as the proto-oncogene c-MET, melanoma antigen encoding genes (MAGE), and mucin-1 antigen (MUC-1), are co-expressed with thyroglobulin and thyroid peroxidase in differentiated TCs (114, 115). A promising study demonstrated that dendritic

cells vaccines targeting the carcinoembryonic antigen (CEA), an antigen highly revealed in MTC, induced a complete regression of metastases in lung and liver (116). Further clinical studies are ongoing to test adoptive cytotoxic T cells that target preferentially expressed antigen in melanoma (PRAME), New York esophageal squamous cell carcinoma 1 (NY-ESO-1), MAGE Family member A4 (MAGEA4), synovial sarcoma translocated gene (SSX), and survivin in patients with advanced tumors, including TCs. Currently, the immune checkpoints (PD-1, CTLA-4) inhibition represents a valid strategy for treatment of different types of cancers. Blocking these pathways strengthens T cells and inhibits T reg suppressor cells. In TC histological subtypes, different levels of PD-L1 expression have been detected (7.6% of FTC, in 6.1% of PTCs, and 22.2% of ATCs) (117). Moreover, PDL-1 has been found expressed in more than the half of 126 cases of primary PTCs, and its expression positively correlates with rich tumor-infiltrating lymphocytes (118). Two antibodies targeting PD-1, the receptor of PD-L1 (Pembrolizumab and Nivolumab), have been FDA-approved for treatment of melanoma, renal carcinoma, and non-small cell lung carcinoma, and recently also for PTC patients. Specifically, clinical trials are ongoing to test the efficacy of Pembrolizumab in association with PLX3397 in TCs to better maximize the clinical results in these tumors (NCT02452424). Although the immunotherapy needs further evaluation in TCSC compartment, it may represent a reliable strategy for TC treatment.

INFLUENCE OF THE TUMOR MICROENVIRONMENT ON TCSCs

TME is composed of different cell types (endothelial cells, fibroblasts, immune cells) and extracellular components (cytokines, chemokines, exosomes, growth factors, hormones, extracellular matrix) that support tumor growth. TME exerts an important role not only during tumor initiation, progression, and metastasis but also in drug resistance. Therapy refractoriness is mediated by a continuous crosstalk between tumor and stromal cells (119, 120). In TCs, an inflammatory TME has been involved in thyroid carcinogenesis. Specifically, the molecular interplay between cytokines and chemokines with a protumorigenic role could explain how the inflammation could favor TC initiation (121). TCSCs survival is crucially modulated by TME cells, through a bidirectional complex network of chemokines/cytokines and the induction of an EMT-like phenotype mediated by tumor-associated fibroblasts (CAFs) and by exosomes release **(Figure 4)**.

TCSCs and the Interplay With Chemokines/Cytokines

The interplay between TCSCs and stromal cells supports tumor progression by recruiting fibroblasts or macrophages to facilitate metastasis. This crosstalk is mediated by different kinds of chemokines, which are low molecular weight peptides with proangiogenic, cytoproliferative, and prometastatic properties (122). Among the main players of thyroid tumorigenesis, many chemokines and cytokines have been proposed such as CXCL8, CCL2, CXCL16, CCL15, and IL-6 (123). Of note, FTC cells produce CCL15, which is a chemokine that binds CCR1 receptor expressed by TAMs to facilitate their recruitment and to favor tumor progression and metastasis (124). Studies of co-culture of macrophages and PTC cells showed that CXCL16, abundantly secreted by TAMs, promotes PTC cells migration and invasiveness. It has been demonstrated that TC progression is arrested by using an antihuman CXCL16 neutralizing antibody (84). CCL2, a chemokine secreted by PTC cells, facilitates TAM recruitment in thyroid TME. CCL2 production has been positively correlated with $BRAF^{v600E}$ mutations in PTC cells and with TAM infiltration (123). Several studies show how CXCL8, a chemokine secreted by TC cells, plays a protumorigenic activity by enhancing mechanisms such as stemness and EMT in TC cells. In fact, *in vitro* data showed that CXCL8, through an auto-paracrine circuit, binds CXCR1 receptor and induces the tumor sphere formation, typical of CSCs (77). CXCL8's role in carcinogenesis is also confirmed by *in vivo* studies that show as the inoculation of TC cell line, BCPAP, in NOD/SCID mouse model induces metastasis if treated with exogenous recombinant human CXCL8 (83). TAM-derived IL-6 has a pivotal role in tumor initiating, survival, proliferation, and drug resistance in solid tumors (125–128). Similarly, IL-6 drives TC progression even if its molecular mechanism is unknown (129, 130). Zheng et al. studied the effect of IL-6 on ATC-derived CSCs showing that the *in vitro* treatment of HTh74 and HTh74R TC cell lines with exogenous IL-6 leads to increased number of tumor spheres and stemness markers, OCT4 and CD133. IL-6 promotes the colony formation and EMT through the upregulation of vimentin and Snail and the downregulation of E-cadherin in HTh74 and HTh74R TSCSs, contributing to TC progression. In addition, IL-6 increases dramatically TSCS growth and proliferation through JAK1/STAT3 pathway activation (131).

TCSCs and Tumor-Associated Fibroblasts (CAFs)

Cancer cells establish a complex relationship with the surrounding stromal cells such as cancer-associated fibroblasts (CAFs). During tumor progression, CAFs create a "reactive stroma" by releasing a series of signals inducing phenotypic changes in cancer cells, which reciprocally communicate with CAFs (132). Many studies have shown a positive correlation between specific mutations in TC cells and fibroblast activation. For instance, $BRAF^{V600E}$ mutation in TC cells has been involved with a metastatic phenotype by modulating TME and the extracellular matrix (ECM) (133). $BRAF^{V600E}$ mutation is associated with upregulation of integrin and fibronectin, which compose the ECM. The upregulation of ECM-associated genes induces the formation of a fibrotic tumor stroma in which an increased number of fibroblasts is recruited to facilitate TC progression (134). Further studies showed that several ATC cell lines are able to reprogram human normal fibroblasts in CAFs to support tumor growth. Treatment of quiescent fibroblasts with conditional medium from ATC cells induces their transformation in CAFs. These findings have been confirmed by the evaluation of fibroblast-specific markers such as platelet-derived growth

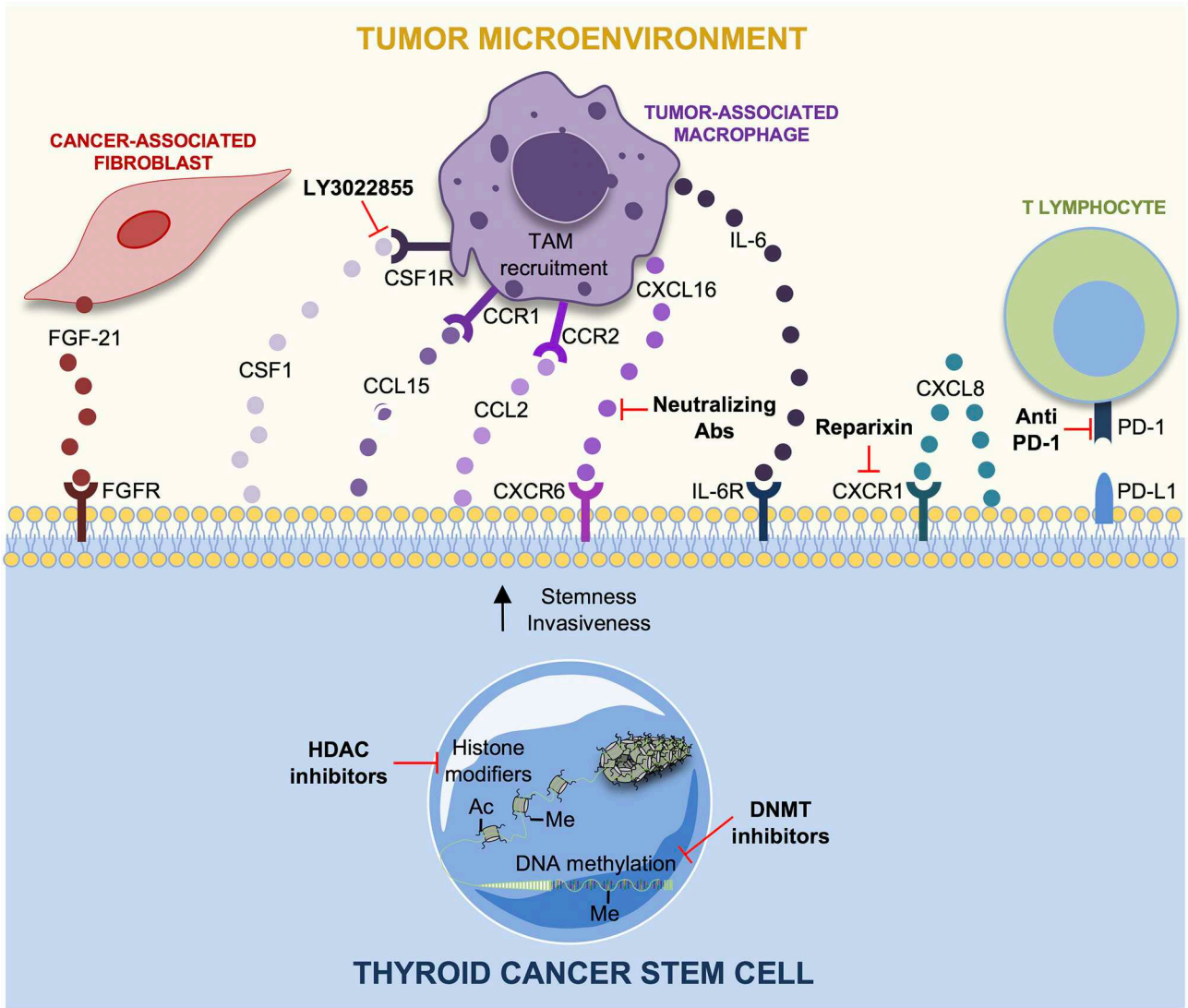

FIGURE 4 | Tumor microenvironment (TME) interactions and therapeutic strategies in thyroid cancer stem cells (TCSCs). The principal interactions between TCSCs and tumor microenvironment (TME) cells, along with the novel potential therapeutic targets, are shown. Cancer-associated fibroblasts (CAFs) produce FGF-21, which favors a more aggressive TC cells phenotype. TC cells secrete chemokines as CSF1, CCL15, and CCL2 to recruit tumor-associated macrophage (TAM) in order to sustain TC progression. CXCL8 secreted by TC cells guarantees stemness through an auto-paracrine loop by binding CXCR1. CXCL16 is secreted by TAMs favoring the migration and invasiveness of TC cells. Interleukin-6 (IL-6) supports stemness, colony formation and proliferation, in TC cells through JAK1/STAT3 phosphorylation. Reparixin (CXCR1 inhibitor) decreases thyrospheroid survival and epithelial–mesenchymal transition (EMT). The use of the CSFR1 inhibitor, LY3022855, hampers TAM recruitment. CXCL16-neutralizing antibody counteracts TC progression. Immunotherapy is based on the use of anti-PD1 antibodies as Pembrolizumab and Nivolumab, whereas epigenetic therapy nowadays is limited to histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors alone or in combination with conventional approaches in TCs.

