THE RNA REVOLUTION IN EMBRYONIC DEVELOPMENT AND CELL DIFFERENTIATION IN HEALTH AND DISEASE

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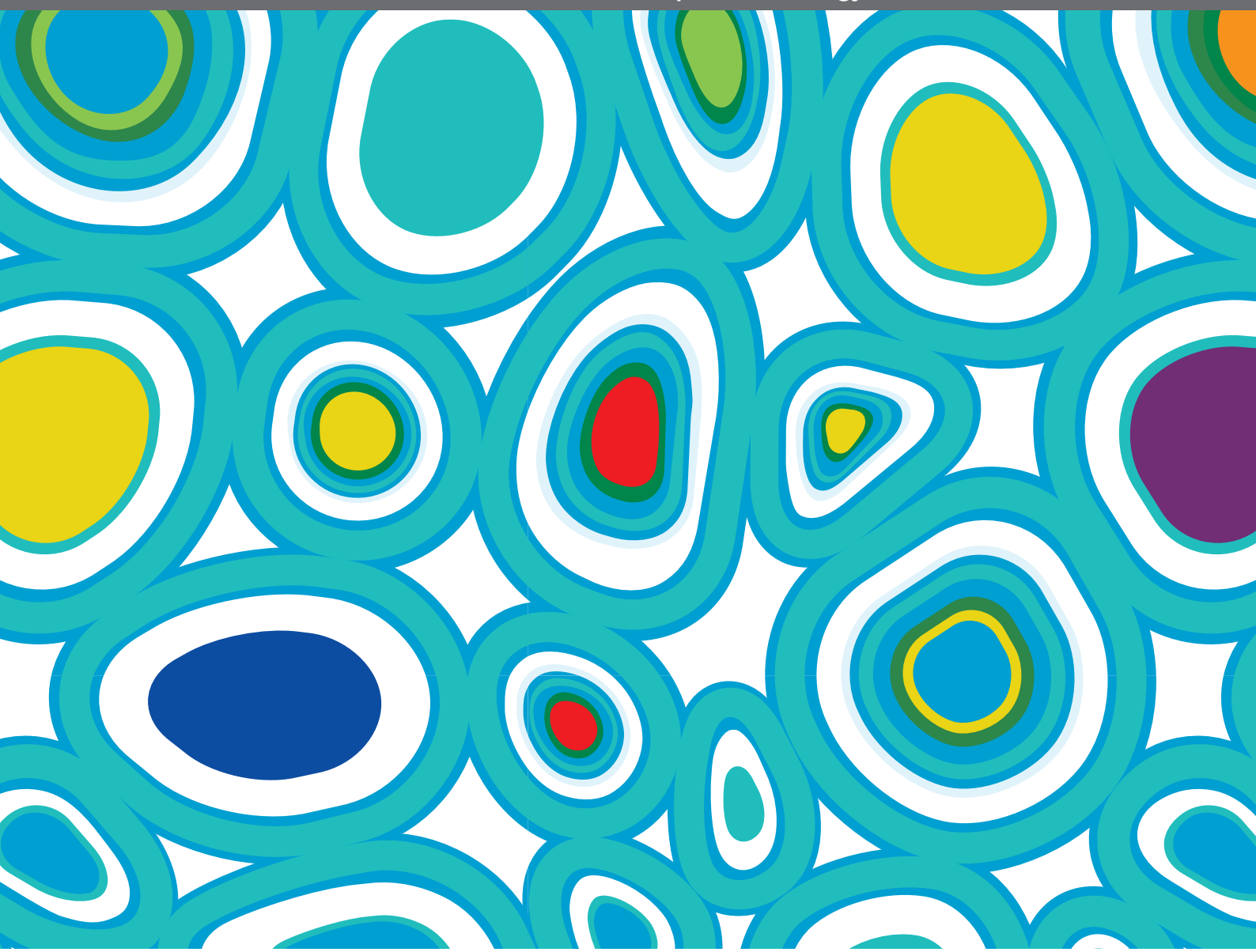

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THE RNA REVOLUTION IN EMBRYONIC DEVELOPMENT AND CELL DIFFERENTIATION IN HEALTH AND DISEASE

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EDITORIAL
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Editorial: The RNA Revolution in Embryonic Development and Cell Differentiation in Health and Disease

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Editorial on the Research Topic

The RNA Revolution in Embryonic Development and Cell Differentiation in Health and Disease

INTRODUCTION

Non-coding RNAs (ncRNAs) and their RNA binding proteins (RBPs) are emerging as crucial molecular players involved in normal and pathological cell fate determination. Among them microRNA (miRNAs), acting as gene expression regulators at post-transcriptional level, are involved in several networks relevant for the regulation of stemness, pluripotency, and cell fate determination during embryogenesis and adult life. Interestingly, the relevance for comprehension of the generation of spatio-temporal specificity of miRNA, their levels and dynamics of expression, and how the animal miRNA repertoire has evolved and diversified, is reported by Dexheimer and Cochella. The understanding of these regulation mechanisms could really help us to understand the contribution of miRNAs to the embryonic development and cell differentiation. Of note, alteration of the molecular mechanisms involving the ncRNA and their effectors in addition to defects in RNA modification and editing may contribute to the pathological cell fate determination in cancer and degenerative diseases.

ncRNAs AND RBPs IN DEVELOPMENT

How cells are able to maintain their identity or change fate specification is a complex problem in biology. The first events of cell fate specification take place during early development and are controlled at multiple levels by non-coding RNAs and RBPs. In the review from Müller et al., the authors discussed the importance of the Argonaute proteins for mammalian development and the recent discovery and implication of their post-translational modifications in the cancer context. Interestingly, these RBPs have functional relevance for miRNA gene regulation but also through their protein-protein interaction mediated *via* specific post-translational modifications. Another example of an RBP, IGF2BP3, is highlighted in an oncogenic context by Mancarella and Scotlandi, demonstrating that many RBPs are involved in several steps of the RNA life cycle. Indeed, in order to assess transcriptome-wide synthesis, processing, and degradation rates of RNAs, novel

methodologies are now available, and Biasini and Marques present in this issue a detailed protocol to achieve metabolic labeling of RNA coupled with sequencing in mouse embryonic stem cells. Stem cells represent an attractive model to mimic early development *in vitro* and such cellular models are very important to allow reproducible research. Many cell types are still nowadays difficult to culture *ex vivo* and a better understanding at the molecular level of cues essential for the maintenance of their cellular identity is essential. Here, the work of Gao et al. unrevealed the role of the TGF-beta/Smad pathway in the production of adult beta cells by regulating the level of specific miRNAs, which is important for the medialization of type 1 diabetes.

ncRNAs IN THE PATHOPHYSIOLOGY OF MUSCLE

ncRNAs are involved in the fine-tuning of gene expression at the basis of tissues homeostasis and regeneration. As reported by Martone et al. specific long ncRNAs (lncRNAs) and circRNAs are able to sustain skeletal and cardiac muscle formation. Of note, together with these ncRNAs, the contribution of Piwi-interacting RNAs (pi)RNAs and tRNA-derived fragments (tRFs) to myogenesis is also emerging. Interestingly, in this review it was discussed not only the nuclear and cytoplasmic activity of selected lncRNAs but also their ability to generate bioactive micropeptides which have been involved in specific muscle-related functions.

As reported by Sweta et al., recent studies suggest that lncRNAs are important for mesodermal specification and further differentiation, development, and function of mesodermal derivatives as skeletal muscle and cardiac lineages. The comprehension of the contribution of lncRNAs to cardiac, endothelial, and vascular smooth muscle cell function could be relevant to highlight how these molecules contribute to cardiac diseases and could be exploited as potential biomarkers. Regarding the clinical impact of ncRNAs for the muscle-related diseases as reported by Donzelli et al. miRNAs are emerging as potential cancer biomarkers in tissues and in body fluids for the early diagnosis of the cancer-related cachexia, a complex metabolic syndrome characterized by a marked reduction in muscle mass.

ncRNAs IN NEUROLOGICAL DISEASE

Regulatory ncRNAs, including miRNAs, lncRNAs and circular RNAs (circRNAs), are particularly enriched in the nervous system, where they play important roles in development, plasticity, and function. Moreover, increasing evidence links ncRNAs to aging, neurological diseases and brain tumors. Salvatori et al. focus on neural lncRNAs and circRNAs, reviewing the literature on their role in regulating neuronal and glial cells differentiation and their involvement in neurodegenerative diseases. The authors highlight the usefulness of ncRNAs as circulating biomarkers in body fluids and the possibility to use them as therapeutic molecules. Development of therapeutic approaches based on antisense oligonucleotides targeting ncRNAs is also discussed. The review by Laneve and Caffarelli concerns the contribution of ncRNAs to medulloblastoma, a common and aggressive pediatric brain tumor. In particular, the authors present extensive evidence on the roles played by miRNAs and lncRNAs as oncogenes or tumor suppressors, together with novel findings on other ncRNA classes, including circRNAs, enhancer RNAs (eRNAs), and small nuclear RNA. Deep characterization of such vast array of RNA species is particularly relevant in the context of medulloblastoma, as it can help decoding the intrinsically heterogeneous character of this severe brain tumor.

ncRNAs IN CANCER AND ANTI-TUMOR RESPONSE

Long and short ncRNA dysregulation has been implicated with all stages of tumor genesis, progression, and therapeutic responses, with some RNAs acting in tumor-specific manners, and others dysregulated and affecting tumorigenesis in a pan-cancer fashion. Turco et al. reviewed the cancer genes and pathways that are affected by upregulation of members of the miRNA family miR-15/107 in multiple tumor types and discussed the diagnostic opportunities presented by the detection of these miRNAs in the blood of breast cancer patients. Tran et al. reviewed the dysregulation of circRNAs in gynecological and breast cancers and discussed the potential effects of circRNA dysregulation. Di Agostino et al. reviewed the regulatory roles of circRNAs in cancer and normal cells and argued that their dysregulation during cell maturation can lead to tumorigenesis, and, importantly, the formation of cancer stem cells, which may be responsible for therapeutic failures in multiple tumor types. The review by Mancarella and Scotlandi compiled evidence that suggests that the dysregulation of RBPs can contribute to aberrant ncRNA biogenesis in cancer, while the review by Fazi and Fatica collected evidence that aberrant N6-Methyladenosine (m^6A) modifications can affect both the biogenesis and function of short and long RNAs in multiple tumor types. Finally, Pesce et al. reviewed the evidence for miRNA regulation of Natural Killer (NK) cell functions and their potential use as predictive biomarkers and effectors of immunotherapies. Their conclusions pointed to new opportunities for miRNA inhibitors and mimics as components in combination therapy strategies for cancer.

AUTHOR CONTRIBUTIONS

AR, CC, PS, and FF equally contributed as Guest Editors of this Research Topic and closely interacted throughout the editorial process, by defining the subjects to be treated and inviting leaders in specific research fields to contribute their work, and by acting as handling editors of the manuscripts submitted to the Research Topic and writing the Editorial. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Interplay Between N^6-Methyladenosine (m^6A) and Non-coding RNAs in Cell Development and Cancer

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RNA chemical modifications in coding and non-coding RNAs have been known for decades. They are generally installed by specific enzymes and, in some cases, can be read and erased by other specific proteins. The impact of RNA chemical modifications on gene expression regulation and the reversible nature of some of these modifications led to the birth of the word epitranscriptomics, in analogy with the changes that occur on DNA and histones. Among more than 100 different modifications identified so far, most of the epitranscriptomics studies focused on the N^6-methyladenosine (m^6A), which is the more abundant internal modification in protein coding RNAs. m^6A can control several pathways of gene expression, including spicing, export, stability, and translation. In this review, we describe the interplay between m^6A and non-coding RNAs, in particular microRNAs and lncRNAs, with examples of its role in gene expression regulation. Finally, we discuss its relevance in cell development and disease.

Keywords: epitranscriptomics, m6A, RNA modifications, non-coding RNAs, microRNAs, lncRNAs, cell reprogramming, ESC development

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INTRODUCTION

To date, more then 100 chemical modifications have been described in non-coding and protein coding RNAs (see The RNA Modification database[1]). The majority of them occur in transfer RNA (tRNA) and ribosomal RNA (rRNA), while a minority of them occur in messenger RNAs (mRNA) and long non-coding RNAs (lncRNAs). In all cases RNA modifications may play important role in RNA folding, stability and function; in view of the fact that, similarly to epigenetics, they can affect gene expression without changing the sequence of the RNA molecules, they are now referred to as "epitranscriptomics." In metazoan, N^6-methyladenosine (m^6A) is the more abundant internal modification in mRNAs and lncRNAs and plays relevant roles in several steps of gene expression, including splicing, export, stability, and translation. Notably, m^6A modification is present in all three different phylogenetic domains, Eukaya, Bacteria, and Archea (Carell et al., 2012), and is also present in viral RNAs (Lavi and Shatkin, 1975) where it has important regulatory functions (Meyer and Jaffrey, 2014). In analogy with DNA and histone modifications, m^6A is a dynamic mark. It is installed by writers, removed by erasers and recognized by reader proteins.

[1] https://mods.rna.albany.edu/mods/

Despite its discovery in the early 1970s (Desrosiers et al., 1974; Perry and Kelley, 1974; Adams and Cory, 1975), the precise function of m^{6}A residues in gene expression regulation remained elusive until recently with the development of high throughput methodologies for mapping of m^{6}A residues in the whole transcriptome (Dominissini et al., 2012; Meyer et al., 2012; Linder et al., 2015). These methods used specific immunoprecipitation of m^{6}A modified RNAs coupled to RNA sequencing. There are currently two different methodologies. The first one was developed by two independent groups (referred to as m^{6}A-Seq or MeRIP-seq) and sequences immunoprecipitated m^{6}A RNA fragment of about 200 nt, thus, does not allow for mapping of m^{6}A residues at single-nucleotide resolution (Dominissini et al., 2012; Meyer et al., 2012). The second one (referred to as miCLIP), uses UV cross-linking to covalently bind the anti m^{6}A antibody to modified RNAs, which induces mutation and truncation during reverse transcription, allowing for identification of single modified nucleotides within RNA species (Linder et al., 2015). m^{6}A in mRNAs and lncRNAs can be installed by two independent complexes (**Table 1**): the heterodimeric complex of METTL3/METTL14 (methyltransferase-like protein 3 and 14), also referred to as MAC (m^{6}A-METTL Complex), and the homodimeric complex of METTL16 (methyltransferase-like protein 16) (reviewed in Zhao et al., 2017; Lence et al., 2019). The MAC complex methylates adenosine during transcriptional elongation within the consensus motif RRACH (R = A/G; H = A/C/U), and is assisted in adenine selection by a multiprotein complex called MACOM (m^{6}A-METTL-associated complex) composed of Wilms tumour 1-associated protein (WTAP), Vir-like m^{6}A methyltransferase-associated (VIRMA), Cbl proto-oncogene like 1 (CBLL1, also known as Hakai), RNA-binding motif 15 (RBM15) or its paralog RBM15B, and zinc finger CCCH-type containing 13 (ZC3H13) proteins (Lence et al., 2019) (**Table 1**). The METTL3 is the catalytic component of the complex while METTL14 is required for RNA binding and stabilization. Recently, METTL14 was also found to interact with histone H3 trimethylation at Lys36 (H3K36me3), a marker for RNA polymerase II (RNA pol II) transcription elongation, thus ensuring modification of nascent RNAs in both intronic and exonic regions (Huang et al., 2019). However, m^{6}A residues in mature mRNA molecules follow precise distribution and are enriched near the stop codon and untranslated regions. On the other hand, the METTL16 complex acts on a specific stem–loop structure of RNA containing the UACAGAGAA sequence. This complex acts on only a few percentages on methylated mRNAs and lncRNAs. However, between targeted RNAs, there is the human *S*-adenosylmethionine (SAM) synthetase MAT2A (Pendleton et al., 2017; Shima et al., 2017; Warda et al., 2017), which regulates cellular levels of the methyl donor SAM. Therein, METTL16 can regulate the activity of all cellular methyltransferases, including METTL3/MELL14. Moreover, it is also responsible for the methylation in the spliceosomal U6 small nuclear RNA (snRNA).

m^{6}A modification can be removed by two demethylase enzymes belonging to the AlkB family of the Fe(II) and α-ketoglutarate-dependent dioxygenases: ALKBH5 (alkB homolog 5) and FTO (fat-mass and obesity associated protein) (Zhao et al., 2017) (**Table 1**). The first one is specific for m^{6}A removal while the second can also demethylate N^6, *2-O-dimethyladenosine* (m^{6}Am), which is installed in mRNA if the first transcribed nucleotide is adenosine, and N^1-methyladenosine (m^{1}A) in tRNA (Wei et al., 2018).