factor receptor α (PDGFR-α) and alpha smooth muscle actin (α-SMA). Therefore, the activated CAFs acquire a more intense metabolic and proliferative activity and a secretory phenotype, which improves the invasiveness and aggressiveness of FTC cells (135). Taken together, these findings demonstrated that factors secreted by CAFs, upon activation by ATC cell-derived conditioned media (CM), represent key modulators in TC progression. Fibroblasts are able to secrete fibroblast growth factor 21 (FGF-21) and to release it into blood circulation by acting at distance on TC cells. High FGF-21 levels increase EMT in PTC through FGFR signaling pathway. Treatment of PTC cell lines with recombinant FGFR1 leads to FGFR pathway signaling activation, leading to ERK and AKT phosphorylation, EMT, and to the development of a more aggressive phenotype of tumor cells. Thus, FGFR1 can promote invasion and migration of PTC cells (136). Parascandolo et al. showed that mesenchymal stem cell (MSC) cultures derived from non-tumorigenic thyroid tissues and from PTCs, exposed to cancer cell-released factors,

are able to differentiate into a multitude of cell types, including CAFs, which eventually support tumor progression. Of note, a subpopulation of PTC cell line, expressing the TCP1 marker, influences the switch from autocrine to paracrine MSC-mediated secretion of superoxide dismutase 3 (SOD3). This leads to MSC activation and differentiation, with the upregulation of fibrotic markers such as FAP, tenascin and Col1 A1, suggesting the presence of CAFs in TC microenvironment. Stromal SOD3 exerts a stimulatory effect on TC cell growth representing a potential target for the treatment of these tumors (137). TIMP family is characterized by a specific structure, with the C-terminal region able to bind specific parts of MMPs in order to form MMP–TIMP complex, which inhibits tumor metastasis and invasion. However, TIMP family components as TIMP-1 and TIMP-2 could play opposite functions as promoting tumor growth, inhibiting apoptosis, and contributing to therapy resistance in different types of cancer, including PTCs (138–140). Consistently, TIMP-1 expression has been detected in fibroblasts of differentiated thyroid carcinoma and thyroid adenoma. Its expression levels are positively correlated to tumor growth, tumor–node–metastasis (TNM) stage, metastasis, and recurrence. In particular, Zhang et al. found that the presurgery peripheral blood levels of TIMP-1 and MMP9 in patients with differentiated TCs are significantly elevated, although TIMP-1 expression levels still remain lower than MMP-9 levels, thus promoting the development of cancer because the balance between the two was broken (141).

TCSCs and Exososomes

Exosomes are a subtype of extracellular vesicles, which originate in the endosomal cell compartment via multivesicular bodies (MVBs). Upon their maturation, exosomes emerge from the extracellular membrane and initiate a cross-talk with bulk tumor cells (142). Cancer cells and cancer-associated cells in TME, as fibroblasts and macrophages, release exosomes through which they transfer specific molecular messages between malignant and non-malignant cells and activate pathways that facilitate tumor initiation and progression (143). Specifically exosomes mediate intercellular communication by the so-called "exosomal cargo," which includes proteins, miRNAs, and mRNAs (144). In TC, exosomes derived from TCSCs have a central role in supporting metastasis by enhancing EMT through non-coding RNA transfer. Hardin et al. used a subpopulation of the TCP1 TC cell line, expressing adult stemness markers, and isolated exosomes from them. Treatment with exosomes of normal thyroid cell lines NTHY-ori-3 leads to the upregulation of long non-coding RNAs (lncRNAs) (MALAT1 and linc-ROR), whose molecular function is unclear, of the EMT marker SLUG and the stem cell marker SOX2 in normal cells, which acquire and increase their proliferative ability. TCP1-derived exosomes are able to activate EMT program in the same cells contributing to a more aggressive phenotype (145).

TCSCs and EMT

EMT is a clear example of cellular plasticity, through which epithelial cells develop a mesenchymal phenotype that enhances their migratory and invasive features (146). Different studies underlined the correlation between EMT process and an increased number of TCSCs in TCs (145, 147, 148). Consistently, Yasui et al. by analyzing thyroidectomy specimens, found that ATC regions coexisted with DTC. ATC regions showed absence of E-cadherin and a dramatic upregulation of the stem cell markers CD44, CD133, and of a neuronal marker nestin. Meanwhile, differentiated TCs and non-neoplastic regions showed a decrease in stem markers expression and nestin, but they were positive for E-cadherin (149). Furthermore, Heiden et al. demonstrated that sonic hedgehog pathway promotes the maintenance of a CSCs pool in ATC. They observed that Gli 1-induced Snail upregulation and increase $ALDH^+$ CSCs number in ATC cell lines (150). Hardin et al. reported that PTC cell lines acquired stem-cell-like features simultaneously to TGF-β-induced EMT. Interestingly, PTC cells upregulated a novel EMT activator paired-related homeobox protein 1 (Prrx1) (151). Ma et al. confirmed the upregulation of SSEA1 in TSCSs, which were positive also for Nanog, Sox2, and Oct4. They found a correlation between stemness and EMT; indeed, TSCSs showed EMT initiation through the increased expression of vimentin and snail and decreased expression of E-cadherin (152). Mato et al. showed for the first time the correlation between the expression of stem markers ABCG2 and the expression of EMT activator genes. They identified the subpopulation of PTC cells named PTC1, expressing ABCG2. PTC1 cells were characterized by an aggressive phenotype and showed high propensity to migration, invasion, and apoptosis inhibition due to *BIRC5* gene expression. For these reasons, PTC1 could have a pivotal role in PTC progression (153). To overcome the lack of nutrients and oxygen within the tumor mass, tumor cells foster the formation of newly synthesized vessels endowed with irregular structure and properties. Recently, it has been demonstrated that patient-derived xenografts of thyroid spheroids obtained from PTCs show "stem-like" features and promote neoangiogenesis in zebrafish *in vivo* model (154). Interestingly, Cirello et al. proposed an additional experimental model resembling the original thyroid tissue, composed of thyroid stem-like cells and endothelial and hematopoietic cells (155). These 3D models allowed to evaluate the therapeutic response to antiangiogenic compounds and other anticancer drugs and to assess the impact of several proangiogenic factors, as VEGF, FGF, and TSH, in TC progression (155, 156). Moreover, also pericytes can regulate the interaction between tumor and endothelial cells. Specifically, pericytes protect tumor cells by releasing proangiogenic factors. In TCs, it has been demonstrated that pericytes confer resistance to $BRAF^{V600E}$ inhibitors and tyrosine-kinase inhibitors (TKI) in $BRAF^{WT/V600E}$-PTCs by secreting trombospondin (TSP-1) and TGF-β (157).

Promising Target Therapy Approach for TME Cells in Thyroid Tumor

New treatment approaches have focused on the development of specific inhibitor compounds and neutralizing antibodies able to block chemokines, CAFs, TCSCs proliferation, and EMT. The aberrant activation of Sonic Hedgehog signaling is a common feature of ATCs, and it represents an important

regulator of cancer cells and CAFs. Cancer cells secrete Shh ligand that encourages the peritumoral stroma, CAFs included, to produce a multitude of factors such as IGF, Wnt, and VEGF, which enhance cancer progression (158). In addition, this pathway is also activated in ATC and PTC cells in the absence of Shh in a Smo-dependent way through the downstream activation of Gli transcription factors caused by the oncogenic RAS/BRAF/MEK. Parascandolo et al. highlighted a specific positive correlation between stromal inhibition and reduction in TC progression. Coculture experiments and stimulation with conditioned medium showed that CAFs support TCP1 TC cells invasion, migration, and non-adherent growth abilities. These effects are both Smo and Shh dependent because they were abolished by the administration of cyclopamine and the 5E1 antibody. Furthermore, an increased Shh secretion has been observed in fibroblasts treated with conditioned medium derived from TC cells highlighting a bidirectional paracrine cross-talk, suggesting that targeting Shh pathway in ATC cells as well as in TME, especially in CAFs, could be exploited as potential target therapy (159). Visciano et al. elegantly studied that CXCL8 is a pivotal mediator of stemness and EMT in TC cells (77). Liotti et al. showed the mechanism through which CXCL8 exerts its autocrine function. The binding of CXCL8 to its receptor, CXCR1, is responsible for the formation of thyrospheres, self-renewal, and tumor-initiating ability. The identification of this autocrine protumorigenic loop could represent a therapeutic approach. Consistently, the treatment with the anti-CXCL8 blocking antibody decreases number and diameter of thyrospheroids, while anti-CXCL1 antibodies did not exert any effect on sphere formation (160). Furthermore, Liotti et al. studied the effect of reparixin, a CXCR1 receptor inhibitor. This treatment dramatically decreases cell survival, proliferation, EMT, and stemness of different TC cell lines (161).

In the last 10 years, different studies have been focused on the development of low molecular weight inhibitor drugs targeting EMT-initiating factors. The combination of BRAF inhibitors, dabrafenib, and MEK1/2 inhibitor, trametinib, have been suggested as a therapy for recurrent TCs (162). Wnt/β-catenin signaling pathway are involved in the EMT program of TC cells, and its inhibition could represent a potential therapy. Indeed, Hardin et al. found that the silencing of β-catenin reduces EMT markers expression in TC (21). Moreover, Gao and Han reported that the silencing of C-Met/PI3K/AKT pathway reverses EMT and metastasis of TC cells (163). The most promising treatments targeting TME in TCSCs are shown in **Figure 4**.