Although m^{6}A modification does not prevent Watson-Crick base pairing of A-U nucleotides, m^{6}A residues can affect tertiary interactions involving Hoogsteen base pairs that use the N^6 atom of A for H-bond (Meyer and Jaffrey, 2014). Therein, m^{6}A residues within RNA molecules can produce local changing in the RNA structure that can alter RNA folding and affect the interaction with proteins and RNAs (Meyer and Jaffrey, 2014; Liu et al., 2015; Edupuganti et al., 2017). However, the function of m^{6}A modifications in gene expression regulation is mainly mediated by m^{6}A readers (reviewed in Patil et al., 2018) (**Table 1**). Proteins of the YT521-B homology (YTH) domain family were the first to be identified. In humans, there are five members: the nuclear YTHDC1, and the cytoplasmic YTHDC2, YTHDF1, YTHDF2, and YTHDF3. Mechanistically, YTHDC1 binding in the nucleus regulates alternative splicing and promotes RNA export while YTHDF2 binding stimulates mRNA decay and YTHDF1 binding promotes translation. The YTHDF3 reader can cooperate with both YTHDF1 and YTFDF2 on modified mRNA while in circular RNAs it can promote translation independently from other YTH proteins. Additional readers, lacking the YTH domains, have been also identified, including the translational regulators eIF3 and ABCF1, which positively regulate translation of modified mRNA and the insulin-like growth factor mRNA-binding proteins IGFBP-1, -2 and- 3, which enhance RNA stability and translation (Huang et al., 2018). Therein, once installed, m^{6}A modifications can produce several different outputs on the regulation of specific RNAs which are not easily predictable.

Notably, m^{6}A plays an important role in embryonic stem cells (ESCs) by controlling cell fate transition and deletion of METTL3 and METTL16 in mouse results in embryonic lethality, indicating essential function for m^{6}A modification in the regulation of gene expression programs required for embryo development (Batista et al., 2014; Wang Y. et al., 2014; Geula et al., 2015; Bertero et al., 2018; Mendel et al., 2018). Moreover, m^{6}A plays also important role in mouse adult brain, by regulating synaptic function and stress-induced responses (Engel et al., 2018; Koranda et al., 2018), and in the hematopoietic system, by controlling stem cell differentiation and homeostasis (Lv et al., 2018; Wang et al., 2018; Yao et al., 2018). Even if most of the m^{6}A studies focused on its direct role on mRNA function, recent evidences showed that m^{6}A can also regulate the synthesis and function of microRNAs and lncRNAs. In addition, microRNAs and lncRNAs can also influence the function of m^{6}A modification in mRNAs. Here, we review the impact of m^{6}A on the regulation of these non-coding RNAs and we discuss the

TABLE 1 | Human m^{6}A proteins.

Protein	Function	References
Writers		
METTL3	Installs m^{6}A residues in mRNAs and lncRNAs	Liu et al., 2014
METTL14	Cooperates with METTL3 in m^{6}A deposition	Liu et al., 2014
METTL16	Installs m^{6}A in U6 snRNA and few mRNAs and lncRNAs	Pendleton et al., 2017; Warda et al., 2017
Erasers		
FTO	Remove m^{6}A and m^{6}Am from mRNA, and m^{1}A from tRNA	Jia et al., 2011
ALKBH5	Remove m^{6}A from mRNA	Zheng et al., 2013
Regulators		
WTAP	Regulates m^{6}A installation by the METTL3/METTL14 complex	Ping et al., 2014
VIRMA	Regulates m^{6}A installation by the METTL3/METTL14 complex	Knuckles et al., 2018; Yue et al., 2018
CBLL1	Regulates m^{6}A installation by the METTL3/METTL14 complex	Wen et al., 2018; Yue et al., 2018
RBM15	Regulates m^{6}A installation by the METTL3/METTL14 complex	Knuckles et al., 2018
ZC3H13	Regulates m^{6}A installation by theMETTL3/METTL14 complex	Knuckles et al., 2018; Wen et al., 2018
Direct readers		
ABCF1	Stimulates cap-independent translation	Coots et al., 2017
eIF3	Stimulates cap-independent translation	Meyer et al., 2015
HNRNPA2B1	Stimulates microRNA processing	Alarcón et al., 2015a
IGF2BPs	Increase mRNA stability	Huang et al., 2018
YTHDC1	Stimulates splicing and mRNA export	Xiao et al., 2016; Roundtree et al., 2017
YTHDC2	Stimulates mRNA decay and translation	Hsu et al., 2017; Wojtas et al., 2017
YTHDF1	Stimulates translation	Wang et al., 2015
YTHDF2	Stimulates mRNA decay	Wang X. et al., 2014
YTHDF3	Stimulates mRNA decay and translation	Li et al., 2017; Shi et al., 2017
Indirect readers		
FMR1	Inhibits translation	Edupuganti et al., 2017
HNRNPC	Regulates splicing	Liu et al., 2015
m^{6}A repelled		
ELAVL1	Increases mRNA stability	Wang X. et al., 2014
G3BPs	Increases mRNA stability	Edupuganti et al., 2017

interplay between m^{6}A, microRNAs and lncRNAs in cell development and disease.

IMPACT OF EPITRANSCRIPTOMICS ON microRNAs BIOGENESIS AND FUNCTION

MicroRNAs (miRNAs) are endogenously encoded short RNAs (∼21 nucleotides, nt) that are produced from long primary transcripts (pri-miRNA) transcribed by RNA Pol II (reviewed in Bartel, 2018). More than 50% of human miRNAs are encoded in introns of coding and non-coding pre-mRNAs and are connected to the expression of their host genes. The pri-miRNA is cleaved co-transcriptionally by a protein complex, named Microprocessor, containing the nuclear RNase III-type endonuclease *Drosha* and the DGCR8 (DiGeorge syndrome critical region gene 8) protein. The recognition of the pri-miRNA by the Microprocessor requires a stem–loop structure formed by the mature miRNA and single-stranded regions flanking the stem–loop. The cleavage by Drosha produces a stem–loop pre-miRNA of about 70 nt that is then recognized and exported to the cytoplasm by the transport receptor Exportin 5. In the cytoplasm, the pre-miRNA is cleaved by the RNase III-type endonuclease Dicer, releasing the miRNA duplex. One strand is then incorporated into the silencing complex containing one Argonaute (AGO) protein (in human AGO1, AGO2, AGO3, and AGO4) and the TNRC6 protein (also called GW182). The miRNA directs the Ago complex to its target mRNAs through perfect complementarity between sequences in the 3′ì untranslated region (3ì-UTR) and a stretch of 6 nucleotides (from nucleotides 2 to 7) in the 5′ region of the miRNA, also referred to as "seed." The Ago proteins recruit TNRC6 protein, which stimulates mRNA deadenylation by interacting with deadenylase complexes and, consequently, produces mRNA destabilization and translational repression (Jonas and Izaurralde, 2015; Bartel, 2018). In addition, TNRC6 also recruits DDX6, a helicase that enhances both the decay and translational repression of

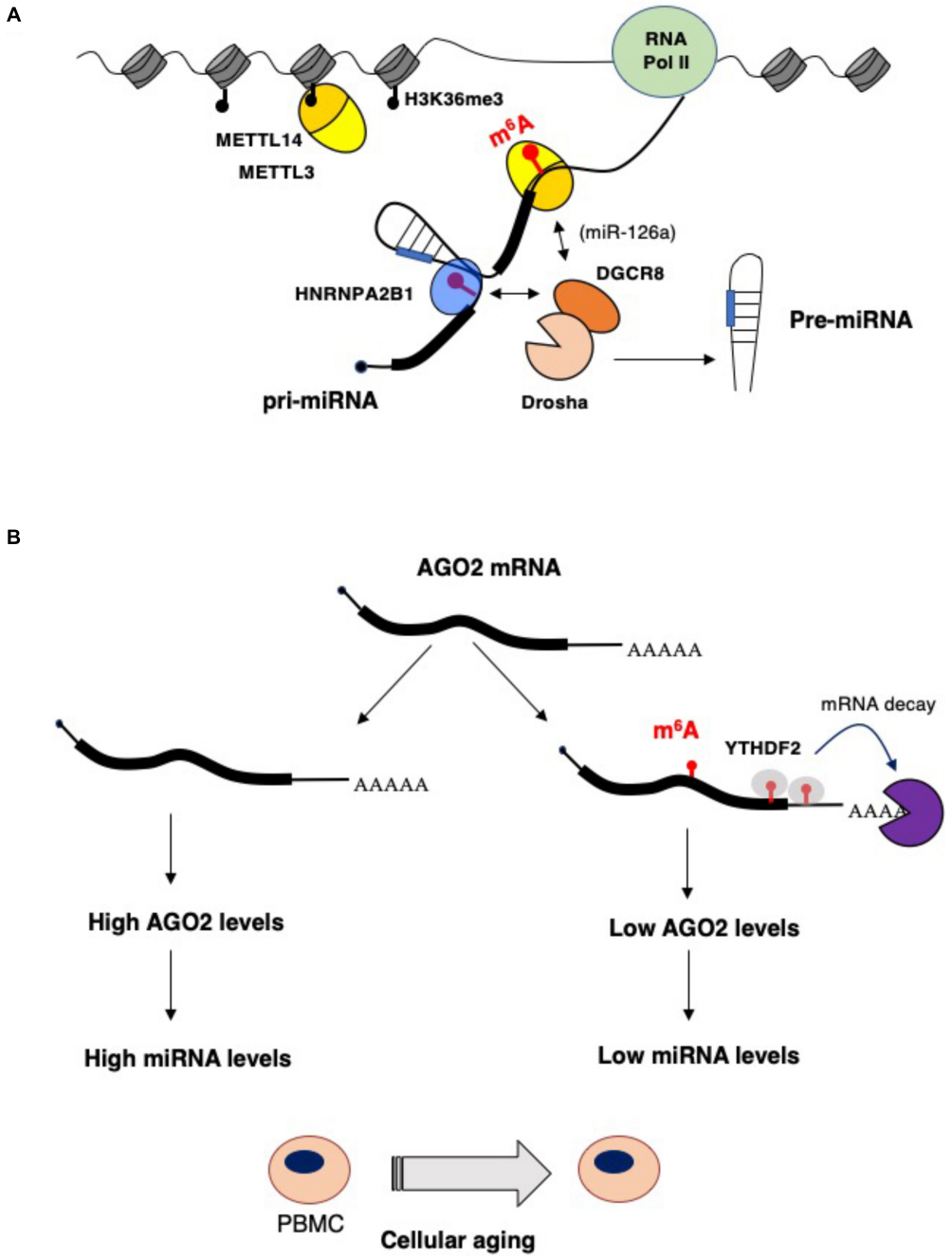

FIGURE 1 | Impact of epitranscriptomics on microRNAs biogenesis and function. **(A)** m^{6}A stimulates microRNA processing by recruiting the Drosha cofactor DGCR8 by the m^{6}A reader HNBRPA2B1 (Alarcón et al., 2015a,b) or, in the case of the miR-126a, by direct interaction with METTL14 (Ma et al., 2017). **(B)** During aging of peripheral blood mononuclear cells (PBMCs), AGO2 and, eventually, miRNA levels are decreased by higher m^{6}A modification of AGO2 mRNA. This results in enhanced mRNA decay that is very likely mediated by the YTHDF2 reader (Min et al., 2018).

target mRNAs (Jonas and Izaurralde, 2015; Bartel, 2018). m^{6}A modification may affect microRNA synthesis and function at multiple levels (**Figure 1A**). A strong correlation between m^{6}A residues in the 3′-UTR and miRNA-binding sites has been identified (Meyer et al., 2012). This has suggested the existence of a functional interaction between m^{6}A modification and miRNAs targeting. In particular, the presence of m^{6}A residues within the complementary region between the 3′-UTR and the miRNA seed might destabilize A-U pairing, thus decreasing duplex stability and affecting miRNA interaction. However, even if alterations in miRNA binding might contribute to some of the observed effects of m^{6}A modification, the impact of m^{6}A modifications within

mRNAs on miRNA targeting is still not clear. On the other hand, m^{6}A can also affect miRNA synthesis. m^{6}A marks are deposited co-transcriptionally on a set of pri-miRNA molecules and are read by the HNRNPA2B1 proteins that, in turn, stimulate nuclear miRNA processing by recruiting the Microprocessor component DGCR8 (Alarcón et al., 2015a,b). Therein, alteration in m^{6}A deposition may unbalance cellular miRNA levels. Moreover, upon acute temperature stress, the METTL3/METL14 complex can co-transcriptionally recruit the DGCR8 protein on stem–loop structures present in heat-shock genes, independently from the presence of an embedded precursor microRNA. Thus, promoting their subsequent nuclear degradation by the microprocessor complex (Knuckles et al., 2017). In view of the recent discovery of the association of METTL14 with chromatin during transcriptional elongation (Huang et al., 2019), we can speculate that the METTL3/METTL14 complex may contribute to the co-transcriptional recruitment of the Microprocessor complex on pri-miRNA transcripts. In addition, it has also been shown that, in hepatocellular carcinoma (HCC), METTL14 can directly recruit DCGR8 on the m^{6}A modified pri-miRNA encoding for oncosuppressor miR-126a (Ma et al., 2017). In particular, low levels of METTL14 in HCC are associated with low levels of miR-126a and increased metastatic capacity (Ma et al., 2017). More importantly, ectopic expression of miR-126a in HCC cells ameliorated the metastatic phenotype induced by METTL14 downregulation (Ma et al., 2017).