CONCLUDING REMARKS

The gold standard therapy for all kinds of TCs is surgery associated with radioactive iodine (RAI) therapy. Unfortunately, a group of "advanced thyroid cancers" including <10% of differentiated TCs, many MTCs, as well as ATCs are not responsive to the standard therapeutic approach and evolve in distant metastatic sites. The 5-years survival rate is <50% in these patients compared with iodine-sensitive well-differentiated TC patients (110). Specific therapeutic targets have been identified for treatment of RAI refractory thyroid tumors. Several genetic alterations in the main molecular pathways, related with TC progression, such as *BRAF* and *RAS* mutations and *RET/PTC* gene rearrangements have been identified and allowed to develop therapeutic strategies for TC patients. To date, FDA has approved the use of different kinase inhibitor drugs, targeting the MAPK pathway (MKIs), such as Levatinib and Sorafenib for advanced RAI-R well-differentiated TCs and Cabozantinib and Vandetanib for MTCs. Due to their systemic toxicity and the ability by tumors to activate alternative proliferation pathways, these drugs partially induce beneficial effects. Moreover, the conventional therapy targets only the proliferating CSCs but no CSCs in a quiescent or slow proliferative state. TCSCs are responsible for TC initiation, progression, relapse, and metastatic dissemination. Given their plasticity, TCSCs are able to remain in a quiescent state within the TME niche. This can explain their persistence after the conventional therapies such as chemo- and radiotherapy. Therefore, a comprehensive knowledge of TCSCs biology may lead to the development of more effective therapeutic strategies. Efficient therapeutic approaches need to be explored to target both TCSCs and their progeny. Immunotherapy and epigenetic drugs in combination with standard therapy could represent promising strategies for TC treatment.

AUTHOR CONTRIBUTIONS

VV, FV, ML, and MT conceptualized and wrote the manuscript. CD'A, GP, AT, MG, SF, DG, and LM contributed to draft the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funding from AIRC under IG 2018 (ID: 14415 project - P.I.) to MT.

ACKNOWLEDGMENTS

VV and AT are research fellows funded by PON AIM line1. We thank Francesco Calo' for his graphical contribution. We apologize to our colleagues whose work we have not been able to include due to space constraints.

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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 Endocrinology

ORIGINAL RESEARCH
published: 24 September 2020
doi: 10.3389/fendo.2020.00631

GFRα 1-2-3-4 co-receptors for RET Are co-expressed in Pituitary Stem Cells but Individually Retained in Some Adenopituitary Cells

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to
Cancer Endocrinology,
a section of the journal
Frontiers in Endocrinology

Received: 30 May 2020
Accepted: 04 August 2020
Published: 24 September 2020

Citation:
Pradilla Dieste A, Chenlo M,
Perez-Romero S,
Garcia-Rendueles ÁR,
Suarez-Fariña M,
Garcia-Lavandeira M, Bernabeu I,
Cameselle-Teijeiro JM and Alvarez CV
(2020) GFRα 1-2-3-4 co-receptors for
RET Are co-expressed in Pituitary
Stem Cells but Individually Retained in
Some Adenopituitary Cells.
Front. Endocrinol. 11:631.
doi: 10.3389/fendo.2020.00631

The RET tyrosine kinase receptor is expressed by the endocrine somatotroph cells of the pituitary where it has important functions regulating survival/apoptosis. However, RET is also expressed by the GPS pituitary stem cells localized in a niche between the adenopituitary and the intermediate lobe. To bind any of its four ligands, RET needs one of four co-receptors called GFRα1-4. It has been previously shown that GFRα1 is expressed by somatotroph cells and acromegaly tumors. GFRα2 was shown to be expressed by pituitary stem cells. GFRα4 was proposed as not expressed in the pituitary. Here we study the RNA and protein expression of the four GFRα co-receptors for RET in rat and human pituitary. The four co-receptors were abundantly expressed at the RNA level both in rat and human pituitary, although GFRα4 was the less abundant. Multiple immunofluorescence for each co-receptor and β-catenin, a marker of stem cell niche was performed. The four GFRα co-receptors were co-expressed by the GPS cells at the niche colocalizing with β-catenin. Isolated individual scattered cells positive for one or other receptor could be found through the adenopituitary with low β-catenin expression. Some of them co-express GFRα1 and PIT1. Immunohistochemistry in normal human pituitary confirmed the data. Our data suggest that the redundancy of GFRα co-expression is a self-supportive mechanism which ensures niche maintenance and proper differentiation.

Keywords: pituitary stem cell niche, GPS, RET, progenitors, cell turnover

INTRODUCTION

RET (HGCN approved name: ret proto-oncogene) is a tyrosine kinase receptor involved in cell survival, proliferation, and differentiation. RET is single pass transmembrane protein essential for tubular kidney and digestive parasympathetic nervous system embryonic development (1). On the other hand, RET signaling pathways have a crucial role in the postnatal pituitary plastic capacity (1). For ligand binding and subsequent activation, RET needs a co-receptor from the GFRα family (GDNF family receptor alpha). There are four RET ligands with special affinity for one specific

co-receptor. GFRα1 has affinity for Glial cell-derived neurotrophic factor (GDNF), GFRα2 for neurturin (NTN), GFRα3 binds artemin (ART), and GFRα4 needs persephin (PSP) [see STRING, https://version-11-0.string-db.org/cgi/network.pl?networkId=lhJBsGLDouUC; reviewed in (1)]. Despite the special affinity of each co-receptor with a specific RET ligand, there is some promiscuity between them. GFRα1 and GFRα2 could be cross-activated by NTN and GDNF, respectively, due to similarities in structure (2–5). GFRα2 can also activate p140NCAM (Neural cell adhesion molecule 1) in neurons lacking RET (6). GFRα4 also responds to NTN in high concentrations (7). On the other hand, GFRα3 seems just activated by ART (3). A fifth RET co-receptor, GFRAL (GDNF Family Receptor Alpha Like), has been described having GDF15 (Growth differentiation factor 15) as ligand but there is no GFRAL interaction with other RET ligands, nor GDF15 binding to GFRα1–4 co-receptors (8–11). The pituitary gland is a neuroendocrine organ performing as the main governor of the endocrine system. The gland is composed by three parts: anterior lobe (adenopituitary, AP), intermediate lobe (IL) and posterior lobe (neuropituitary, NP). The five hormone-secreting endocrine cells are localized in the AP, somatotrophs (growth hormone, GH), thyrotrophs, (thyroid stimulating hormone, TSH), lactotrophs (prolactin, PRL), corticotrophs (adrenocorticotropin, ACTH), and gonadotrophs (follicle stimulating hormone, FSH; and luteinizing hormone, LH). Each of these cell lineages express a characteristic transcription factor that induce and maintain cell differentiation (12–14). The adult pituitary gland contains an adult stem cell niche formed by two rows of cells located in the marginal zone (MZ), between the AP and the IL (15, 16).

Within the adenopituitary, somatotrophs express both RET and GFRα1, together with GDNF and this is maintained in somatotroph-derived pituitary tumors causing acromegaly (17–19). RET is implicated in two pathways that control the number of somatotroph cells: in the absence of ligand GDNF, the RET/PIT1/CDKN2A-p14ARF/p53 pathway leads to apoptosis while in the presence of ligand the RET/GDNF/AKT leads to survival (1, 19–22). Thus, RET can act as a dependence receptor (absence of ligand) or tyrosine-kinase (presence of ligand) (23). RET could be also expressed in female lactotrophs during the different pregnancy/lactation stages, in a similar pathway regulating lactotroph apoptosis after weaning (24).

Interestingly, postnatal pituitary stem cells also express RET together with high levels of the co-receptor GFRα2, and with the ontogeny transcription factor PROP1 (HGCN approved name: PROP paired-like homeobox 1; previously called Prophet of Pit1), and many stem-cell markers, defining its name as the GPS cells (GFRα2, PROP1, Stem) (15). As other stem cells, pituitary stem cells express stemness related markers like SOX2 (SRY-box transcription factor 2), SOX9 (SRY-box transcription factor 9), KLF4 (Kruppel like factor 4), and POU5F1 or OCT4 (POU class 5 homeobox 1) (15, 25, 26). GPS are organized in a pituitary stem-cell niche associated to supportive cells from where they are recruited for transit-amplification, commitment, and differentiation (13, 15, 27–29).

On the other hand, GFRα4 was originally described as not expressed in human pituitary (30). However, *GFRA4* mRNA has recently been shown to be expressed in somatotroph pituitary tumors causing acromegaly (19). *GFRA4* was significantly correlated with poorer prognosis and resistance to first-line therapy. These somatotroph tumors also expressed some *PROP1* mRNA, a stem-cell transcription factor that is not detected in normal somatotroph cells.

The apparent contradictions related to *GFRA4* expression in the altered somatotroph adenomas while it seems not expressed in the normal pituitary, the possibility that GFRα co-receptors can function independently of RET together with the possibility that co-expression of the RET co-receptors could be essential to define stemness in the pituitary drove us to make a comparative study of the four GFRα receptors in the pituitary. RNA and protein expression of each co-receptor was assessed in human and rat pituitary, aiming to describe their distribution among the lobes of the pituitary gland.

MATERIALS AND METHODS

Pituitary Samples

Male and female young adult *Sprague-Dawley* (90 days old) rats were purchased in the registered facility of our institution (CEBEGA). Male and female 90 days old *Wistar Han* rats were purchased from Janvier Labs. Rats were perfused and the pituitary was immediately dissected and post-fixed overnight in 4% paraformaldehyde. Procedures were carried out under license to CVA granted by the corresponding legal authority in animal research within the Galicia Regional Government.

The human pituitary sample was obtained after informed consent from the Biobank of Complejo Hospitalario Universitario de Santiago de Compostela (CHUS). It was a 55 y.o. male patient dead from colon cancer immediately upon admission and did not received any previous therapy.

RNA Extraction

The rat pituitary was dissected after perfusion, discarding the neuropituitary. Adenopituitary (AP) together with Intermediate Lobe (IL) were frozen at −80°C. RNA extraction was performed using the TRIzol™ reagent (15596026, Invitrogen), following manufacturer instructions. A commercial human Pituitary Gland Poly A+ mRNA pool (1305204A, Clontech, USA) was used. The pool comes from 88 normal pituitary glands of Caucasian men and women aged 16–68 years who died from sudden death.

qRT-PCR Assay

One microgram of total RNA were incubated with 1 IU RNase free DNase I (EN0521, Thermo), 5 μL 10X buffer with $MgCl_2$ and water for a total volume of 50 μL, at 37°C for 30 min. The reaction was terminated by inactivating DNase and then RNA was purified with an affinity column using the GeneJET RNA Cleanup and Concentration micro kit (K0842, Thermo Fisher). RNA was finally quantified by spectrophotometry (Nanodrop 2000, Thermo Fisher). Previous to cDNA synthesis, we performed a pre-treatment with DNase incubating 1 μg of RNA with 1 IU of RNase-free DNase I (EN0521, Thermo Fisher),

1 μL of $MgCl_2$ buffer and water to a final volume of 10 μL for 30 min at 37°C. DNAse was then inactivated by adding 1 μL of EDTA and incubating for 10 min at 65°C. cDNA was synthesized following the supplier's protocol, adding 1.5 μL of 300 IU MMLV (28025-013, Invitrogen, USA), 6 μL 5X First-Strand Buffer, 1.5 μL 10 mM dNTPs, 0.1 μL Random Primers, 3 μL 0.1 M DTT, 1 μL RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/μL) and H_2O for a total 30 μL reaction. For human samples, 50, 25, and 12.5 ng of Poly A+ mRNA was similarly treated.

Expression was detected by qPCR using 1 μL of the cDNA reaction plus 6 μL 2x TaqMan Gene Expression MasterMix (4369016 Applied Biosystems) and 6 μL diluted primers in 96 well-plates in a 7500 Real-Time PCR System (4351105, Applied Biosystems, USA). Primers and TaqMan assays used for each gene were designed in contiguous exons and are summarized in **Supplementary Table 1**. As control for general gene expression we used human or rat *TBP* based in published works (19, 31, 32). Negative controls of the reverse-transcription step (all reagents and RNA sample but without reverse transcriptase) and the PCR step (all reagents but no reverse-transcribed sample) were included in each assay plate.

Rat Pituitary Immunofluorescence

For rat pituitary we used coronal sectioning of formalin-fixed paraffin-embedded male pituitaries in 4-micron sections. For dewaxing sections were incubated overnight at 52°C. Antigen retrieval was done using the PT-Link system in high pH buffer (DAKO-Agilent). Sections were incubated with primary antibodies overnight at 4°C and then 1 h with secondary antibodies at room temperature. For nuclei staining we used DAPI. Concentrations and conditions of antibody incubations are listed in **Supplementary Table 2**. Slides were photographed using a confocal microscope TC-SP5-AOBS with a white laser (470–670 nm) and a UV laser (Leica) with LAS AF (*Leica Application Suite Advanced Flourescence*) software, using serial sections (Z) every 1 μm for the 20X (PL APO 20X/N.A.0.70 CS) objective and 0.3 μm for the 63X objective (oil PL APO 63x/N.A.1.4-0.6 CS). Data were collected using Sequential Mode with the following order: first, Channel 00 (DAPI); second, Channel 02 (Alexa 488); third, Channel 03 (Cy3). Data were collected at resolution 1,024 × 1,024 pixels, with zoom 1x-3x, giving an XY field of a range from 775 × 775 μm till 288.5 × 288.5 μm for objective 20x and of a range of 246 × 246 μm till 90.4 × 90.4 μm for objective 63x. Thus, the final resolution was between 0.75 and 0.28 μm/pixel (20x) and 0.24–0.08 μm/pixel (63x).

Immunohistochemistry

We used 3 μm sagittal sectioning of the formalin-fixed paraffin-embedded pituitary. Sections were deparaffinised and retrieved as above. Sections were blocked with EnVision® FLEX Peroxidase-Blocking Reagent (DAKO-Agilent). Staining was performed incubating with primary antibody in diluent buffer followed by incubation with EnVision™ FLEX/HRP system (DAKO-Agilent) followed by DAB. Nuclei were counterstained with diluted Hematoxylin. Concentrations and conditions of antibody incubations are listed in **Supplementary Table 2**.

Graphs and Descriptive Statistics

Results are presented as Mean ± SEM using GraphPad Prism v.7 (GraphPad Software, California USA). Normality for each group of quantitative data was assessed using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie for "*p*" value. Quantitative variables were compared by Mann-Whitney tests when non-parametric or unpaired *t*-tests when parametric.

RESULTS

The Four GFR Alpha co-receptors of RET Are Expressed in Rodent and Human Pituitary

To clarify which of the four co-receptors were expressed in the pituitary we performed quantitative RT-PCR for each co-receptor. In rats, we used AP of young adult male and female rats (90 days old) of the two most common strains, *Sprague-Dawley* and *Wistar*. As expected from our previous results in Sprague-Dawley rats (15, 17), expression of the co-receptor *Gfra1* and *Gfra2* was abundant in rat AP without differences between male and female (**Figure 1A**). No differences were found when compared to *Wistar* rats. We could detect expression of the two other co-receptors *Gfra3* and *Gfra4*, this last being the least expressed in both rat strains. We did not find sex differences in expression except for *Gfra3* significantly less expressed in *Wistar* females (**Figure 1A**).

Next we studied the expression of the four RET co-receptors in the human pituitary (**Figure 1B**). A mixed pool of male and female adult normal adenopituitaries was used. To establish the quality of the pool, we detected the expression of hormones and receptors for some hypothalamic factors. As expected the most abundant detection corresponded to GH and PRL, since the normal pituitary has a ratio of more than 50% of somatotrophs and around 35% of lactotrophs, with certain variations in women (proportionally more lactotrophs) respect to men (13, 15, 18, 21, 33). The next hormone expressed was POMC corresponding to corticotrophs being the next in number (around 15%). Gonadotrophs are around 10% of endocrine cells and express both *LHB* and *FSHB*; *LHB* expression is as abundant as *POMC* but *FSHB* is less expressed. Thyrotrophs are the least abundant endocrine population but *TSHB* is as well-expressed as *FSHB*. This indicates that in addition to the proportion of cells of each type, other factors must be considered, such as the direct regulation of the expression of that gene. Both *GHRH*, *SSTR2*, and *SSTR5* were abundant.

RET and the four *GFRA* co-receptors are expressed in the human AP at levels slightly under the housekeeping gene *TBP*. Interestingly, the co-receptor GFRA4 that previously had been suggested to be absent, is clearly expressed although as happened in the rat AP, it is the least abundant GFRα co-receptor.

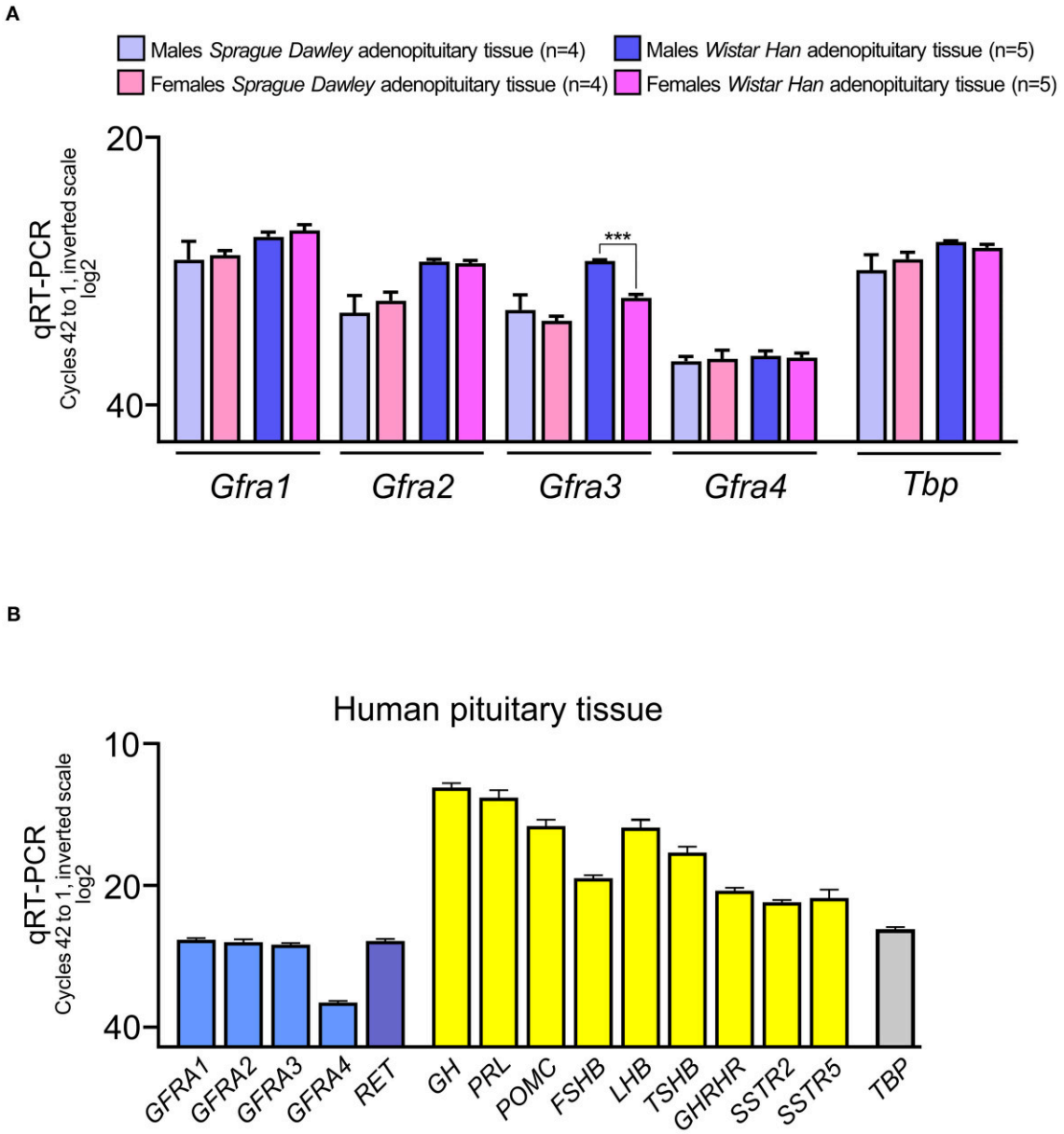

FIGURE 1 | RNA expression of the *GFRA* co-receptors of RET in the adenopituitary. For comparative purposes among different genes, expression is shown in cycles of the qRT-PCR compared to the housekeeping control gene, *Tbp* (in rat pituitary) or *TBP* (in human pituitary). The total number of cycles is 40. Less cycles required for amplification, closer to 20 cycles, higher expression since the scale is log2. **(A)** The four co-receptors are expressed in male and female adenopituitary from the two most common strains used in biomedical research, Sprague-Dawley, and Wistar. The most abundant co-receptor is *Gfra1* and the least expressed is *Gfra4*. Female Wistar adenopituitary express significantly less *Gfra3* than males. **(B)** Expression of *GFRA* co-receptors and *RET* in human normal pituitary, pooled from men, and women. For comparative purposes hormones and receptors for hypothalamic factors were also measured. As in rat pituitary, *GFRA4* is the less abundant in human adenopituitary.