Another example highlighting the role m^{6}A modification on miRNAs processing has been recently reported for miR-25-3p. Zang and colleagues indeed described the impact of cigarette smoking on miR-25-3p maturation by m^{6}A modification in pancreatic ductal adenocarcinoma (Zhang et al., 2019). In this manuscript, the authors described how the cigarette smoking induced the upregulation of METTL3 expression by affecting METTL3 promoter epigenetic regulation. This results in a METTL3-dependent modification of pri-miR-25-3p and an increase of miR-25-3p processing. The induction of miR-25-3p affects the expression of its target PH domain leucine-rich-repeats protein phosphatase 2 (PHLPP2), with an consequential impact on the AKT-p70S6K signaling pathway (Zhang et al., 2019). These results suggest that a METTL3-miR-25-3p-PHLPP2-AKT regulatory axis could be relevant for the transformation process induced by cigarette smoking in pancreatic tissue.

Interestingly, an additional mechanism leading to the modulation of m^{6}A miRNAs modification is represented by the DDX3-dependent network. Indeed, DDX3, a member of the family of DEAD-box RNA helicases, has been shown to be able to interact with the RNA m^{6}A demethylases, such as ALKBH5, resulting in m^{6}A RNA demethylation. With specific regard to miRNAs, DDX3, thanks to its ability to also interact with AGO2 protein, may contribute to miRNAs demethylation. In summary, the functional contribution of DDX3 to the control of cell growth and proliferation may be at least in part mediated by its interaction with ALKBH5 and AGO2, relevant for the demethylation of mRNAs and miRNAs (Shah et al., 2017).

miRNA levels can also be controlled by m^{6}A modification of AGO2 mRNA (**Figure 1B**). In a study performed on human peripheral blood mononuclear cells (PBMCs) from young and old donors, the AGO2 mRNA was found highly m^{6}A methylated in young PBMCs and this correlated with a lower level of AGO2 mRNA in old PBMCs during aging (Min et al., 2018). AGO2 levels are important for both miRNA synthesis and function. Indeed, a lower level of AGO2 in old PBMCS resulted in an altered level of miRNA expression (Min et al., 2018), indicating that m^{6}A modification on AGO2 mRNA contributes to cellular aging by regulating global miRNAs synthesis.

IMPACT OF EPITRANSCRIPTOMICS ON lncRNA REGULATION AND FUNCTIONS

LncRNAs are generally defined as transcripts longer than 200 nt without coding potential (reviewed in Fatica and Bozzoni, 2014). The human genome contains 16,193 genes encoding for lncRNAs (Gencode v30), which can produce more than 30,000 lncRNA transcripts. The majority of lncRNAs, but not all, share several features with coding mRNAs; they are 5′ capped, spliced and, in most of the cases, polyadenylated. Similar to mRNAs, lncRNAs are also m^{6}A methylated and the levels of m^{6}A residues strongly depend on the cell line, tissue type and growth condition (Meyer et al., 2012; Han et al., 2019; Xiao et al., 2019). In cell lines, the enrichment score of m^{6}A peaks within mRNAs and lncRNAs is very similar (Han et al., 2019). However, it has been shown recently that in human fetal tissues, a lower proportion of lncRNA is m^{6}A modified compared to mRNA (Xiao et al., 2019). In contrast to mRNAs, m^{6}A residues in lncRNAs are distributed along the whole body of the transcript and are more present in lncRNAs that undergo alternative splicing (Xiao et al., 2019). Thus, this indicates a possible function for m^{6}A modification in regulation of lncRNA isoforms. Many lncRNAs are retained and function in the nucleus. Nuclear lncRNA may regulate gene expression by several mechanisms, such as modulating the activity of regulatory protein complexes, regulating chromosomal conformations and, more generally, nuclear organization (reviewed in Engreitz et al., 2016). In particular, different lncRNAs regulate gene expression by guiding regulatory complex to specific gene *loci*. This is generally achieved by lncRNA interaction with chromatin associated proteins, local chromosomal architecture or by forming an RNA-DNA triple helix (Engreitz et al., 2016; Li et al., 2016). LncRNA local structure and interaction with specific proteins plays an important role in lncRNA function. Therein, m^{6}A modification might regulate lncRNA function by providing binding sites for m^{6}A reader proteins or, alternatively, might regulate local RNA structure to allow access for specific RNA-binding proteins to nearby m^{6}A residues. Furthermore, m^{6}A modification may also influence RNA–DNA triple helix formation, in which a lncRNA binds with sequence specificity through Hoogsteen base pairs in the major groove of a Watson–Crick base-paired DNA duplex. Therein, m^{6}A modification can potentially affect lncRNA interaction with specific DNA *loci*.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as nuclear-enriched abundant transcript 2 (NEAT2), is a highly expressed nuclear lncRNA, frequently upregulated in cancer, that contains several m^{6}A modifications

(Dominissini et al., 2012). Even if MALAT1 is transcribed by RNA pol II, it lacks a canonical poly-A tail. The high stability observed for MALAT1 transcript is ensured by a triple-helix at its 3′-end that specifically binds METTL16 (Brown et al., 2014, 2016). MALAT1 accumulates in the nuclear speckels, which are nuclear domains enriched in splicing factors, and associates with different splicing regulators such as the serine/arginine-rich (SR) proteins and the protein heterogeneous nuclear ribonucleoprotein C (Tripathi et al., 2010; Zhou et al., 2016). In cell lines, MALAT1 silencing alters the alternative splicing of specific pre-mRNAs (Tripathi et al., 2010). However, MALAT1 knock-out in mouse has no effect on the alternative splicing nor on the formation of nuclear speckles (Nakagawa et al., 2012; Zhang et al., 2012). A further study showed that MALAT1 can also bind to the Polycomb 2 protein (Pc2), a component of the Polycomb Repressive Complex -1 (PRC1), and that it can act as scaffold in distinct subnuclear compartments required for coordinated regulation of gene transcription (Yang et al., 2011). Notably, m^{6}A modifications identified in MALAT1 can alter the accessibility of the RNA motif to which proteins bind, through changing its local structure (Liu et al., 2015; Spitale et al., 2015; Zhou et al., 2016), a mechanism known as "m^{6}A switch" (Liu et al., 2015). Therein, m^{6}A might affect the function of MALAT1 in splicing and transcription by regulating RNA-protein interactions.

MALAT1 can also interact with microRNAs and act as a competing endogenous RNA (ceRNA), thus affecting microRNA binding to target mRNAs, in different cell types (Leucci et al., 2013; Han et al., 2015; Hirata et al., 2015; Xiao et al., 2015). Even in some cases, the interaction of MALAT1 with microRNAs has been reported to occur in the nucleus (Leucci et al., 2013), whereas in other cases, a specific translocation of MALAT1 in the cytoplasm is required (Han et al., 2015). However, the mechanism responsible for its translocation is still not known. Notably, m^{6}A modification is directly involved in RNA nuclear export (Zheng et al., 2013; Roundtree et al., 2017; Lesbirel et al., 2018). Therein, the level of m^{6}A modifications within MALAT1 might directly control its cellular localization and, eventually, its ceRNA activity. Alternatively, m^{6}A modifications might directly regulate the RNA-RNA interactions between MALAT1 and targeted microRNAs, as recently reported for another ceRNA (Yang et al., 2018).

The X-inactive specific transcript (Xist), an important regulator of X-chromosome inactivation in mammals, is another highly m^{6}A methylated lncRNA. An initial shRNA screening performed in mESCs identified three components of the MACOM complex, WTAP, VIRMA (also known as KIAA1429) and RBM15 proteins, which are all regulators of Xist activity (Moindrot et al., 2015). Moreover, knockdown of RBM15 and WTAP greatly impaired Xist mediated epigenetic silencing (Moindrot et al., 2015). In addition, WTAP protein was also identified as a stable interactor of Xist RNA (Chu et al., 2015). More recently, it has been confirmed that m^{6}A modification is strictly required for Xist-mediated transcriptional repression and that knockdown of the METTL3 writer inhibits X chromosome silencing (Patil et al., 2016). The RBM15 protein was identified as the MACOM components that interacts with and guides the METTL3/METTL14 complex for the formation of the 78 m^{6}A present in Xist RNA (Patil et al., 2016). Furthermore, it was also shown that the YTHDC1 reader recognized the m^{6}A marks in mESCs and is required for Xist activity even if the mechanism has not yet been clarified (Patil et al., 2016). Notably, tethering of YTHDC1 on Xist in the absence of m^{6}A residues is sufficient for its repressive function, indicating that m^{6}A *per se* is not required for Xist activity. YTHDC1 is the only nuclear reader of the YTH family and is usually involved in the regulation of pre-mRNA spicing through recruiting splicing factors (Xiao et al., 2016). Similarly, YTHDC1 binding on Xist might function by bridging *cis*- acting regulatory elements on XIST RNA with *trans*-acting proteins required for transcriptional silencing.

Recently, it has also been found that the enhancer RNAs (eRNAs), non-coding transcripts produced from enhancer regions that act as regulators of transcription, are highly m^{6}A modified (Xiao et al., 2019). This has suggested that m^{6}A modification might contribute to the enhancer function of eRNAs during transcription.

In the cytoplasm, lncRNAs may regulate mRNA stability and translation by recruiting regulatory proteins to interacting mRNAs or by acting as ceRNAs (reviewed Fatica and Bozzoni, 2014). Therein, m^{6}A residues might affect cytoplasmic lncRNA function with the same mechanisms described above. A recently identified cytoplasmic lncRNA whose function is regulated by m^{6}A is lincRNA 1281 (linc1281) (Yang et al., 2018). Linc1281 is required for mESC differentiation and acts as a ceRNA by sequestering miRNAs of the let-7 family (Yang et al., 2018). Notably, linc1281 contains different m^{6}A marks in its 3′-end region that are required for the binding of let-7 (Yang et al., 2018). It has been proposed that the presence of m^{6}A in linc1281 can act as m^{6}A-switch for specific RNA binding proteins, which will eventually regulate the interaction with let-7. However, the identity of such proteins has not yet been discovered. A similar mechanism has been already proposed for the binding of HuR (ELAVL1) protein and miRNAs to mRNAs encoding developmental regulators in mESCs (see below).

In view of the fact that many lncRNAs can be m^{6}A modified and that m^{6}A can affect their expression levels and functions, it very likely that other examples of lncRNAs regulated by m^{6}A modification will follow soon.

INTERPLAY BETWEEN NON-CODING RNAs AND m^{6}A EFFECTOR PROTEINS

As highlighted above, m^{6}A is a relevant modification for non-coding RNA biogenesis and functional activity. However, it has been also reported that lncRNAs may control the function of the epitranscriptomics machinery. For example, the expression of the nuclear lncRNA FOXM1-AS allows the interaction between the FOXM1 nascent RNA and the m^{6}A demethylase ALKBH5, that results in the demethylation of FOXM1 transcripts. This promotes the binding of HuR protein (also known as ELAVL1) with FOXM1 pre-mRNA, resulting in an elevated expression of FOXM1. Interestingly, it has also been shown that, in glioblastoma stem-like cells (GSCs), the m^{6}A demethylase

ALKBH5 is highly expressed, and the depletion of ALKBH5 and FOXM1-AS disrupts GSC tumorigenesis through the reduction FOXM1 expression (Zhang et al., 2017) (**Figure 2A**).

Recently, an additional function of non-coding RNAs in the control of m^{6}A modification has emerged. In particular, it has been reported that the expression of epitranscriptomics machinery components may be controlled by miRNAs through the targeting of their corresponding mRNAs. Here, we include some representative examples of such modulation.

miR-145, broadly reported as a tumor suppressor miRNA, was shown to control the expression of the YTHDF2 reader. YTHDF2 is involved in the deadenylation and decay of m^{6}A-containing RNAs through a direct interaction and recruitment of the CCR4-NOT deadenylase complex (Du et al., 2016).

Specifically, in liver cancer cells, miR-145 downregulates YTHDF2 mRNA expression with an increase on the overall levels of mRNAs containing m^{6}A residues, as evaluated by dot-blot and immunofluorescence analyses with anti m^{6}A antibodies,

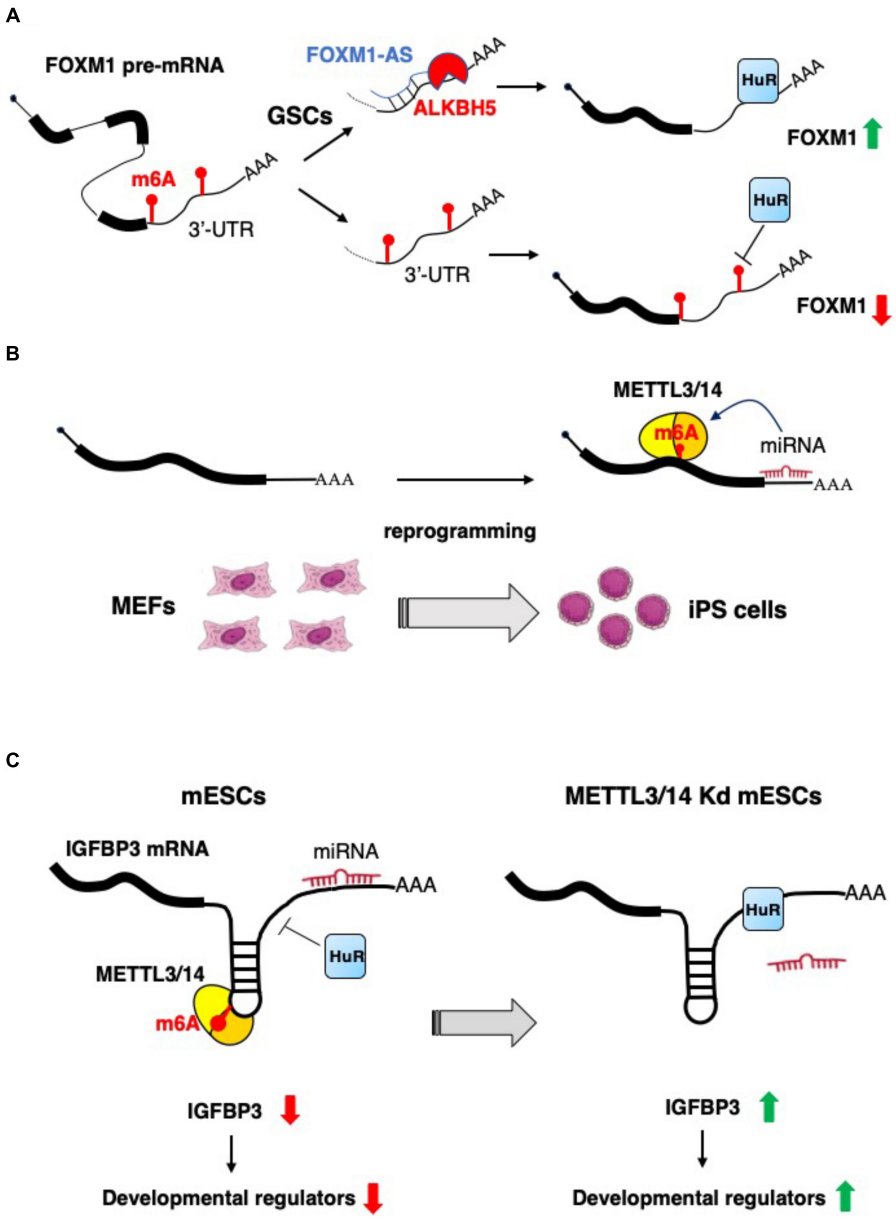

FIGURE 2 | Examples of the interplay between non-coding RNAs and epitranscriptomics. **(A)** In glioblastoma stem-like cells (GSCs) the expression of FOXM1 is increased by the concomitant expression of the antisense transcript FOXM1-AS, which, in turn, promote m^{6}A demethylation by recruiting ALKBH5 (Zhang et al., 2017). **(B)** m^{6}A RNA methylation is positively regulated by microRNAs, which recruit METTL3 on specific mRNA and promotes reprogramming to pluripotency (Chen et al., 2015). **(C)** m^{6}A modification decreases the IGFBP3 mRNA levels by inhibiting the binding of HuR and promoting the interaction with microRNAs. IGFBP3 protein positively regulates the stability of different developmental regulators. This mechanism ensures low level of IGFBP3 in mESCs (Wang Y. et al., 2014).

which do not allow m^{6}A mapping in specific RNA species (Yang et al., 2017). Interestingly, this increase is inhibited by YTHDF2 overexpression, supporting the central role of this protein in this regulation (Yang et al., 2017). Accordingly, miR-145 expression levels are negatively correlated with those of YTHDF2 in HCC tissues. Functionally, miR-145 is able to suppresses the proliferation of HCC cells through the modulation of m^{6}A-modified mRNA levels by targeting the 3′-UTR of YTHDF2 mRNA.