Functional Localization of RET and Its Four co-receptors in Rat Pituitary

Once we were sure that all four RET co-receptors were expressed at the mRNA level, we studied its protein expression within the pituitary gland. For the rat pituitary, we used immunofluorescence and confocal microscopy. Since we did not find differences in mRNA expression, we used male pituitaries. The major cell population in the pituitary are the endocrine cells grouped in acini within the AP. A second population, the pituitary stem cells are localized at a niche comprising the first cell layer between the IL and AP (13, 15, 16, 25). In this niche, the parenchymal cells are the GPS cells called, as described above, for the acronym of some co-expressed proteins: GFRα2, PROP1 and Stem Cell factors (SOX2, SOX9, and OCT4). The

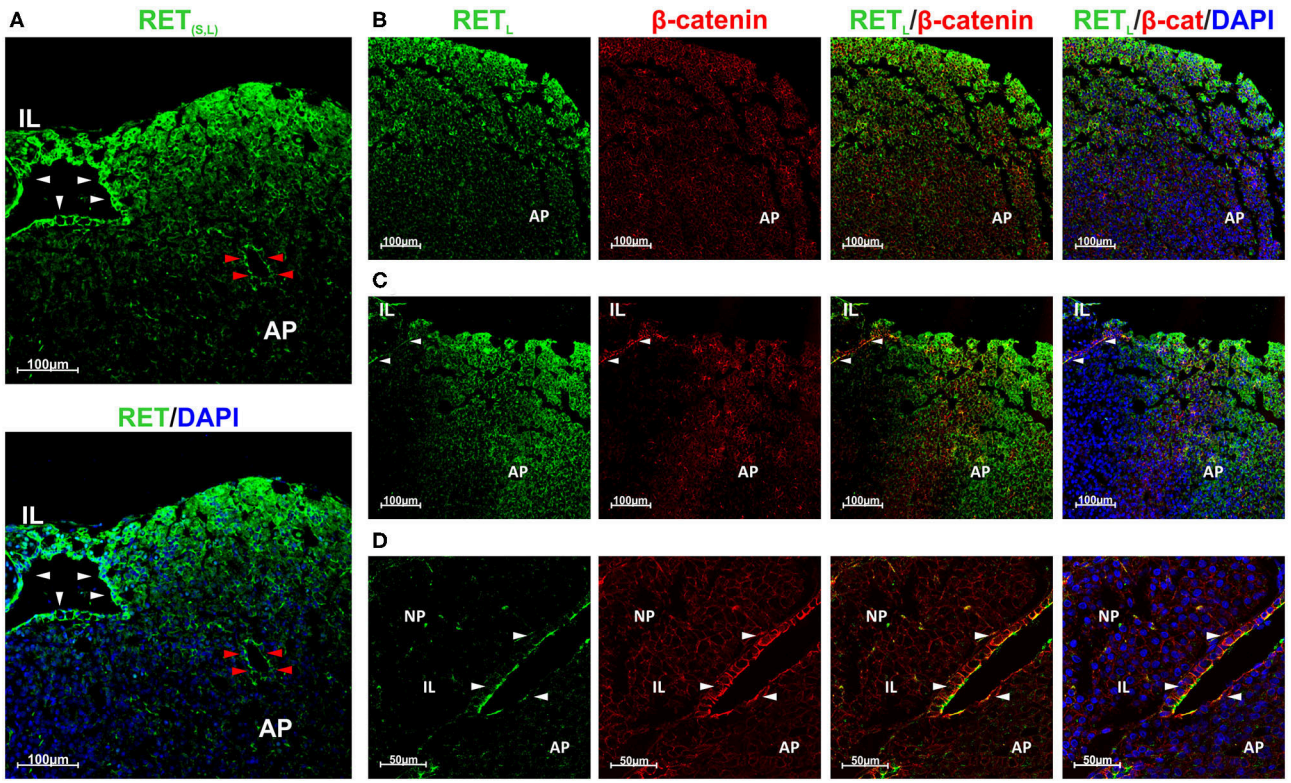

FIGURE 2 | GPS cells at the pituitary stem cell niche co-express RET and β-catenin. RET is also expressed by endocrine cells at the periphery of the AP lobes. **(A)** High RET expression (green) in the stem cell niche (white arrowheads) and in one follicle within the AP (red arrowheads). It was detected with an antibody recognizing both isoforms (S and L). RET is also expressed at the periphery of the AP lobe. **(B–D)**: RET was detected with an antibody for RET_L isoform. **(B)** At the AP, RET (green) is expressed at the periphery of the lobe and do not colocalize with β-catenin (β-cat, red). DAPI (blue) is used to reveal the nuclei. **(C)** At the marginal zone between the IL and the AP the GPS cells reside, forming the pituitary niche. RET (green) and β-catenin (red) are co-expressed in GPS showing yellow blended color (arrowheads). In the AP, RET does not colocalize with β-catenin. **(D)** Higher magnification at the niche, shows colocalization of RET (green) and β-catenin (red) at the GPS cells (yellow at the combined projection, indicated by arrowheads). AP, Adenopituitary; IL, Intermediate Lobe; NP, Neuropituitary.

niche also contains other cell types supporting the GPS (15). The niche projects into the AP as finger like projections appearing as rounded structures in a section (13, 34).

We have previously studied RET expression in the endocrine cells of the AP restricting it to the somatotrophs (17). However, we had not compared the expression between the niche and the somatotrophs. Shown in **Figure 2** is a coronal rat pituitary section. RET is expressed at the outside cell layers of the AP lobe where somatotrophs are located but it is expressed more intensely in the stem cell niche (**Figure 2A**). Moreover, the follicles within the AP also present higher RET expression. Here it was detected with an antibody recognizing the two RET_L and RET_S isoforms. There are two RET isoforms, RET_L and RET_S with a small difference in protein length at the end of the cytoplasmic C-terminal tail. Both are equally expressed in the pituitary (15, 19, 20). To further associate this high intensity of expression with the stem cell niche we co-stained for RET_L and β-catenin. It is known that GPS cells of the pituitary niche express huge levels of β-catenin a characteristic shared with embryonic stem cells (33, 35–37). On the other hand, β-catenin is expressed at low levels by all epithelial cells including the endocrine AP cells (**Figures 2B,C**) (38, 39). At the niche, RET –detected with RET_L antibody- was co-localized with β-catenin both expressed at high levels (**Figure 2C**). Intriguingly, RET expression was concentrated at the apical pole of the GPS cell layer (**Figure 2D**).

Our next step was to establish the staining for each of the GFRα co-receptors, looking at whether there was staining at the GPS niche or at the adenopituitary or both. GFRα1+ was expressed in the stem cell niche as demonstrated by its colocalization with β-catenin (**Figure 3A**). In the adenopituitary, cells expressing GFRα1 negative for β-catenin were localized at the AP lobes outside the niche (**Figure 3B**).

The GFRα2 co-receptor shows a continuous staining of the apical pole of the first cell layer in the marginal zone of in the cleft surrounding IL and AP (**Figure 3C**). It is fully colocalized with β-catenin (**Figure 3C**). GFRα2 is also expressed in a layer of cells surrounding groups of IL cells, at what we consider an extension of the pituitary niche (**Figure 3C**). GFRα2 is considered one of the principal markers for pituitary stem cells, those known as GPS (15). Only a small population of weak GFRα2 positive cells are scattered within the adenopituitary (**Figure 3D**). This expression is related to partial sectioning through pituitary follicles since they show blended signal with β-catenin (**Figure 3D**).