Another example is represented by miR-33a. In non-small-cell lung carcinoma (NSCLC) cells, it was recently described that miR-33a, by targeting the 3′-UTR of METTL3 mRNA, reduces the expression of METTL3 at both mRNA and protein levels and, eventually, global m^{6}A mRNA methylation, with a functional reduction of cellular proliferation and anchorage-independent growth (Du et al., 2017).

Recently, it has been reported that the oncogenic properties of glioblastoma stem cells (GSCs) in terms of proliferation, migration, and invasion could be influenced by a novel miR-29a/QKI-6/WTAP molecular axis (Xi et al., 2017). Of note, the overexpression of miR-29a inhibits WTAP expression and the activation of the ERK and PI3K/AKT pathways by downregulating QKI-6 expression and impairing the oncogenic abilities of GSCs (Xi et al., 2017). The impact of this regulatory network on m^{6}A levels have not been addressed in this study. However, considering the relevance of WTAP in regulating the methylation activity of the METTL3-METTL14 complex (Ping et al., 2014), it might be speculated that miR-29a, besides regulating DNA methylation during cell reprograming by targeting DNA-methyl-transferases (DNMTs) (Hysolli et al., 2016), could also have a crucial role in the modulation of RNA m^{6}A methylation during neoplastic transformation processes.

Lately, a novel role of miRNAs in the regulation of m^{6}A modification has emerged. Specifically, an enrichment of seed sequences for miRNAs has been observed in transcripts presenting m^{6}A residues using next generation sequencing (NGS) approaches (Chen et al., 2015). This study evaluated the contribution of miRNAs to the *ab initio* induction of m^{6}A methylation by depletion or overexpression of Dicer to modulate the overall miRNAs activity. In particular, Dicer expression favors the induction of m^{6}A methylation on target mRNAs, highlighting the tight connection between miRNAs activity and m^{6}A modification. Mechanistically, Dicer promotes the localization of METTL3 in nuclear speckles, enhancing its interaction with the transcript subjected to m^{6}A modification. In the same study, by using m^{6}A-Seq, the authors identified m^{6}A levels specific to different degrees of pluripotency by analyzing various experimental models, such as ESCs, induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), and testicular sertoli cells (SCs). They revealed the existence of both cell-common and cell-specific modified transcripts associated with biological processes such as stem cell maintenance and cell differentiation. Moreover, to explore the contribution of m^{6}A modification in cell reprograming, the authors overexpressed human METTL3 into mouse embryonic fibroblasts (MEFs), expressing the reprograming factors Oct4, Sox2, Klf4, and c-Myc, and evidenced that the reprograming efficiency of MEFs was significantly improved by the increase METTL3-dependent m^{6}A levels (Chen et al., 2015) (**Figure 2B**).

Of note, a role for m^{6}A as a signal for miRNA-dependent degradation of transcripts encoding developmental regulators in mESCs has also recently emerged. Indeed, during mESCs normal development, the METTL3/METTL14-dependent m^{6}A methylation of transcripts encoding developmental regulators blocks HuR protein binding and results in miRNAs-mediated transcript destabilization (Wang Y. et al., 2014). The loss of m^{6}A modification, in METTL3 and METTL14 knocked-down cells, allows for HuR-mRNA interaction and reduction of miRNA functional activity, improving transcripts stability and promoting loss of the mESC ground state (Wang Y. et al., 2014) (**Figure 2C**).

On the contrary, it has also been shown that the presence of m^{6}A modification may serve as a protective signal, which inhibits mRNA degradation through the binding of the IGF2BPs readers. In the case of SRF transcript, m^{6}A modification allows interaction with IGF2BP inhibiting miRNA-mediated decay in cancer cells (Müller et al., 2019). SRF induction is associated with tumor cell phenotype and poor prognosis (Muller et al., 2019). In addition, IGF2BP1 can also stabilize different oncogenic mRNAs by inhibiting general mRNA degradation, as reported for MYC mRNA (Huang et al., 2018).

CONCLUSION

We are witnessing an impressive increase in the number of studies elucidating the role of m^{6}A modification in cell development and cancer. However, the majority of these studies mainly focused on the impact of m^{6}A marks on coding RNAs, while an important contribution of ncRNA molecules is emerging, such as lncRNAs and microRNAs, on the function of the epitranscriptome. Moreover, lncRNA and miRNA function can be itself regulated by m^{6}A. Nevertheless, different questions still need to be answered. In particular, mapping of m^{6}A modification in mRNAs and lncRNAs showed a different distribution of m^{6}A marks within these two types of RNA pol II transcripts. However, it is still not known how this is achieved and, above all, if specific regulatory factors act differentially in controlling the m^{6}A methylases activity on mRNA and lncRNA molecules. Moreover, these studies have mainly used polyA$^+$ RNA for m^{6}A mapping, therein excluding many regulatory ncRNAs missing a polyA tail. Other important remaining questions in the field will be to determine if small ncRNAs such as miRNAs or the recently functionally characterized Y RNAs contain m^{6}A residues and if these are relevant for their regulatory function in normal and pathological conditions. Furthermore, another important issue concerns the impact of the interplay between m^{6}A modification and non-coding RNA molecules on cell fate determination and development, and which are relevant for these biological processes. In this review, we have described some examples, but the general impact of this emerging molecular network will be clarified after extensive further investigation.

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

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REVIEW
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Importance of Long Non-coding RNAs in the Development and Disease of Skeletal Muscle and Cardiovascular Lineages

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The early mammalian embryo is characterized by the presence of three germ layers-the outer ectoderm, middle mesoderm and inner endoderm. The mesoderm is organized into paraxial, intermediate and lateral plate mesoderm. The musculature, vasculature and heart of the adult body are the major derivatives of mesoderm. Tracing back the developmental process to generate these specialized tissues has sparked much interest in the field of regenerative medicine focusing on generating specialized tissues to treat patients with degenerative diseases. Several Long Non-Coding RNAs (lncRNAs) have been identified as regulators of development, proliferation and differentiation of various tissues of mesodermal origin. A better understanding of lncRNAs that can regulate the development of these tissues will open potential avenues for their therapeutic utility and enhance our knowledge about disease progression and development. In this review, we aim to summarize the functions and mechanisms of lncRNAs regulating the early mesoderm differentiation, development and homeostasis of skeletal muscle and cardiovascular system with an emphasis on their therapeutic potential.

Keywords: non-coding RNA, skeletal muscle, endothelial cell, vascular smooth muscle cell (VSMC), differentiation, mesoderm, myogenesis, cardiovascular diseases

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INTRODUCTION

Gastrulation results in the formation of the three germ layers - ectoderm, mesoderm and endoderm. The mesoderm is a middle layer between the innermost, endoderm and the outer ectoderm. The transition from epithelial cells to mesenchymal cells marks the formation of mesoderm which is further organized into the paraxial, intermediate and lateral mesoderm (Nakaya and Sheng, 2008; Evseenko et al., 2010). The three parts of the mesoderm are acted upon by several lineage commitment programs and differentiate into the progenitor cells that give rise to musculoskeletal, urogenital and cardiovascular structures of the body (Doss et al., 2012). Cells of these organs have the same genome, but the differences in transcriptionally active and inactive regions of genome guide the precursor cells toward different cell fates (Iwafuchi-Doi and Zaret, 2016). The differences in genomic organization, followed by activation or silencing of genes, are the result of complex gene regulatory networks (GRNs) (Materna and Davidson, 2007). For many years these GRNs were thought to be controlled exclusively by protein coding genes until the discovery

of functional non-coding RNA transcripts (ncRNAs) which form an integrated network to shape the cellular environment during different developmental and metabolic processes (Kim and Sung, 2012). These ncRNAs are divided into two categories based on the transcript length—small ncRNAs (<200 nucleotides) and long ncRNAs (>200 nucleotides) (Mercer et al., 2009). Currently, miRNAs are the best-characterized ncRNAs that are well conserved and repress the expression of target mRNA by binding to its 3′ UTR (Majoros and Ohler, 2007). On the other hand, long ncRNAs (lncRNAs) constitute a less characterized but highly diverse class of ncRNAs. lncRNAs are structurally similar to protein-coding genes as most of them are transcribed by RNA polymerase II, 5′ capped and polyadenylated at 3′ end (Bunch et al., 2016). Regardless of their close similarity to the protein-coding mRNAs, lncRNAs lack the potential to code functional proteins. Although there are many lncRNAs that contain putative open reading frames and indeed some have been re-classified to protein-coding genes (Anderson et al., 2015; Nelson et al., 2016; Matsumoto et al., 2017). The number of *bona-fide* lncRNAs identified in human genome is, in general, comparable to that of protein-coding genes, but only a few have been functionally characterized. Functionally, lncRNAs can either act in *cis* by regulating expression of neighboring genes, or in *trans*, regulating the expression of distant genes (Ulitsky and Bartel, 2013). lncRNAs regulate the gene expression by mending the 3-dimentional genome organization, mediating the binding of chromatin modifying proteins or by sequestering the bound regulatory factors or miRNAs by acting as molecular decoys or sponge (Morriss and Cooper, 2017). A summary of different mechanisms of lncRNA mediated genome regulation is represented in **Figure 1**. A further class of lncRNAs emerging from regulatory regions of the genome such as enhancers can initiate chromatin looping by recruiting chromatin modifying factors to activate or repress transcription at distant genomic location (Wang et al., 2011). In the past decade several research groups have speculated on the functions of lncRNAs in different biological and pathological systems. More specifically, many lncRNAs have been reported to play crucial roles in the development of skeletal muscle and cardiac lineages, and connected diseases. Here, we discuss the current understanding of the roles of lncRNAs in skeletal muscle and cardiac derivatives emphasizing on their therapeutic potential in the associated pathological conditions.

lncRNAs DURING EARLY MESODERMAL SPECIFICATION

Recent studies suggest that lncRNAs are important for mesodermal specification and further differentiation, development and function of mesodermal derivatives (**Figure 2**). For instance, antisense RNA *Evx1as* regulates mesendodermal differentiation toward the mesoderm/posterior streak fate through *cis* regulation of Even-skipped homeobox 1 (Evx1) (Luo et al., 2016). Evx1 is a homeodomain TF that promotes mesoderm differentiation by inhibiting the endoderm/anterior streak gene *GSC* (Kalisz et al., 2012). The expression of the divergent lncRNA *Evx1as* was highly correlated with the expression of *Evx1*. Interestingly, *Evx1as* knockdown exhibited a higher impact on the expression of mesendoderm markers than the knockdown of Evx1, suggesting the possible trans regulation by *Evx1as* independent of Evx1 (Luo et al., 2016). lncRNA *HoxBlinc* is involved in early differentiation and is transcribed from the Homeobox B (Hoxb) locus marking the formation of Flk+ mesoderm with the potential to form hematopoietic and cardiac cells (Deng et al., 2016). As in the case of *Evx1as* and *HoxBlinc*,

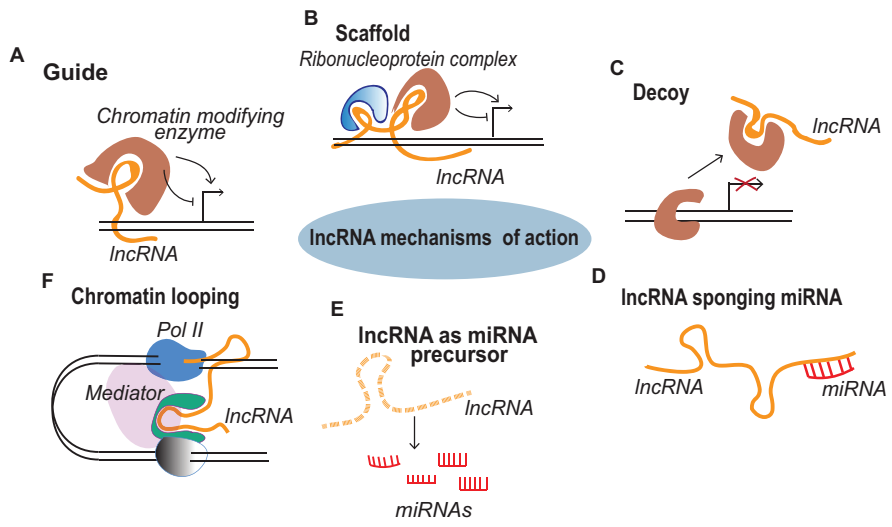

FIGURE 1 | lncRNA mechanisms of action. **(A)** Guide lncRNAs activate or repress gene expression through relocalization of regulatory factors. **(B)** Scaffold lncRNAs aid in the formation of Ribonucleoprotein (RNP) complexes. **(C)** Decoy lncRNAs remove the regulatory factor bound to the genome thereby terminating its regulation. **(D)** lncRNAs sponge the miRNAs, thus inhibiting the miRNA mediated gene repression. **(E)** miRNA precursor lncRNAs function as primary miRNA precursors that are processed into mature miRNAs. **(F)** lncRNA transcription from regulatory regions of the genome initiate long range gene regulation.