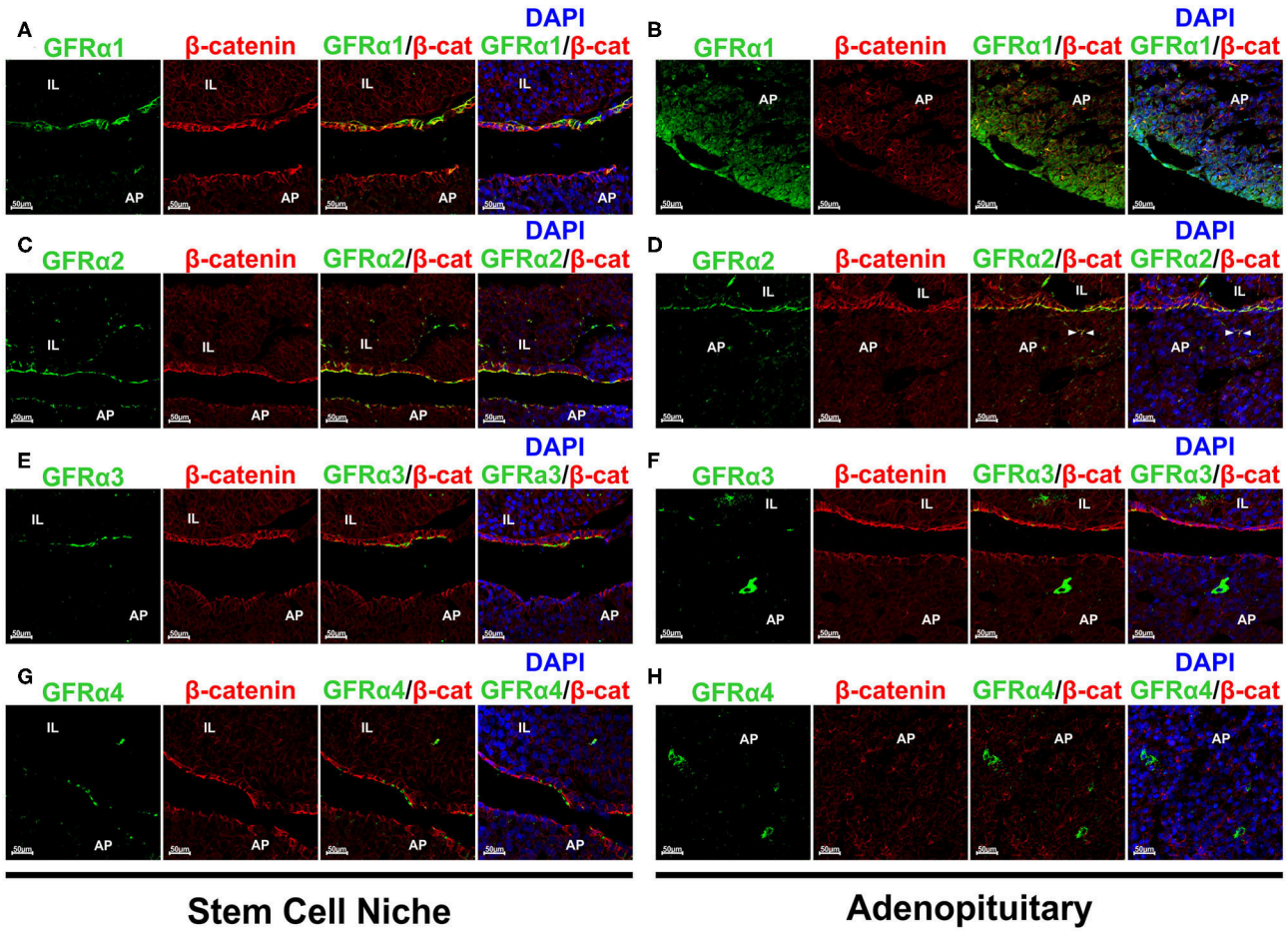

FIGURE 3 | Expression of the GFRα 1-2-3 and 4 co-receptors in rat pituitary. Sections were double stained for each one of the co-receptors and β-catenin to reveal the pituitary stem cell niche. **(A)** GFRα1 (green) colocalizes with β-catenin (β-cat, red) at the GPS niche (yellow blended color in the combined projection). However, GFRα1 cells at the adenopituitary do not co-express β-catenin **(B)**. DAPI is shown to reveal the nuclei. **(C)** GFRα2 is continuously expressed along the pituitary stem cell niche, at the apical pole of the GPS cells. It is co-expressed with β-catenin. **(D)** There are few GFRα2 cells through the AP and are also GPS cells located at the pituitary follicles as shown by co-expression with β-catenin (arrowheads). **(E)** GFRα3 is also co-expressed with β-catenin at the apical pole of the GPS cells. **(F)** There are isolated GFRα3 expressing cells that do not express β-catenin. **(G)** GFRα4 is also colocalized with β-catenin at the niche. **(H)** Isolated GFRα4 cells negative for β-catenin were found in the AP (Left) but also—and numerous- in the IL (Right). AP, Adenopituitary; IL, Intermediate Lobe; NP, Neuropituitary.

GFRα3 was also expressed at the apical pole by the niche cells co-expressing beta-catenin (**Figure 3E**), as previously published (39). However, contrary to GFRα2, GFRα3 expression seems less abundant in comparison to GFRα2 since its expression was not continuous. Isolated GFRα3 positive cells, with intense cytoplasmic staining and negative for β-catenin were dispersed throughout the AP (**Figure 3F**). These cells did not express β-catenin suggesting that they were differentiated cells or cells in the process of differentiation.

The last of the RET co-receptors was GFRα4 that presented a pattern similar to the one found for the other co-receptors at the niche (**Figure 3G**). GFRα4 was in the extracellular aspect of the plasma membrane in GPS cells co-expressing β-catenin. Regarding the AP, GFRα4 was expressed in isolated cells negative for β-catenin showing a cytoplasmic staining (**Figure 3H**, left). Moreover, abundant cells expressing GFRα4 were found at the IL (**Figure 3H**, right).

To further confirm these results we performed new stainings and recorded it at higher magnifications, adding an overlay with transillumination using Differential Interference Contrast (DIC) that reveals tissue structure. The space between the niche containing the GPS cells and the AP, the MZ, is thin and present some actual contacts between both layers. However, fixation and embedding retracts the tissue separating both layers, MZ and AP, allowing a more detailed study of the GPS cells. We know this by our previous studies in GFRα2, the most well-studied co-receptor (13, 15). **Figure 4A** shows GFRα2 and β-catenin in an unusual pituitary that remained with a thin MZ. **Figure 4B** shows the usual open MZ where the niche remains stuck to the IL. GFRα2 is localized at extracellular aspect of the plasma membrane in the apical pole of the GPS cells, with some cells (or fragments) remaining at the AP side after the retraction, indicating its proximity in the pituitary *in vivo*. Another layer of GFRα2 is present at the apical pole of the IL (see below). In

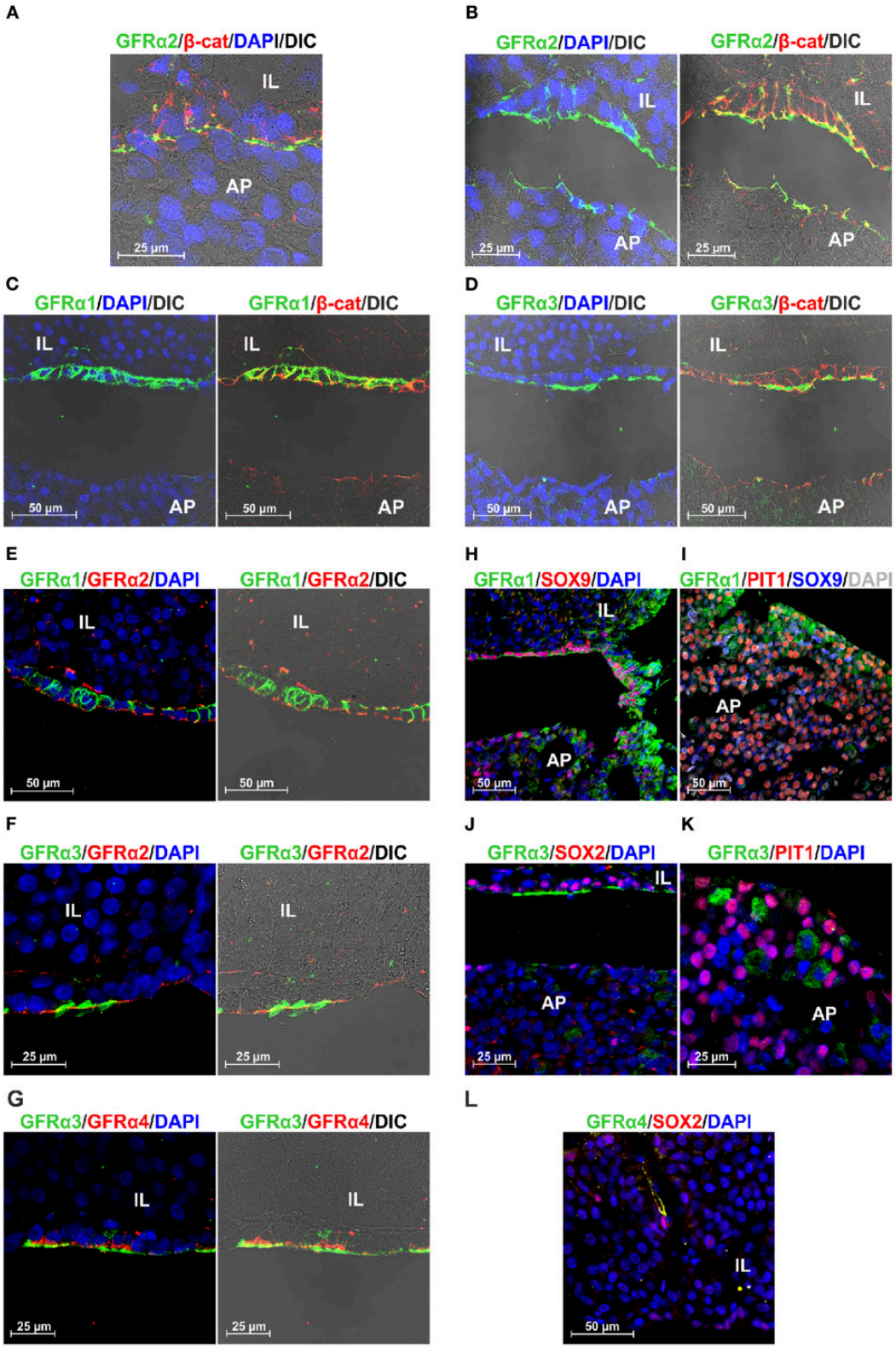

FIGURE 4 | Polarization and colocalization of the 4 GFRα at the niche but not in the AP. GFRα1 is also expressed in PIT1+ cells. **(A,B)** Colocalization of GFRα2 and β-catenin (β-cat) at higher magnification in a rat pituitary sample that maintain its structure with a thin MZ **(A)**, compared to another that presents the usual fixation-induced retraction opening the MZ **(B)**. **(B)** GFRα2 accumulates in the apical pole of the GPS cells residing in the stem cell niche, oriented toward the AP. **(C)** In contrast to GFRα2, GFRα1 is distributed along the plasma membrane of GPS cells. **(D)** GFRα3 coincides with GFRα2 in the apical pole oriented toward the AP.

(Continued)

FIGURE 4 | (E) Colocalization of GFRα1 and GFRα2 in the GPS cells of the niche. **(F)** Colocalization of GFRα3 and GFRα2 in the GPS cells of the niche. GFRα3 appears outside of GFRα2 in what resembles cilia in the DIC. **(G)** Colocalization of GFRα3 and GFRα4 in the GPS cells of the niche. GFRα4 is also polarized toward the AP. GFRα3 is outside of GFRα4. **(H)** In the niche, GPS cells contain nuclear SOX9 and GFRα1 highly enriched in the plasma membrane. As we enter the AP, a border area known as the wedge, GFRα1 staining spreads throughout the cell, which is no longer SOX9 positive. **(I)** The periphery of the AP lobes is a characteristic zone of somatotroph cells. There we found abundant GFRα1+ (green) /nuclear PIT1+ (red) cells but very few GFRα1+/nuclear SOX9+ (blue). Some cells show nuclear PIT1 and cytoplasmic SOX9, which is sometimes combined with GFRα1. Some cells are double negatives for PIT1/SOX9 (gray nucleus). **(J)** GFRα3+ cells colocalize with nuclear SOX2 in the niche, but are negative for SOX2 in the AP. **(K)** GFRα3+ cells do not co-express PIT1. **(L)** In IL, an elongated GFRα4+ cell with cytoplasmic SOX2 just next to a cell with intense nuclear SOX2.

contrast to GFRα2, GFRα1 is distributed all around the plasma membrane of the GPS cell delimitating its shape like β-catenin (**Figure 4C**). GFRα3 (**Figure 4D**) and GFRα4 (see below) are less expressed but coincide in the apical pole with GFRα2. Thus, RET, GFRα2, GFRα3, and GFRα4 are all of them apical proteins.

The localization of these proteins suggested that all co-receptors were co-expressed together with RET in GPS niche cells. We carried out new stains aimed specifically at studying this to the extent of technical possibilities: two of the antibodies had rabbit origin (GFRα2 and GFRα4) and the other two goat origin (GFRα1 and GFRα3), precluding double stainings among themselves. We observed colocalization of GFRα1 and GFRα2 the two most expressed receptors all along the GPS cell layer (**Figure 4E**). GFRα3 is colocalized with GFRα2 at the apical pole, and it is concentrated in structures suggesting cilia (**Figure 4F**). GFRα4, the less expressed receptor, is colocalized with GFRα3 at the apical pole but, with an appearance similar to GFRα2, in the plasma membrane behind GFRα3 (**Figure 4G**).

We could not find any colocalization of these receptors outside the niche in the adenopituitary although, as shown above in **Figure 3**, there were cells expressing GFRα co-receptors. To explore the possibility that these cells were differentiating or already differentiated we stained with some nuclear markers. SOX2 and SOX9 are transcription factors highly co-expressed by the GPS cells at the niche, but remaining in cells that leave the niche committed to differentiation (27, 28). PIT1 is the transcription factor characteristic of differentiated somatotrophs, lactotrophs, and thyrotrophs. The most abundant co-receptor within the AP is GFRα1 that is expressed together with RET and GDNF in many somatotroph cells (18). **Figure 4H** shows the transition between the MZ and the AP, so-called the wedge. At the niche, SOX9+ GPS cells express GFRα1 at the plasma membrane; as the AP is reached, GFRα1 cells show more diffuse staining throughout the cytoplasm, and most are negative for SOX9. Somatotroph cells are concentrated on the periphery of the AP lobes. In this zone, most of the GFRα1 cells are PIT1+ (**Figure 4I**); few GFRα1 cells have a SOX9+ nucleus. Interestingly, many PIT1+ cells have cytoplasmic SOX9, and in some this is added to cytoplasmic GFRα1 (**Figure 4I**).

GFRα3 colocalized with SOX2 at the niche but not in the AP (**Figure 4J**). GFRα3 also did not colocalized with PIT1 (**Figure 4K**). GFRα4 did not colocalize with PIT1 or SOX9 in the pituitary gland. We found at the IL some elongated cells co-expressing GFRα4 and cytoplasmic SOX2 in the vicinity of another intense SOX2+ (**Figure 4L**).

Functional Localization of RET and Its Four co-receptors in Human Pituitary

The results obtained in the rat pituitary indicated that the GPS cells of the marginal zone co-expressed all co-receptors GFRα 1-2-3 and 4 together with RET plus the stemness markers (SOX2, SOX9, PROP1, OCT4). On the other hand, within the AP, isolated cells outside the niche expressed only one of those co-receptors, either while differentiating or during transiently amplifying after recruitment. Previous studies have shown that the human pituitary expresses RET together with GFRα1 in the somatotrophs (18) and the co-receptor GFRα2 together with OCT4 at the stem cell niche (15). As indicated above, human somatotroph adenomas have expression of the GFRα4 co-receptor (19). This led us to also study the expression of the four RET co-receptors in the normal human pituitary.

It is not easy to obtain human pituitary glands from healthy and middle-aged adults in hospital tissue banks. The pituitary must be preserved whole and well-oriented to study the stem cell niche, the patient must not be elderly because the niche may no longer be functional, and the cause of death or previous treatments should not affect the pituitary or to the conservation of the sample. Our case was a 55-year-old male patient whose death was upon admission to the hospital due to causes other than pituitary pathology, and no previous treatments.

The human pituitary has a small IL comprised between the NP and the AP, the main endocrine portion (**Figure 5A**). The stem cell niche is located between the IL and the AP at the center of the pituitary and is only visible using sagittal sections through a central longitudinal plane from top to bottom. In the niche, cysts known as Rathke's Cysts (RC) were surrounded by the stem cells (**Figure 5A**). Immunohistochemistry (IHC) for Synaptophysin delineated the NP and the IL, with less intensity at the AP (**Figure 5B**). The RC appeared weakly stained. Chromogranin A stained exclusively the AP (**Figure 5C**). PIT1 the transcription factor common to somatotrophs, lactotrophs, and thyrotrophs stained cells within the AP but none in the marginal zone where the RC were located, nor in the IL or NP (**Figure 5D**). Cytokeratins are expressed by the all endocrine epithelial cells but its expression was very high in the stem cells that surround the RC in the marginal zone (**Figure 5E**). GDNF the most abundant RET ligand in the pituitary was expressed exclusively at the AP, with no staining in the IL or NP (**Figure 5F**). It is known that GDNF is expressed by normal somatotrophs together with GFRα1 and its expression

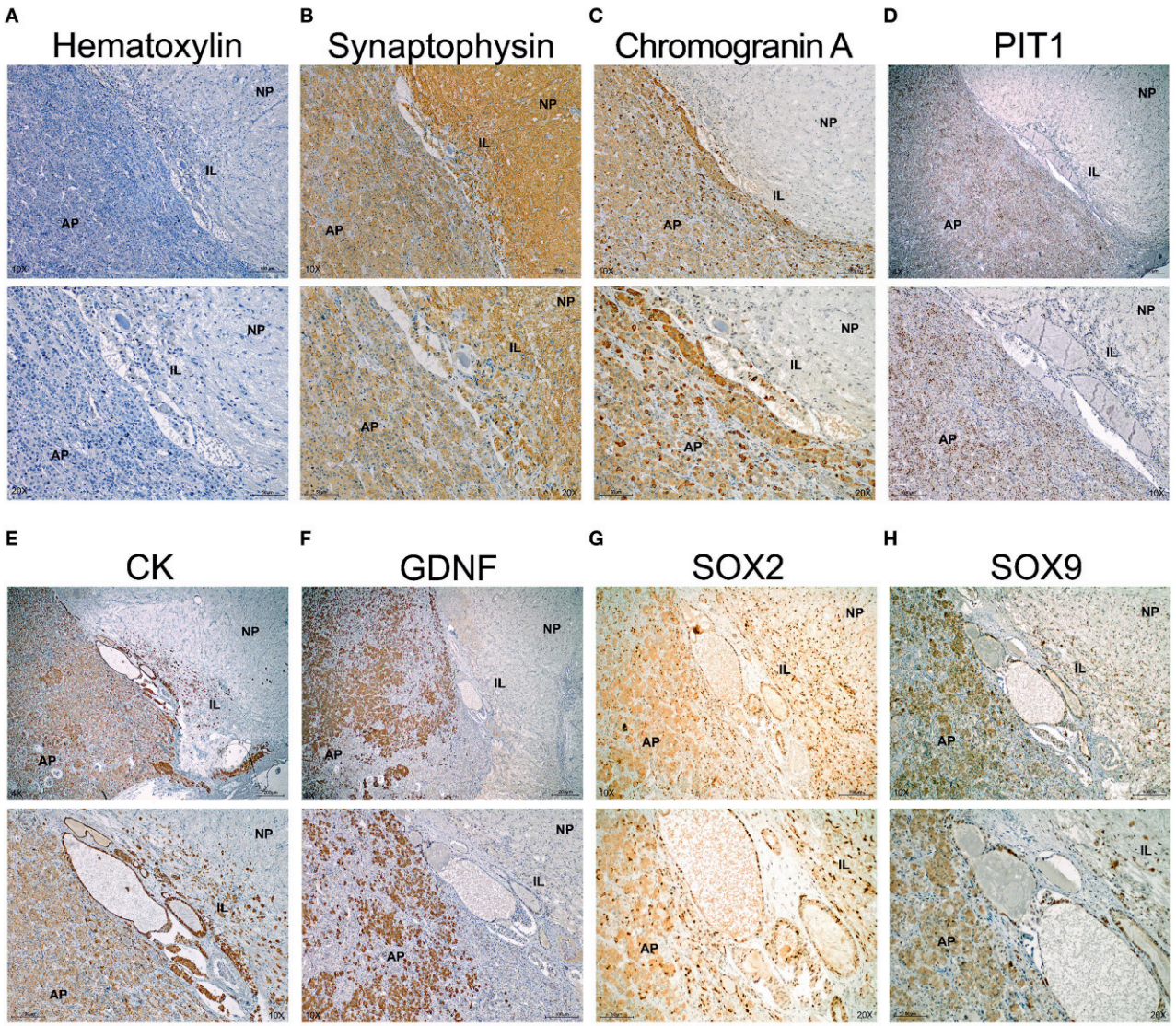

FIGURE 5 | The normal human pituitary co-express GPS stem cell markers at the epithelium of the RC. Immunohistochemistry in sagittal sections of human pituitary counterstained with Haematoxylin to reveal nuclei. Two magnifications are shown per field. **(A)** Negative control omitting primary antibody. No signal is seen and only remains the haematoxylin counterstain revealing the three portions of the pituitary. At the border between the IL and AP appears cysts (Rathke's Cysts, RC), surrounded by a single layer of cells considered the GPS in humans. **(B)** Synaptophysin stains the NP, IL and less the AP. **(C)** Chromogranin A stains the AP. **(D)** PIT1 is a transcription factor express by differentiated somatotrophs, lactotrophs and thyrotrophs at the AP. **(E)** Cytokeratins (CK) are expressed by the epitheial endocrine cells. However, at the niche, the pituitary stem cells overexpress cytokeratins. **(F)** GDNF is the most abundant ligand of RET expressed at the adenopituitary. **(G)** SOX2 is a transcription factor expressed in many cells throughout the NP, IL, and AP. However, intensity is high at the stem cell niche formed by RC. **(H)** SOX9 is a transcription factor expressed by some cells at the NP and AP. Intensity is higher at the RC epithelium. AP, Adenopituitary; IL, Intermediate Lobe; NP, Neuropituitary.

is preserved in somatotroph adenomas (18, 19, 22). SOX2 was intensely detected within the nuclei of the RC epithelium, as corresponds to a marker of GPS stem cells (**Figure 5G**). However, SOX2 was also expressed by some cells of the NP and IL, and some cells in the AP (**Figure 5G**). It has been previously shown that recruited SOX2 positive cells migrate from the niche into the AP (27, 28). Similar staining was found for SOX9 (**Figure 5H**), although its intensity was higher in the RC cells than the AP, IL, or NP.