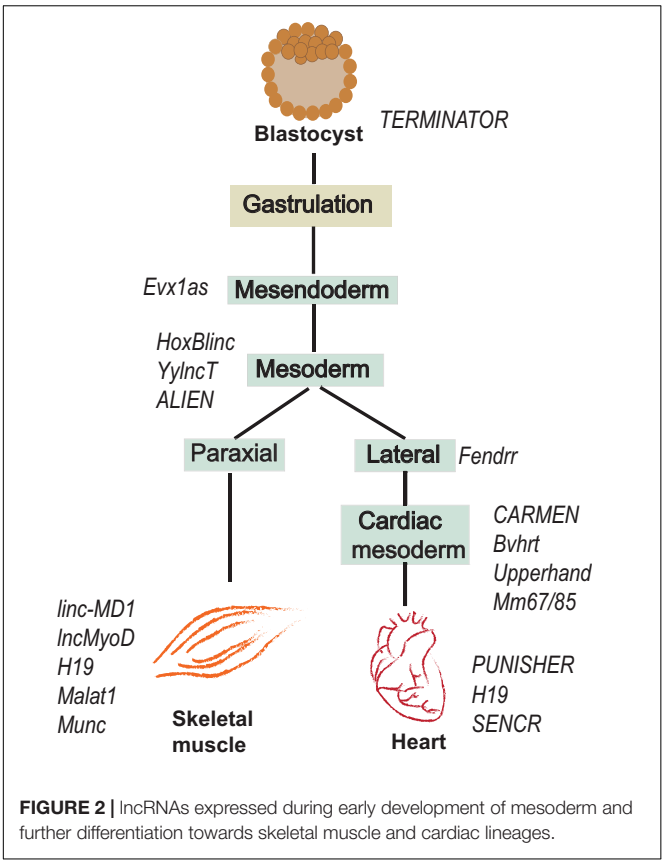

FIGURE 2 | lncRNAs expressed during early development of mesoderm and further differentiation towards skeletal muscle and cardiac lineages.

genomic loci of many other key developmental regulators were found to transcribe divergent lncRNAs, collectively termed as Ying Yang lncRNAs (yylncRNAs). Ying Yang lncRNAs follow tissue-specific expression patterns similar to that of their protein-coding counterparts (Frank et al., 2019). The active locus of the mesoderm specifier BRACHUARY (T) encodes *yylncT* and the expression patterns of the two were nearly identical during mesodermal commitment. The depletion of *yylncT* specifically abolished the differentiation of human embryonic stem cells (hESCs) to mesoderm without affecting the differentiation toward ectoderm and endoderm, emphasizing the mesoderm specific role of *yylncT* (Frank et al., 2019). A summary of lncRNAs regulating early mesodermal differentiation is illustrated in **Figure 2**.

lncRNAs REGULATING MYOBLAST PROLIFERATION AND MUSCLE DEVELOPMENT

During embryonic development, the paraxial mesoderm develops into segmented somites, organized into ventral sclerotome and dorsal dermomyotome which form the axial skeleton, the skeletal muscle and the dermis of the adult body, respectively (Chal and Pourquié, 2017). Myogenic progenitor cells (MPCs) are formed in the myotome and subsequently become myoblasts that proliferate and differentiate to form myotubes maturing into skeletal muscle fibers. The MPCs are also responsible for the formation of quiescent satellite cells that contribute to regeneration in adult muscles (Chal and Pourquié, 2017). Upon injury, satellite cells in the adult body get activated and become proliferative myoblasts further differentiating to form new muscle fibers. Alterations in regulatory circuitry of myogenesis leads to muscle disorders and diseases such as, hypertrophy and atrophy. This makes it advantageous to identify novel molecular regulators of myogenesis and injury induced regeneration which will aid to discern new therapeutic targets.

Before differentiating into mature myofibers, the myoblasts proliferate with the activation of cell cycle genes. lncRNA *Sirt1AS* is an antisense RNA that promotes myoblast proliferation by protecting Sirt1 mRNA—a suppressor of cell cycle inhibitors—from miR-34a mediated degradation (Wang G.-Q. et al., 2016). *lnc-31* also promotes proliferation by maintaining the expression of critical cell cycle genes, cyclin D1 (Ccnd1), cyclin E (Ccne1) and Cdc25a (Ballarino et al., 2015). *lnc-31* harbors miR-31 precursor sequence, but works independently of miR-31. Despite the poor sequence conservation, both *lnc-31* and its human homologue, *has-lnc-31*, are upregulated during proliferation antagonizing the differentiation process. Furthermore, *lnc-31* and *has-lnc-31* are abundantly expressed in Duchenne muscular dystrophy (DMD) in mice and humans (Ballarino et al., 2015).

Recently, lncRNA *Syisl* was reported to supress myoblast differentiation, promoting cell proliferation and fusion. *Syisl* escorts the EZH2 of PRC2 to the promoter of cell cycle inhibitor p21 and core myogenic genes like MyoG, Mck, Myh4 (Jin et al., 2018). The same study reported increase in muscle density upon *in vivo* knockout of *Syisl*. lncRNA *Syisl* presents an example of lncRNAs activated in a stage specific manner in regulation of early myogenesis. A schematic representation of lncRNAs regulating the early and late phases of myogenesis with their downstream and upstream effector targets is provided in **Figure 3**.

lncRNAs REGULATING MYOBLAST DIFFERENTIATION

Myoblast differentiation leads to the formation of multinucleate myotubes that form the mature myofiber (Chal and Pourquié, 2017). A number of stage specific factors, such as MyoD, Myf5, MyoG, and MRF4 act in coordination with epigenetic and transcriptional regulators to regulate the myoblast differentiation (Braun and Gautel, 2011). Enhancer regions of MyoD and MyoG were shown to give rise to eRNAs (enhancer RNAs) which in turn regulate their expression. Two such examples are core enhancer eRNA (^{CE}*eRNA)* and *MUNC* – both of which are transcribed from the upstream regulatory region of MyoD (Mousavi et al., 2013). ^{CE}*eRNA* is transcribed from the core enhancer of MyoD regulating its expression in *cis* by facilitating the chromatin accessibility to RNA polymerase ll. Whereas, *MUNC* (also called as ^{DRR}*RNA*) is transcribed from Distal Regulatory Region (DRR) of MyoD, enhances the expression of MyoD in *cis* and of distantly located myogenic genes, MyoG and Myh3, in *trans* (Mueller et al., 2015). Albeit *MUNC* overlaps the DRR of MyoD, *MUNC* acts

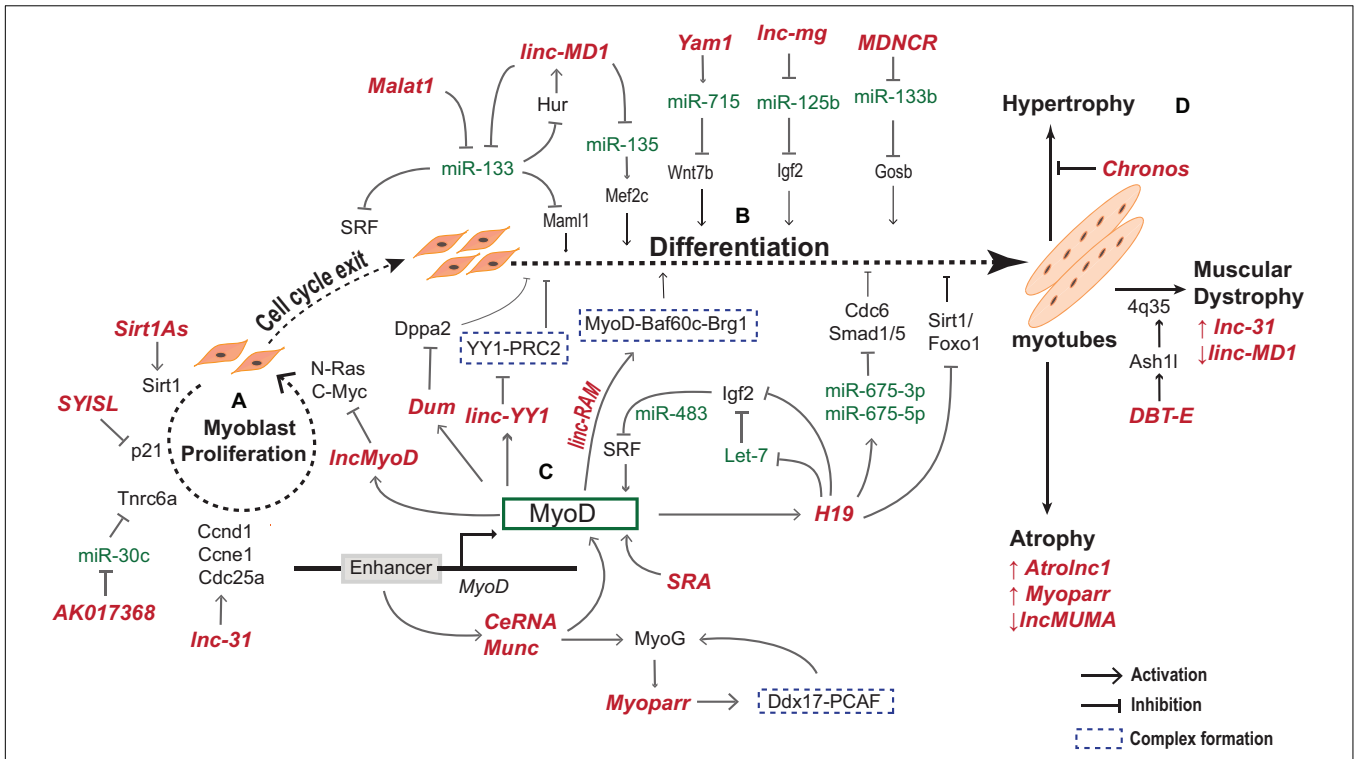

FIGURE 3 | lncRNAs regulate myogenesis at different stages. During myogenesis, myoblast cells proliferate and differentiate into myocytes that fuse together to form multinucleate myotubes. The proliferative and differentiation stages of myogenesis are regulated by several lncRNAs. **(A)** lncRNAs regulating the myoblast proliferation by activating downstream cell cycle genes. **(B)** lncRNAs expression and function during cell cycle exit and differentiation to form myotubes. **(C)** MyoD activated lncRNAs regulate other myogenic factors including MyoD and MyoG to form a complex MyoD–lncRNA–miRNA–mRNA regulatory network during differentiation of myoblasts. **(D)** Examples of lncRNA expressed and regulating various skeletal muscle disorders.

on multiple myogenic gene promoters. Similar to eRNAs, the promoter region of mouse and human MyoG transcribes lncRNA *Myoparr* essential for cell cycle withdrawal by activating multiple myogenic factors including the neighboring TF MyoG (Hitachi et al., 2019). *Myoparr* regulates the interaction between MyoD coactivator Ddx17 and histone acetyl transferase and promotes denervation caused atrophy (Hitachi et al., 2019). The discovery of eRNAs and promoter associated RNAs highlight an additional role of regulatory regions of genome, such as enhancers, in genome regulation.

lncRNA and mRNA microarray analysis identified 997 differentially expressed lncRNAs upon MyoD knockdown in C2C12 cells (Guo et al., 2017). Gene ontology predicted that most of these lncRNAs are associated with pathways involved in muscle formation and cell cycle regulation. The study also identified *lncRNA-AK143003* to be significantly regulated by MyoD. In silencing and overexpression experiments *AK143003* acts as differentiation antagonist, but further investigation is required to assess its role and mechanism during myogenesis. This study provides a repertoire of lncRNAs in MyoD network for further validation. Another lncRNA involving MyoD in the regulatory circuitry is *linc-RAM* (Linc-RNA Activator of Myogenesis). It binds to MyoD and facilitates the assemblage of MyoD-Baf60c–Brg1 complex onto the regulatory regions of myogenic genes (Yu et al., 2017). In addition, lncRNA *SRA* acts as a coactivator for master regulator of muscle differentiation, MyoD and *SRA* Knockdown prevented proper expression of muscle genes and differentiation (Caretti et al., 2006). Taken together, these lncRNAs present a second layer of regulation in the MyoD regulatory network. **Figure 3C**, depicts an overview of MyoD regulated lncRNAs and in turn the lncRNAs modulating MyoD and other myogenic genes.

lncRNAs are known to act as guides and scaffolds by recruiting chromatin or DNA modifying complexes. *DUM* silences Dppa2, an anti-myogenic regulator, by recruiting Dnmts to CpG sites of Dppa2 promoter (Wang L. et al., 2015). *Dum* acts as a promyogenic factor transcriptionally induced by MyoD with highest expression during proliferation and early myogenesis. Ectopic overexpression of *DUM* improved regeneration of muscle mass (Wang L. et al., 2015). *lncMyoD* is another lncRNA activated by MyoD in terminal muscle differentiation suppressing the IGF2-mRNA-binding protein 2 (IMP2) mediated translation of proliferation genes (Gong et al., 2015).

The maternally imprinted lncRNA *H19* is expressed exclusively in embryonic tissues and strongly repressed after birth in all tissue types but skeletal muscles (Brannan et al., 1990; Poirier et al., 1991). *H19* knockdown decreased the differentiation of myoblast cells and mouse satellite cells by

derepressing the mRNAs targets of miR-675-3p and miR-675-5p, encoded by *H19*, emphasizing a crucial role of *H19* during skeletal muscle differentiation (Dey et al., 2014). During myoblast differentiation, miR-675-3p downregulated the BMP pathway by targeting anti-differentiation smad1 and smad5, whereas miR-675-5p repressed the DNA replication initiation factor Cdc6 (Dey et al., 2014). Another study suggests that *H19* decrease the expression of myoblast inhibitory genes Sirt1/FoxO1, thus reinforcing the role of *H19* in muscle differentiation (Xu et al., 2017). In addition, *H19* has been shown to act as a molecular sponge for microRNAs belonging to let-7 family, preventing premature myoblast differentiation (Kallen et al., 2013). More recently, a study of double mutant mice lacking MyoD and Igf2 genes elucidates a complex loop of H19 mediated Igf2 repression by MyoD in development of diaphragm muscle (Borensztein et al., 2013). The authors demonstrate that MyoD stabilized the interaction of CS9 mesodermal enhancer with *H19* promoter that accounts for increased expression of *H19* in the presence of MyoD. Furthermore, *H19* represses Igf2 expression in trans (Wilkin et al., 2000). MyoD is in turn negatively regulated due to downregulation of SRF by Igf2 encoded miR-483. Conclusively, H19-Igf2-MyoD are tightly regulated in a negative feedback loop during embryonic myogenesis (Qiao et al., 2011; Borensztein et al., 2013). Overall, it appears that *H19* has a crucial role in myoblast differentiation by mechanistically regulating the key genes such as IGF-2, Sirt1/FoxO1, and microRNAs miR-675 and let-7 during adult as well as embryonic myogenesis.

Similar to *H19*, lncRNA *MALAT1* targets multiple factors during myogenesis. Malat1 is incessantly upregulated during the differentiation of myoblasts to myotubes and its downregulation results in cell cycle arrest in G0/G1 phase, suppressing myoblast proliferation (Watts et al., 2013). A recent study demonstrated a new mechanism of microRNA mediated degradation of *MALAT1* transcripts in myoblast nucleus by miR-181 through Ago2-dependent nuclear RNA-induced silencing complex (RISC) (Chen et al., 2017). *MALAT1* was also found to influence miR-133 mediated SRF targeting during myogenesis (Han et al., 2015). Conclusively, these mechanistic studies provide evidence that a single lncRNA can act at different levels in GRN through multiple of modes of action.