Next, we performed staining for the RET co-receptors in this human pituitary. IHC for GFRα1 showed an abundant signal in the AP but also specific and intense staining in the epithelium forming RC, which are considered the GPS stem cells (**Figure 6A**). GFRα2, GFRα3, and GFRα4 were detected at high intensity in the epithelium of the RC (**Figures 6B–D**). Furthermore, the three co-receptors presented isolated cells within the AP positive for one of these co-receptors (**Figures 6B–D**). GFRα3 and GFRα4 –but not

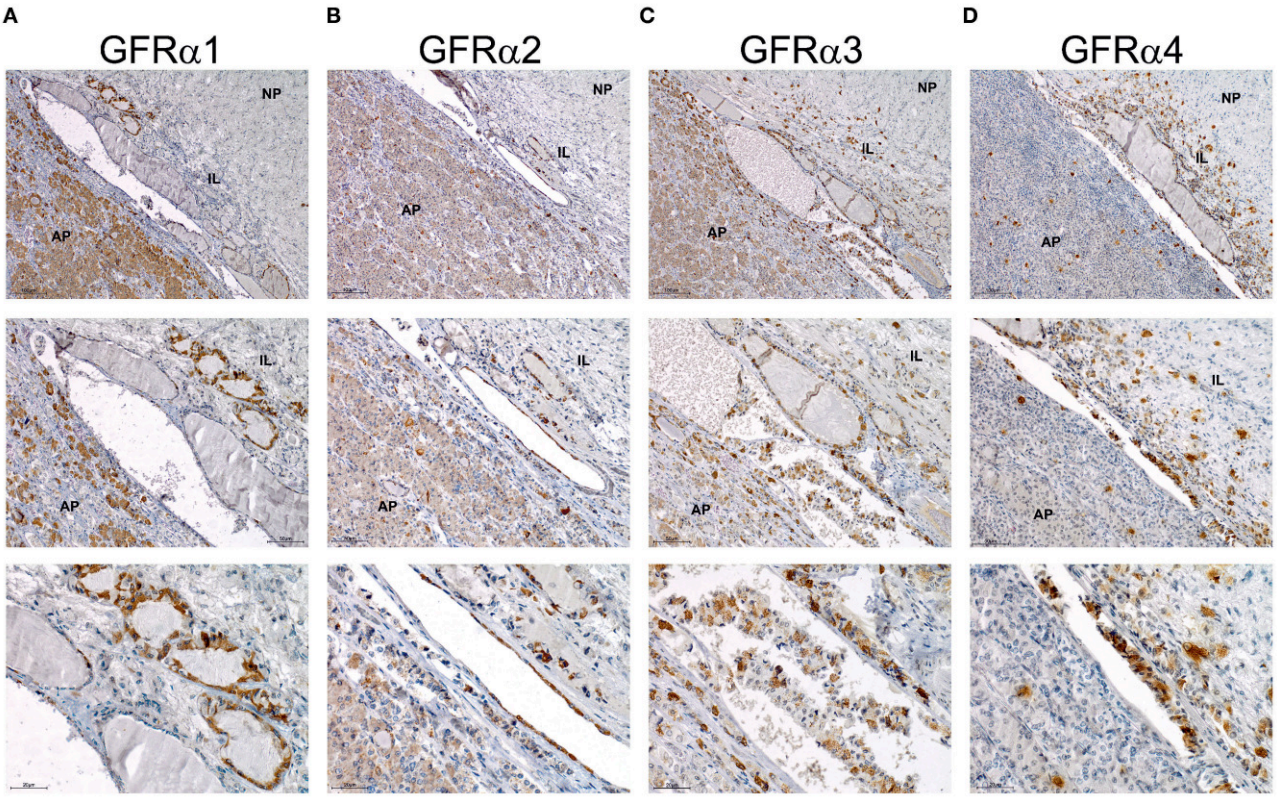

FIGURE 6 | Expression of the GFRα 1-2-3 and 4 co-receptors in human pituitary. Immunohistochemistry of each co-receptor in sagittal sections of human pituitary counterstained with Haematoxylin to reveal nuclei. Three magnifications are shown per field. Top row pictures show a global vision of pituitary and general distribution of the four GFRα co-receptors. Middle row has a higher magnification in order to focus specially in intermediate lobe and adenopituitary. Bottom row shows a close-up view of the stem cell niche composed of RC. **(A)** GFRα1 is abundantly expressed at the AP but intensity at the niche is higher. At highest magnification intensely stained RCs are shown. **(B)** GFRα2 is highly expressed at the niche with some isolated cells through the AP. At highest magnification intensely stained RCs are shown. **(C)** GFRα3 is highly expressed at the niche. Few isolated cells are present at the AP but also at the IL. At highest magnification intensely stained RCs are shown. **(D)** GFRα4 presents a similar staining with high intensity at the niche, and isolated cells in the AP and IL. At highest magnification intensely stained RCs are shown. AP, Adenopituitary; IL, Intermediate Lobe; NP, Neuropituitary.

GFRα1 or GFRα2 also showed individual cells stained within the IL (**Figures 6C,D**).

DISCUSSION

In this study we have found that the four RET co-receptors GFRα1-2-3 and 4 are co-expressed in the pituitary stem cells of the rat and human pituitary. These results suggest that the co-expression of the complete RET/GFRα system is important to maintain the stemness condition and that when migrating from the niche toward differentiation, the expression of the co-receptors is progressively lost. Moreover, in both human and rat pituitaries, few isolated cells positive for one or the other receptor could be observed, especially in the human pituitary. There are SOX2 cells in the adenopituitary that are negative for stem cell markers, acting as transit amplifying cells (15, 26, 34, 40, 41). The cells scattered throughout the AP expressing one of the four GFRα co-receptors could be transit amplifying cells, cells committed to differentiation but not yet fully differentiated, or differentiated cells. The last two possibilities could be applied to GFRα1+ cells within the AP since they express PIT1 and GH (18), but not nuclear SOX9. However, GFRα3 or GFRα4 individual cells are negative or present cytoplasmic SOX2, therefore the possibilities are still open.

The importance of GFRα1 in the AP was previously described (1, 20). GFRα1 plays a key role as a co-receptor for GDNF by mediating the action of this ligand on the survival and apoptosis of somatotroph cells through the RET/GDNF-AKT survival pathway (1, 20). However, we have demonstrated expression of GFRα1 in stem cells from the pituitary niche in human and rat, co-expressing RET and the GPS marker β-catenin (15, 39). β-catenin is a well-known marker of epithelial stem cell niches in many organs (38). Our data reveal the expression and colocalization of RET and the four GFRα co-receptors with β-catenin in the stem cell niche of human and rat pituitary. We know that RET and GFRα3 expression is lost in craniopharyngiomas (39), a pituitary benign neoplasia possibly derived of some stem cell population where β-catenin activation and nuclear localization (28), either through mutation (42) or BRAF kinase activation (43), has been described. There is no

description of a direct interaction between RET and β-catenin in pituitary cells. However, molecular studies in human HEK293 and neuroblastoma cell lines demonstrated a GDNF/GFRα1-induced interaction between RET and β-catenin at the plasma membrane, resulting in tyrosine phosphorylation of β-catenin which prevents its proteasomal degradation, and results in cell survival and proliferation (44). Future studies will have to explore the RET-β-catenin interaction in pituitary GPS cells.

Stem cell niches are structures where postnatal stem cell reside surrounded by other population that help to maintain their integrity and its proper activation and turnover. The expression of GFRα1 in pituitary stem cells and also somatotrophs (18, and this manuscript) suggest that GFRα1 is conserved during somatotroph differentiation but lost in the rest of hormone-producing cells. Moreover, GFRα1 expression together with its ligand GDNF-remains relevant in somatotroph derived tumors causing acromegaly (18, 19, 45). The increased expression of GDNF, blocks the RET /PIT1/p14ARF/p53 apoptosis pathway inducing AKT activation and survival contributing to cell growth and tumor development (19, 21, 22). Pituitary stem cells are not the unique expressing GFRα1, since is expressed in Sertoli and Leydig cells in testis (Leydig cells also co-express GFRα2), where it arises as essential in spermatogonial stem cells (46). In the eye, the basal layer from the bulbar conjunctiva epithelium express several stem cell markers, including GFRα1, postulating them as a stem cell niche in the conjunctive (47).

GFRα2 in highly and continuously expressed by the GPS (GFRα2, PROP1, Stem) cells at the pituitary niche (13, 15, 39). However, the AP is less enriched in this co-receptor where many of the scattered GFRα2 positive cells belong to follicles that are niche extensions into the AP, and co-express β-catenin (13). Cells from follicles also co-express the other GPS stem cell markers and S-100. Differentiated secretory cells in the adenopituitary are negative for GFRα2, but they are derived from the stem cell niche (27, 28). The mechanism involved in differentiation from stem cell to hormone-producer cell remains unknown. GFRα2 is also transiently expressed in cardiac progenitors which are pluripotent stem cells. Interestingly, GFRα2 knockout ESC cell lines complete cardiomyocyte differentiation. Ishida and collaborators described the redundant role of the GFRα co-receptors, since GFRα1 together with neurturin are able to substitute the lack of GFRα2 in this cardiac progenitors (48). The same could happen in the pituitary and the redundancy of GFRα co-expression is a self-supportive mechanism which ensures proper differentiation.

GFRα3 and GFRα4 were discovered later than GFRα1-2 and thus, they are less studied. GFRα3 and GFRα4 expression patterns found in the pituitary were similar, especially in human pituitary, where scattered cells expressing any of the two co-receptors were not only found in AP but also in IL. It has been described that human pituitary cells do not express GFRα4 (30). However, we demonstrate here that GFRα4 is expressed at both the RNA and protein levels in human and rat pituitary. Moreover, GFRα4 is co-expressed with the other co-receptors in stem cells and is retained in apparent progenitors both at IL and at the AP. All co-receptors are co-expressed together with RET at the niche. Our results with some nuclear markers of commitment or differentiation like PIT1 indicate that cells expressing one single co-receptor in the AP could be marked for commitment to differentiation.

Further studies in individual GFRα co-expression with markers of differentiation would help to delineate the process of recruitment and differentiation leading to a wider comprehension of cell lineage differentiation within the pituitary and to a deeper knowledge of the differentiation process. This has implications in pituitary pathology as demonstrated for the role of RET and GFRα4 in acromegaly tumors (19).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biobanco, Complexo Hospitalario Universitario de Santiago de Compostela (CHUS). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Servicio de Ganderia, Delegación Territorial da Coruña, Xefatura Territorial da Conselleria do Medio Rural e do Mar, Xunta de Galicia.

AUTHOR CONTRIBUTIONS

AP performed animal experiments and stainings. MC performed human staining. MG-L performed stainings. SP-R and MS-F performed the RNA experiments. AG-R performed animal experiments. JC-T collected human samples. IB and CA conceptual design and funding. AP, MC, IB, and CA manuscript and figure preparation. AP, MC, SP-R, AG-R, MS-F, MG-L, JC, IB, and CA final corrections and revision of Ms. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by national grants from Agencia Estatal de Investigación (AEI) and Instituto de Salud Carlos III, with participation of European FEDER funds, to CA (BFU2016-76973-R), IB (PI/150056), and JC-T (PI19/01316). This work has received financial support from the Xunta de Galicia (Centro singular de investigación de Galicia accreditation 2019–2022) and the European Union (European Regional Development Fund - ERDF).

ACKNOWLEDGMENTS

We thank Ana Senra for her technical expertise.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00631/full#supplementary-material

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