As seen in *H19* and *MALAT1*, sponging of miRNA appears to be a common mechanism of lncRNA action in muscle differentiation. For instance, *lnc-mg* (myogenesis associated lncRNA) promoted Igf2 mediated myogenesis by titrating miR-125b (Zhu et al., 2017). *lnc-mg* was enriched in skeletal muscle and its silencing caused muscle atrophy and loss of muscular endurance during exercise and its overexpression led to hypertrophy. Furthermore, its expression was dynamically induced during differentiation of muscle stem cells (Zhu et al., 2017). Similarly, *linc-MD1* modulates the time of muscle differentiation by favoring the expression of MAML1 and MEF2C by sponging miR-133 and miR-135, respectively (Cesana et al., 2011). In addition to sponging of miR-133, *linc-MD1* is also the host of miR-133 enabling its alternate synthesize from *linc-MD1*, controlled by a feedforward positive loop of HuR protein and *linc-MD1* (Legnini et al., 2014). *linc-MD1* sponges miR-133b derepressing the expression of HuR protein which in turn physically interacts with *linc-MD1* to prevent Drosha cleavage of pri-miR-133 sequence (Legnini et al., 2014).

TF Yin Yang 1 (YY1) is an important regulator of myogenesis that silences multiple genes in myoblasts by recruiting Ezh2 (Enhancer of ZesteHomologue2) (Caretti et al., 2004; Wang et al., 2007). Promoter region of YY1 gives rise to *linc-YY1* exhibiting low expression in proliferating myoblasts, increased at the beginning of the myogenic program with gradual decrease in the late stages of myogenesis (Zhou et al., 2015). Interestingly, YY1 follows a similar expression profile during the process. When myoblasts undergo differentiation, *linc-YY1* is activated by MyoD, which then destabilizes the YY1/PRC2 complex activating pro-differentiation genes (Zhou et al., 2015). Knockdown of *linc-YY1* delayed the expression of many myogenic markers which are direct targets of YY1/PRC2, such as MyoG, MyHC, Tnni2 and a-Actin, and miR-1 and miR-29. This indicates that *linc-YY1* is a pro-myogenic factor whose knockdown in C2C12 cells delayed myogenic differentiation. Genome wide ChIP-Seq in myoblasts revealed that YY1 regulates several lincRNAs in skeletal muscle collectively called as *Yams* (YY1-associated muscle lincRNAs) (Lu et al., 2013). *Yam1* is a lncRNA regulated by YY1 and acts as an anti-myogenic factor and exerts its function in cis through regulation of miR-715 and via targeting Wnt7b expression (Lu et al., 2013). Downregulation of Wnt7b inhibits myogenic differentiation (von Maltzahn et al., 2012). Among other Yams, *Yam-3* also inhibits differentiation while, *Yam-2* and *Yam-4* facilitates early differentiation (Lu et al., 2013). These observations indicate that lncRNAs are not only regulated by key TFs but in turn regulate the function of other TFs. A list of lncRNAs regulating myogenesis is represented in **Table 1**.

THERAPEUTIC POTENTIAL OF lncRNAs IN MUSCLE REGENERATION AND DISEASES

Given the known importance of lncRNAs in skeletal muscle myogenesis, it is not surprising to know that they also regulate the process of muscle regeneration. lncRNAs such as *H19*, *DUM*, *MUNC*, *Yam1* and *lncMyoD* have been shown to regulate regeneration in cardiotoxin (CTX) model of muscle injury (Shiekhattar, 2013; Dey et al., 2014; Gong et al., 2015; Mueller et al., 2015; Wang L. et al., 2015). Upon CTX mediated injury, the expression of *H19* was decreased at days 1–3, followed by increase at days 5–7 and again decreased after the formation of new myofibers at day 14. Interestingly, miR-675-3p and miR-675-5p are co-expressed with *H19* throughout the course of regeneration, making it evident that the pro-differentiation action of *H19* is mediated by microRNAs generated from it (Dey et al., 2014). While *H19* is implicated in regulating regeneration at several stages, expression of *Yam1* appears to be stage specific with highest expression at day 2 followed by a sharp reduction for the rest of the regeneration process (Lu et al., 2013). Likewise, *lncMyoD* is upregulated at days 3–5 and decreased for the remaining regeneration process (Gong et al., 2015). In contrast, *MUNC* regulates late stage muscle regeneration as its knockdown decreased the average diameter of myofiber at day 14 (Gong

TABLE 1 | List of lncRNAs regulating skeletal muscle myogenesis and their regulatory mechanisms.

lncRNA	Target	Mechanism	Function/disease relevance	Species	References
AK143003	Unknown	Unknown	Negative regulation of differentiation	Mouse	Guo et al., 2017
AK017368	Tnrc6a	Sponge miR-30c	Promotes myoblast proliferation	Mouse	Liang et al., 2018
Atrolnc1	NF-kb Murf-1	Decoy	Promotes muscle wasting in CKD mice	Mouse	Sun et al., 2018
Charme	Unknown	Unknown	Regulates robustness of skeletal and cardiac myogenesis. *In vivo* depletion in mice resulted in cardiomyopathy	Mouse	Ballarino et al., 2018
Chronos	Bmp7	Unknown	Repressor of skeletal muscle hypertrophy	Mouse	Neppl et al., 2017
^{CE}RNA	MyoD	eRNA, regulate pol II occupancy at MyoD	Promotes myogenesis	Mouse	Mousavi et al., 2013
DRR/MUNC	MyoD, MyoG, Myh3	*Cis* and Trans regulation, Pol II recruitment at myogenic promoters	Promotes myogenesis And late stage regeneration	Mouse, Human	Mousavi et al., 2013; Mueller et al., 2015
DBT-E	Ash1l	Guide chromatin remodeling at D4Z4 locus	Expressed in FSHD patients	Human	Cabianca et al., 2012
Dum	Dppa2	*Cis*, recruits Dnmt 1, 3a and 3b at Dppa2 promoter	Promotes differentiation and regeneration	Mouse	Wang L. et al., 2015
H19	Let-7, Igf2, MyoD miR-675, Sirt1/Foxo1	Cis and trans miRNA Sponge, Precursor of miRNA	Promotes myogenic differentiation	Mouse, Human and Cattle	Wilkin et al., 2000; Borensztein et al., 2013; Kallen et al., 2013; Dey et al., 2014; Xu et al., 2017
lnc-31	Cyclins, Cdc25a	*Trans* acting	Maintenance of myoblast proliferation, Upregulated in mdx mice and DMD patient myoblast	Mouse, Human	Ballarino et al., 2015
linc-MD1	miR-133, miR-135, Hur protein	*Cis*, miRNA sponge	Controls time of muscle differentiation, Reduced in DMD patients	Mouse, Human	Cesana et al., 2011
lnc-mg	miR-125b	miRNA sponge	Promotes myogenesis, Knockout and overexpression resulted in atrophy and hypertrophy in mice, respectively	Mouse	Zhu et al., 2017
lncMyoD	IMP2 mediated mRNA translation	Decoy	Terminal muscle differentiation	Mouse, Human	Gong et al., 2015
lncMUMA	miR-762	miRNA sponge	Promotes differentiation, protects against atrophy	Mouse, Human	Zhang et al., 2018
linc-RAM	Myogenic genes	Scaffold, assembly of MyoD-Baf60c-Brg1complex	Promotes differentiation, Impaired muscle regeneration in vivo knockout mice	Mouse	Yu et al., 2017
linc-YY1	PRC2	Trans, Decoy	Promotes differentiation, impaired regeneration upon knockdown	Mouse, Human	Zhou et al., 2015
Malat1	miR-133, Myogenic genes	miRNA sponge, Guide Suv39h1 to myoD binding loci	Promotes myogenesis, Improved regeneration in knockout mice	Mouse, Human	Han et al., 2015; Chen et al., 2017
MDNCR	miR-133b	miRNA sponge	Promote differentiation	Cattle	Li H. et al., 2018
Myoparr	MyoG	Scaffold	Promotes differentiation and muscle	Mouse, Human	Hitachi et al., 2019
Sirt1AS	Sirt1	*Cis*-acting, protects Sirt1 mRNA from miR-34a degradation	Promotes myoblast proliferation	Mouse	Wang G.-Q. et al., 2016
SRA	MyoD	Unknown	Promotes muscle differentiation	Mouse	Caretti et al., 2006
SYISL	P21, myoG, Mck	Guides EZH2 to promoter of target genes	Promotes myoblast proliferation and fusion	Mouse	Jin et al., 2018
Yam 1	Wnt7b	*Cis*-acting, Activates miR-715	Inhibits differentiation	Mouse	Lu et al., 2013

et al., 2015; Mueller et al., 2015). Among others, the knockout of *linc-RAM* and lncRNA *IRM* in mice displayed impaired muscle regeneration, while the knockdown of *MALAT1* enhanced the regeneration (Chen et al., 2017; Yu et al., 2017; Sui et al., 2019). Paradoxically, lncRNA *LINC00961* encodes a conserved polypeptide, SPAR, downregulated during the regeneration process (Matsumoto et al., 2017). The downregulation of SPAR activates mTORC1 important for skeletal muscle regeneration and hypertrophy. The functional importance of SPAR emphasizes that not all lncRNA-encoded peptides are translational noise and also raises concern regarding the classification of lncRNAs.

Impaired regeneration process leads to conditions like atrophy and hypertrophy characterized by muscle wasting and increase in size of muscle cells, respectively. Cachexia is a condition that involves atrophy and muscle wasting, commonly associated with chronic kidney disease (CKD) (Morley et al., 2006). lncRNA *Atrolnc-1* is elevated in atrophic muscles of mice with cachexia and its inhibition in mice with CKD attenuated muscle wasting (Sun et al., 2018). Targeting *Atrolnc-1* could possibly help ameliorate the severity of CKD. *lncMUMA* was also regulated during atrophy with minimum expression during atrophy development in hindlimb suspension (HLS) mouse model (Zhang et al., 2018). A study on the role of lncRNA in age-associated atrophy identified lncRNA *Chronos* as a repressor of hypertrophic growth through negative regulation of BMP7 (Neppl et al., 2017). The dysregulation of lncRNAs during atrophy and hypertrophy further strengthens their importance in development of muscle fibers.

Similar to atrophy and hypertrophy, lncRNA expression is dysregulated in muscular dystrophy. DMD is the most prevalent type of dystrophy caused by lack of functional dystrophin protein that connects muscle fibers to extracellular matrix (Hoffman et al., 1987). A study using tilling array designed for dystrophin locus identified novel lncRNAs with expression profiles similar to those of dystrophin gene (Bovolenta et al., 2012). These lncRNAs have a repressive role on the full-length dystrophin isoform and their expression is inversely correlated with dystrophin long isoform in the muscle of female dystrophinopathy carriers. Among other lncRNAs, *lnc-31* is upregulated in mdx mice muscle and human DMD patients (Ballarino et al., 2015). On the contrary, *linc-MD1* was reduced in DMD patients (Cesana et al., 2011). Facioscapulohumeral muscular dystrophy (FSHD) is another type of dystrophy characterized by wasting of facial, upper arm and shoulder girdle muscle. In 95% of FSHD cases the defect is a deletion in D4Z4 microsatellite repeat, leading to loss of repressive mark (Wijmenga et al., 1990, 1992). This de-repression is coordinated by a chromatin associated lncRNA *DBT-E* transcribed from D4Z4 repeat through the recruitment of Trithorax group protein at FSHD locus (Cabianca et al., 2012). Although, a few lncRNAs have been reported and studied in muscular dystrophies, they haven't been studied in non-dystrophic muscle diseases.

Given that lncRNAs are dysregulated in various muscular diseases, they can possibly be novel biomarkers or targets for therapeutic interventions. However, studies investigating lncRNAs in patients with muscular diseases are much languished. Genome wide association studies on different cohorts of muscular disorders may help identify lncRNA loci closely associated with genetic disorders. lncRNAs regulating myogenesis has been well explored in *in vitro* and *in vivo* mouse models, nonetheless their role in humans needs to be investigated in depth.

lncRNAs IN CARDIOVASCULAR DEVELOPMENT, PROLIFERATION AND DIFFERENTIATION

The heart is the first organ to be formed during mammalian embryogenesis. It consists of a multitude of cell types that are formed through complex lineage commitment programs acting upon lateral plate and paraxial mesoderm (Bruneau, 2013; Stone and Stainier, 2019). The intricate network of signaling pathways and the core transcriptional networks in cardiovascular biology have been extensively investigated for many years. On par with the skeletal muscle development and function, lncRNA discovery has unraveled a new layer of regulation in cardiac biology. A number of studies have identified several lncRNAs crucial for cardiac commitment, differentiation and dysfunction leading to diseased conditions. Many groups have discovered new platforms to identify and catalog lncRNAs that regulate cardiac commitment and pathologies (Grote et al., 2013; Kumarswamy et al., 2014; Ounzain et al., 2014; Viereck et al., 2016). Furthermore, the involvement of lncRNAs as therapeutic targets for cardiovascular diseases (CVDs) is beginning to be understood. In this section, we discuss the role of lncRNAs that specifies and regulates the function of cardiomyocytes, smooth muscle cells and endothelial cells and their roles in CVDs and therapeutics.

lncRNAs REGULATING EARLY CARDIAC FATE AND DISEASE

The minimal regenerative capacity of cardiomyocytes makes it difficult to overcome the damage caused by cardiac diseases. Hence, novel strategies are needed that can improve the regenerative potential of the damaged myocardium. lncRNAs have emerged as novel modulators in cardiac development and regeneration in recent years. *Braveheart (Bvht)* is the first lncRNA identified in mouse cardiac commitment and its depletion in differentiating mouse embryonic stem cells (mESCs) reduced the potential to form cardiac tissue (Klattenhoff et al., 2013). More specifically, *Bvht* functions upstream of Mesp1, a master regulator of cardiac differentiation and depletion of *Bvht* decreased the expression of early cardiac cell surface markers (such as PdgfRa and Flk-1) with consistent increase in hematopoietic markers, suggesting its involvement in regulating cell fate decisions (Klattenhoff et al., 2013). However, the role of *Bvht* in hematopoietic differentiation is unclear and needs further investigation. *Bvht* lacks a human ortholog and this loss in the due course of evolution represents the species-specific differences in heart development.

lncRNA *Fendrr* with its expression restricted to the nascent lateral plate mesoderm, regulates cardiac differentiation by interacting with the PRC complex (Grote et al., 2013). *Fendrr* is imperative for the development of the heart and the body wall. Mechanistically, *Fendrr* binds to two major chromatin modifiers, PRC2 and TrxG/MLL, and recruits these complexes to the promoters of genes involved in inception and differentiation of the lateral plate mesoderm, thus regulating cardiac lineage commitment (Grote et al., 2013).

CARMEN, (CAR)diac (M)esoderm (E)nhancer-associated (N)oncoding RNA) is a super enhancer (SE)-associated lncRNA identified in the transcriptome of cardiac precursor cells (CPCs) obtained from the human fetal heart (Ounzain et al., 2014, 2015). The *CARMEN* locus is upstream of two microRNAs known to direct differentiation toward SMC—MiR-143 and -145. *CARMEN* is a conserved lncRNA and shelters a highly active, notch-repressive, SRF/NKX2.5 bound cardiac enhancer, responsible for restricted expression of miR-143 and miR-145 during cardiac development. Notably, the mouse ortholog of *Carmen* and previously mentioned lncRNA *Bvht* are co-located in the mouse genome. Both of the lncRNAs showed maximum expression between cardiac mesoderm and cardiac precursor stages during induced cardiac differentiation of P19CL6 cells, indicative of their involvement in early cardiac specification. While *Bvht* works in *trans*, *CARMEN* functions in *cis* as well as in *trans* and both were found to be essential for maintaining the cardiac identity in neonatal cardiomyocytes (Klattenhoff et al., 2013; Ounzain et al., 2015). Thus, *CARMEN* represents a SE-associated lncRNA that can potentially be manipulated for initiating neocardiogenesis for treating a heart damage.

Global transcriptomic profiling of enhancer transcribed lncRNAs during ESC differentiation into cardiomyocytes in mouse and human reveal co-expression of many of lncRNAs and their predicted downstream targets (Ounzain et al., 2014). The human enhancer transcripts of mm-67, -85, and -130 were significantly upregulated at different time points of cardiac differentiation. More specifically, knockdown of mm-85 derived lncRNA in P19CL6 mouse embryonic carcinoma cells significantly decreased myocardin expression and upregulated mm-67 present within the myocardin gene (Ounzain et al., 2014). This shows the crucial role of mm-85 in regulating myocardin expression in mouse P19CL6 embryonic carcinoma cells.

The growing body of experimental evidence suggests that enhancer derived lncRNAs are important for expression of proximal target genes in cardiac development. lncRNA *Uph* (also named *Upperhand* or *Hand2os1*) is transcribed from upstream enhancer of HAND2, a regulator of heart development and reprogramming of fibroblast to cardiomyocytes (Anderson et al., 2016). Knockout of *Uph* in mouse embryos resulted in the inability of the embryo to develop a right ventricular chamber and the cardiac phenotype emulated by HAND2 knockout embryos (Anderson et al., 2016). More recently, deletion of *Uph* in the mouse upregulated the expression of Hand2 accompanied by the dysregulated cardiac gene program, congenital heart defects and prenatal lethality (Han et al., 2019). While *Uph* is transcribed upstream, lncRNA *Handsdown* (*Hdn*) is located downstream of *Hand2* (Ritter et al., 2019). The genetic analysis in mice demonstrated that *Hdn* is haploinsufficient and *Hdn*-heterozygous mice presented right ventricular hyperplasia with increased levels of *Hand2* (Ritter et al., 2019). Thus, *Uph* and *Hdn* regulate *Hand2* expression in cis thereby playing a crucial role in cardiac development.

A study in human ESCs and zebrafish developmental models identified three lncRNAs implicated at different stages of mesoderm and cardiovascular development, namely, *TERMINATOR*, *PUNISHER* and *ALIEN* (Kurian et al., 2015). These three lncRNAs are conserved from zebrafish to humans and manifest similar stage-specific expression. *TERMINATOR* is essential for early embryonic survival, pluripotency and early mesendodermal differentiation. *TERMINATOR* knockdown in zebrafish resulted in >70% lethal embryos, and developmental arrest and severe cardiovascular defects in the surviving embryos (Kurian et al., 2015). Mesodermal specification is marked by the expression of *ALIEN*. Loss of *ALIEN* resulted in mesoderm-related defects including defective vascular patterning and cardiac chamber formation, alluding toward specific role of *ALIEN* in the early developmental of the progenitor stage common to vascular and cardiomyocyte fates. Silencing of *PUNISHER* was also accompanied by severe impairments in vasculature and cardiac development and function (Kurian et al., 2015). These results were extrapolated to mouse embryos and human ESCs by knockdown of the three lncRNAs followed by microarray hybridization to check for the differential expression of different genes at different stages upon knockdown of these individual lncRNAs (Kurian et al., 2015). Albeit the mechanisms employed by these lncRNAs in controlling the developmental processes remains elusive.

lncRNA *H19* besides its role in early embryonic development, is also critical for late stage cardiac differentiation. During the late stage cardiac differentiation of P19CL6 mouse cells, *H19* knockdown promoted cell proliferation and inhibited apoptosis (Han et al., 2016). *H19* curbs the expression of miR-19b, thereby increasing the expression of miR-19b target Sox-6. Thus, *H19* presents a classic example of lncRNAs having tissue specific roles and targets and provides an explanation for how lncRNAs are capable of regulating a wide variety of cellular processes at different stages in various tissues. **Table 2** consolidates the function of lncRNAs involved in cardiovascular development, homeostasis and their relevance to CVDs.

With the expanding knowledge about the importance of lncRNAs in cell fate decisions and heart development, it is evident that they are operational in maintaining the homeostasis of cardiovascular system. High throughput RNA-sequencing has steered the identification of differentially regulated lncRNAs in cardiac pathologies. One of the common heart conditions is cardiac hypertrophy, characterized by the increase in cardiomyocyte size to compensate for inappropriate cardiac function leading to heart failure (HF). lncRNA *CHAST* was upregulated in murine and human cardiac hypertrophy and *in vivo* depletion of this lncRNA in mouse hypertrophic model prevented and reverted the condition (Viereck et al., 2016). *CHAST* promotes hypertrophy by blocking autophagy and its inhibition prevented HF, thus presenting a promising target for treatment. *Chaer* is another pro-hypertrophic

TABLE 2 | List of lncRNAs with their regulatory mechanisms and physiological impact in cardiovascular biology.

lncRNA	Target	Mode of action	Function	Species	References
ALIEN	Unknown	Unknown	Cardiovascular commitment	Human, Mouse and Zebrafish	Kurian et al., 2015
ANRIL	CDKN2A/B ADIPOR1, VAMP3, C11ORF10	Scaffold	Genetic risk factor for CAD Pro-atherogenic	Human	Bochenek et al., 2013; Holdt et al., 2013
Apf	Atg7	Sponge miR-188-3p	Controls autophagy and MI	Mouse	Wang K. et al., 2015
Bvht	Hand1, Hand2, Mesp1, Nkx2-5, Tbx20	Decoy	Cardiac lineage commitment	Mouse	Klattenhoff et al., 2013
Carl	Pbh2, Bax, Caspase3, Bcl-2	miR-539 sponging	Inhibits mitochondrial fission and apoptosis in cardiomyocyte	Mouse, Human and Rat	Wang et al., 2014b; Li L. et al., 2018
CARMEN	Gata4, mesp1, Nkx2-5, Myh6	*Cis* and *trans* regulation SE-associated	Cardiac specification	Mouse Human	Ounzain et al., 2015
Chast	Plekhm1	*Cis*-regulation	Promotes hypertrophy	Mouse, Human	Viereck et al., 2016
Chaer	Hypertrophy genes	Guides PRC2 to hypertrophic gene loci	Promotes hypertrophy	Mouse, Human and Rat	Wang Z. et al., 2016
Chrf	Myd88	Sponge miR-489	Promote hypertrophy, Elevated in HF tissues	Mouse, Human	Wang et al., 2014a
Fendrr	Foxf1, Gata6, Nkx2-5, Pitx2	Guide PRC2 and TrxG/MLL to promoters of target genes	Cardiovascular development	Mouse, Human and Rat	Grote et al., 2013
Ftx	Bcl2l2	miR-29b-1-3p sponge	Inhibits cardiomyocyte apoptosis	Mouse	Long et al., 2018
GAS5	ANNEXIN A2	Guide	Supress SMC proliferation and migration	Human	Li et al., 2015
GATA6-AS	LOXL2	Decoy	Induced in ECs during hypoxia, involved in EndMT	Human	Neumann et al., 2018
H19	Sox6, MAPK, NF- kB, PTEN, VCAM-1, p21, TGF-β1	Sponge miR-19b, positively regulates miR-675, inhibition of phosphorylation of STAT3.	Proliferation and apoptosis during late stage cardiac differentiation, pro-atherogenic, promotes VSMC proliferation and restenosis, negatively regulates EC aging, prevents glucose induced EndMT	Mouse, Human and Rat	Han et al., 2016; Pan, 2017; Lv J. et al., 2018; Hofmann et al., 2019
MALAT1	TGFBR2/SMAD3, Cyc or CCN A2, B1, B2, Cdk1, ATG7	Sponge miR-145	EndMT, Controls proliferation and migration of ECs	Human	Michalik et al., 2014; Song et al., 2018
LEENE	eNOS	eRNA, recruits Pol II at eNOS promoter	EC homeostasis	Human, Mouse	Miao et al., 2018
MEG3	PTEN, AMPK and JAK-STAT signalling	Sponge miR-21, sponge miR-9	SMC proliferation and migration, supress EC proliferation and angiogenesis	Human	He et al., 2017; Zhu et al., 2018
Mdrl	miR-484	miR-361	Regulates cardiomyocyte mitochondrial fission and apoptosis	Mouse	Wang et al., 2014c
MIAT	VEGF	Sponge miR-93 and miR-150	Pro-hypertrophic	Human, mouse	Zhu et al., 2016; Li Y. et al., 2018
Mm67/77/85/130/132	Unknown	Cis-regulation, eRNAs	Cardiac development and remodelling	Mouse	Ounzain et al., 2014
Mhrt	Brg1	Decoy	Prevents hypertrophy and HF	Mouse, Human	Han et al., 2014
MYOSLID	MYOCD, MRTF-A, TGF-β (SMAD)	Cis and trans-regulation	SMC differentiation and proliferation	Human	Zhao et al., 2016
linc-p21	P53	Mdm2 mediated ubiquitination of p53	Repress VSMC proliferation and induce apoptosis, downregulated in atherosclerotic plaques in mice model	Human, Mouse	Wu et al., 2014
SENCR	MYOCD, *CCL5, CX3CL1, CDH5*	Decoy	SMC contractibility, potentiates mesodermal and endothelial commitment, regulates proliferation and migration of ECs, stabilize EC adherens junction, dysregulation associated with premature CAD and limb ischemia	Human	Bell et al., 2014; Boulberdaa et al., 2016; Lyu et al., 2019

(Continued)

TABLE 2 | Continued

lncRNA	Target	Mode of action	Function	Species	References
SMILR	HAS2, CENPF	*Cis*-acting	SMC proliferation, Increased expression in unstable atherosclerotic plaque and in plasma of high plasm CRP	Human	Ballantyne et al., 2016; Mahmoud et al., 2019
RNCR3	KLF2	Sponge miR-185-5p	Athero-protective	Human	Shan et al., 2016
ROR	ANP and BNP	Sponge miR-133	Pro-hypertrophic	Mouse, Human	Loewer et al., 2010
PANCR	PITX2	*Cis*-acting	Induced during early differentiation of hESCs to cardiomyocytes	Human	Gore-Panter et al., 2016
PLSR4	Mfn2	Sponge miR-214	Anti-hypertrophic	Mouse	Lv L. et al., 2018
PUNISHER	FOXC1, TAL1	Guide	Endothelial commitment	Human, Mouse and Zebrafish	Kurian et al., 2015
Upperhand	Hand2	*Cis*-acting, eRNA	Heart development	Mouse	Anderson et al., 2016

lncRNA which transiently interacts with PRC2 complex and attenuates hypertrophy upon its silencing in pre-stressed heart (Wang Z. et al., 2016). Unlike *CHAST* and *Chaer*, *Mhrt* is a cardioprotective lncRNA regulated by the stress activated Brg1-Hdac-Parp chromatin repressive complex (Han et al., 2014). Pathological stress activates Brg1 leading to aberrant gene expression including inhibition of *Mhrt.* Ultimately, this leads to cardiomyopathy and hypertrophy (Hang et al., 2010; Han et al., 2014). *Mhrt* forms a feedback loop with chromatin remodeling factor Brg1 and its repression results in cardiomyopathy. Restoring the pre-stress levels of *Mhrt* prevented hypertrophy and heart failure. Human ortholog of lncRNA *Mhrt* is repressed in various myopathic hearts demonstrating a conserved lncRNA dependent mechanism in cardiomyopathy (Han et al., 2014). These studies not only highlight the role of lncRNAs in diseases but also emphasize their therapeutic potential.

lncRNAs REGULATING ENDOTHELIAL CELL FUNCTION

New blood vessel formation is obviously an important aspect of heart regeneration. In the adult body, perturbed vessel formation may lead to inappropriate blood supply accompanied by shortage of oxygen to the heart resulting in diseased conditions such as myocardial infarction (MI). Endothelial cells (ECs) are important for the formation of the blood vessels and EC dysfunction is one of the early steps in the development of vascular pathologies. Many studies have identified lncRNAs regulating ECs function (**Figure 4A**) and lncRNAs differentially regulated during vascular diseases. One such example is *MALAT1* which is elevated in TGF-β1-induced endothelial-to-mesenchymal transition (EndMT) where it regulates TGFBR2 and SMAD3 by negatively regulating miR-145 (Xiang et al., 2017). EndMT is a hallmark of various pathological conditions including CVDs, fibrosis and cancer and *MALAT1* regulation of EndMT is a potent target for the development of gene therapy approaches. *MALAT1* is also involved in the regulation of proliferation of ECs. *MALAT1* knockdown increased basal sprouting and migration, inhibiting the proliferation. The reduced proliferation was due to the switch of ECs from a proliferative to promigratory phenotype. In addition, there was a simultaneous increase in the expression of cell cycle inhibitors, such as p21 (Michalik et al., 2014). Furthermore, *Malat1* expression was elevated in hypoxic conditions and its inhibition in a hind limb ischemia mouse model showed reduced capillary density and blood flow recovery (Michalik et al., 2014). Similarly, the inhibition of lncRNA *MANTIS* also prevented the angiogenic sprouting and alignment of ECs subjected to sheer stress (Leisegang et al., 2017). *MANTIS* controls the angiogenesis by activating the ATPase activity of BRG1, facilitating the assemblage of RNA polymerase II onto key endothelial genes like SOX18, SMAD6, and COUP-TFII (Leisegang et al., 2017). Unlike *MALAT1* and *MANTIS*, overexpression of lncRNA *MEG3* suppressed EC proliferation and *in vitro* angiogenesis through negative regulation of miR-9 (He et al., 2017).

Recently, Fiedler et al. (2015) generated an expression atlas of human hypoxia-sensitive lncRNAs with identification of two lncRNAs—*LINC00323* and *MIR503HG*—important for sustaining EC function. The expression levels of growth factor signaling and endothelial TF GATA2 were alleviated upon silencing of these two lncRNAs, accompanied by impaired cell cycle control and blocked capillary formation (Fiedler et al., 2015). Another study aiming to determine hypoxia influence on lncRNA expression in HUVECs identified lncRNA *GATA6-AS* to be upregulated during hypoxia (Neumann et al., 2018). *In vitro* EndMT was reduced upon *GATA6-AS* silencing along with impaired vascular sprouting and endothelial cell migration (Neumann et al., 2018). Additionally, EndMT was regulated by *H19* during diabetic retinopathy where overexpression of *H19* prevented glucose mediated EndMT through regulation of TGF-β1 signaling in a Smad-independent manner (Thomas et al., 2019). Moreover, *H19* was shown to regulate EC aging via negative regulation of age induced inflammatory activation (Hofmann et al., 2019).

lncRNA *SENCR* is important for endothelial cell commitment and function. *SENCR* overexpression substantially enhanced the mesodermal and endothelial commitment of hESC (Boulberdaa et al., 2016). In HUVECs, the upregulation of *SENCR* instigated migration, proliferation and formation of capillary-like structures. Concomitantly, silencing of *SENCR* had reducing effects on these processes. Known migratory and angiogenesis genes were downregulated upon *SENCR* silencing, with no effect on the expression of neighboring *FLI1* gene. Vascular

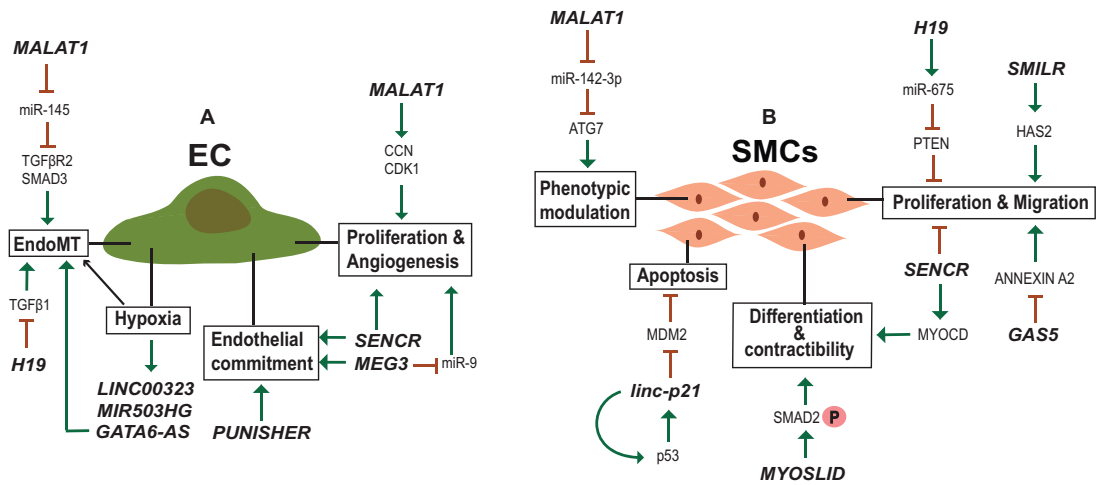

FIGURE 4 | lncRNAs regulating endothelial (EC) and smooth muscle cell (SMC) biology. **(A)** Examples of lncRNA regulating Endothelial cell identity, physiology and activated in hypoxic conditions. **(B)** Examples of lncRNA regulating SMC proliferation, migration, contractibility and phenotypic modulation between contractile and synthetic phenotype.

cells derived from patients with limb ischemia and premature coronary artery disease (CAD) showed a reduced level of *SENCR* as compared to control samples (Boulberdaa et al., 2016). Recently, *SENCR* was found to be important for maintaining the membrane integrity of ECs to control the vascular permeability (Lyu et al., 2019). Another important aspect of EC biology is Nitric Oxide synthesis (eNOS) that controls the vasodilation. RNA-seq and chromatin capture study identified eRNA *LEENE* enhancing the eNOS expression by recruiting RNA pol II to the eNOS promoter (Miao et al., 2018). These results suggest the importance of lncRNA in EC homeostasis and endothelial dysfunction which is one of the key triggers of vascular diseases.

lncRNA IN VASCULAR SMOOTH MUSCLE CELL (VSMC) FUNCTION

In addition to ECs, VSMC development and function is important in vascular setting. In contrast to terminally differentiated skeletal muscles, VSMCs can undergo reversible phenotypic change between contractile and synthetic phenotypes (Rensen et al., 2007; Davis-Dusenbery et al., 2011). The phenotypic diversity of VSMCs provide the necessary flexibility to the blood vessels to function under different physiological (**Figure 4B**) and pathological conditions. In normal adult animals, VSMCs exist as highly specialized and differentiated cells with a contractile phenotype. During several vascular pathologies the differentiated VSMCs switch to a proliferative phenotype with surged synthetic activity (Iyemere et al., 2006). Recent studies have identified lncRNA *MALAT1* and a novel lncRNA *SMILR* as important regulators of the switch from a differentiated to a proliferative phenotype of VSMCs (Ballantyne et al., 2016; Song et al., 2018; Mahmoud et al., 2019). Both were shown to promote proliferation and migration of VSMCs, but the mechanism employed and target genes are different for each.

MALAT1 knockdown facilitated the conversion of SMCs from a proliferative phenotype to a differentiated one by inhibiting autophagy (Song et al., 2018). Mechanistically *MALAT1* was found to compete with miR-142-3p to regulate ATG7 mediated activation of autophagy that results in the conversion of VSMCs from a contractile to a proliferative state. While *MALAT1* acts in trans, *SMILR* influences cellular proliferation by regulating the expression of proximal gene HAS2, involved in atherosclerotic lesion formation (Ballantyne et al., 2016). More specifically, *SMILR* affects the late mitotic and cytokinesis phases of cell cycle by interacting with CENPF and this *SMILR*:*CENPF* interaction is in turn regulated by Staufen1 RNA binding proteins (Mahmoud et al., 2019). Alteration of mature VSMC from a contractile phenotype to an osteoblastic phenotype is another major aspect of VSMC biology, which leads to vascular calcification (Iyemere et al., 2006). The transcriptome analysis during calcification of rat VSMCs identified lncRNA *Lrrc75a-as1* as a negative regulator of vascular calcification (Jeong et al., 2019).

Thirty one lncRNAs were identified from RNA-Seq in human coronary artery SMCs (HCASMC), one of which was *SENCR*, that helps maintain the normal SMC differentiated state (Bell et al., 2014). SENCR is transcribed antisense to the Friend Leukemia Integration virus1 (FLI) gene, whereas, *SENCR* knockdown had no effect on the expression of FLI1 or other neighboring genes ruling out *cis*-acting effects of *SENCR* on local gene expression. Attenuation of *SENCR* significantly reduced the expression of many SMC contractile genes, including MYOCD, at both mRNA and protein levels with significant increase in genes inducing motility (Bell et al., 2014). Collectively, these results confirm the regulatory importance of *SENCR* in human coronary artery SMC (HCASMC) differentiation and migration. A study searching for lncRNAs regulated by myocardin (MYOCD/SRF), the master switch for VSMC differentiation, identified lncRNA *MYOSLID* (MYOcardin-induced Smooth muscle Long non-coding RNA, Inducer of Differentiation) as

a direct target for MYOCD/SRF and TGFβ/SMAD pathways (Zhao et al., 2016). *MYOSLID* promoted VSMC differentiation and inhibited VSMC proliferation. lncRNAs regulating SMC phenotypic modulation, proliferation and migration might be used as molecular targets in therapies for diseases aggravated by vascular remodeling.

Excessive proliferation of VSMCs is a major attribute of restenosis. *In vitro* overexpression of *H19* accelerated VSMC proliferation by positively regulating miR-675, which in turn downregulates PTEN expression (Lv J. et al., 2018). *H19* and miR-675 were upregulated in injured arterial walls in a rat balloon injury model (Lv J. et al., 2018). lnc-*GAS5* is another lncRNA implicated in proliferation and migration of SMCs. Overexpression of lnc-*GAS5* inhibited the proliferation, migration and reduced apoptosis of human saphenous vein SMCs (HSVSMCs) and conversely, its silencing promoted these cellular behaviors (Li et al., 2015). lnc-*GAS5* function is mediated through a Ca^{2+}-dependent RNA binding protein, Annexin A2. Thus, low expression of lnc-*GAS5* increases proliferation and migration of HSVSMCs through AnnexinA2 facilitating in the pathogenesis of the Great Saphenous Veins. *linc-p21* is also shown to regulate SMC proliferation but its expression is downregulated during atherosclerosis (Wu et al., 2014). *linc-p21* silencing in a carotid artery injury mouse model resulted in neonatal hyperplasia due to dysregulation of multiple P53 targets (Wu et al., 2014). The angiotensin II regulated *lnc-Ang362* is a host for miR-221 and miR-222, two known miRNAs implicated in SMC proliferation (Leung et al., 2013). lnc-Ang362, miR-221 and miR-222 were increased in the lung tissues derived from pulmonary arterial hypertension (PAH) patients (Wang et al., 2019). Thus, lnc-Ang362 could be a target for treating PAH.

DIAGNOSTIC POTENTIAL OF lncRNAs IN CVDs

lncRNA with prognostic and diagnostic potential are of particular interest in the clinical arena. A Genome wide association study (GWAS) has identified chromosome 9p21 locus exhibiting the highest association with atherosclerosis (Holdt et al., 2013). This risk locus encodes for lncRNA *ANRIL*, associated with atherosclerotic severity. Further investigations revealed that *ANRIL* functions in *trans*, leading to pro-atherogenic effects such as cell proliferation, increased cell adhesion and decreased apoptosis (Holdt et al., 2013). The association of increased *ANRIL* level with LV dysfunction further highlights its diagnostic and prognostic importance. Similarly, another GWAS study identified that the single nucleotide polymorphism (SNP) associated with MI altered the expression of lncRNA MIAT (Ishii et al., 2006).

Circulating lncRNAs can serve as biomarkers of the diseases making the diagnosis much easier. A transcriptome study identified lncRNA *LIPCAR* in the plasma of patients with heart failure as a novel biomarker which can predict heart remodeling and future death in the patients (Kumarswamy et al., 2014). Another study performed the microarray analysis in the plasma of CAD patients and reported that lncRNA *CoroMarker* as a sensitive biomarker for CAD (Yang et al., 2015). Likewise, circulating *ZFAS1* and *CDR1AS* were found to be decreased and increased in acute myocardial infarction (AMI), respectively (Zhang et al., 2016). Another study reported low levels of lncRNA *GAS5* in the plasma of 30 CAD patients as compared to 30 healthy individuals (Yin et al., 2017).

Promising results for several kinds of lncRNAs were reported similarly (Schlosser et al., 2016). However, the authors observed difficulties in reproducibility of detection. This could be due to the low sensitivity of the conventional lncRNA detection methods. Regardless of the technical challenges the low abundancy of lncRNAs cannot undermine their reported functional importance in many instances highlighted in this review. A summary of lncRNAs as potential biomarkers and their roles in cardiac diseases is represented in **Figure 5**.

lncRNA–miRNA INTERACTIONS IN SKELETAL MUSCLE AND CARDIOVASCULAR DEVELOPMENT AND DISEASE

The crosstalk between lncRNA–miRNA–mRNA appears to be common in different facets discussed so forth. lncRNAs inhibit the function of miRNA by binding to them in a sequence specific manner, thereby increasing the number of target mRNAs that would otherwise be suppressed by miRNAs. In this regard, comprehensive knowledge of complex lncRNA–miRNA–mRNA-networks would help develop novel RNA-based therapeutics for different diseases (**Figure 6**).

As seen in skeletal muscle proliferation and differentiation, lncRNA *H19*, *linc-MD1*, *lnc-mg* and *Malat1* inhibit the action of miRNAs by the sponging mechanism (Cesana et al., 2011; Kallen et al., 2013; Han et al., 2015; Chen et al., 2017; Zhu et al., 2017). However, lncRNAs can regulate the function of miRNAs in many different ways. For instance, lncRNA *Sirt1As* shields the miR-34a binding site on Sirt1 mRNA by binding to the transcripts (Wang G.-Q. et al., 2016). lncRNAs can positively regulate miRNAs by acting as precursor miRNAs as in the case of *H19* giving rise to miR-675-3p and miR-675-5p (Dey et al., 2014).

These RNA networks are not restricted to skeletal muscle but are also noticeable in cardiovascular physiology and disease. *CHRF*, *Plscr4*, *MIAT* and *ROR* were involved in miRNA mediated cardiac hypertrophy regulation. lncRNA *CHRF* triggers cardiomyocyte hypertrophy by sponging antihypertrophic miR-489, which targets Myd88 (Wang et al., 2014a). Similarly, pro-hypertrophic lncRNA *MIAT* regulates anti-hypertrophic miR-93 and miR-150 (Zhu et al., 2016; Li Y. et al., 2018). *ROR* is another hypertrophic inducer that sponges miR-133 (Jiang et al., 2016). In contrast, *Plscr4* is an anti-hypertrophic lncRNA downregulating miR-214 to promote the expression of Mfn2 (Lv L. et al., 2018). Apoptosis and autophagy are among other pathways targeted by competing-endogenous RNAs (ceRNAs). lncRNA *CARL* has been proposed to inhibit cardiomyocyte apoptosis by sequestering miR-539,

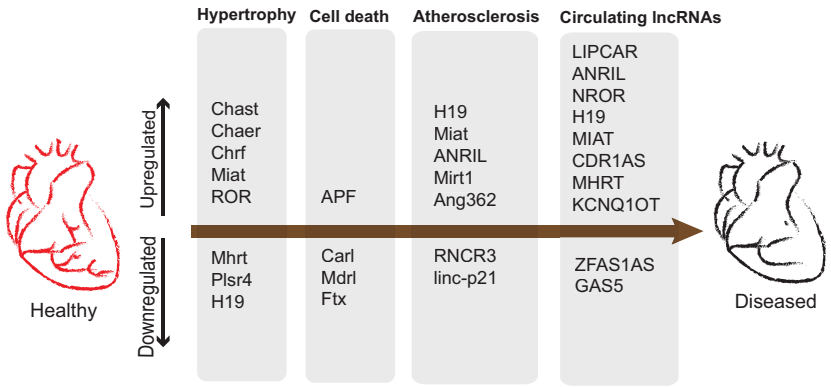

FIGURE 5 | lncRNAs upregulated and downregulated during development of cardiac diseases and potential biomarkers. Cardiac remodeling is characterized by aberrant myocardium growth along with apoptosis and vascular remodeling leading to atherosclerosis.

a microRNA targeting PHB2 (Wang et al., 2014b), a subunit of mitochondrial membrane protein Prohibitin involved in mitochondrial homeostasis (Tatsuta et al., 2005). Cardiomyocyte apoptosis and mitochondrial fission was also inhibited by *Mdrl* via downregulation of miR-361, counteracting the inhibition of miR-484 processing (Wang et al., 2014c). lncRNA *Ftx* inhibited cardiomyocyte apoptosis by preventing miR-29b-1-5p mediated downregulation of Bcl2 (Long et al., 2018). Likewise, *Apf* triggers autophagy by targeting Atg7 through negative regulation of miR-188-3p, an inhibitor of autophagy and myocardial infarction (Wang K. et al., 2015). It is interesting to note that some of the above mentioned networks are well conserved across species, suggesting the crucial roles, they have in cardiovascular biology. Hence, modulating lncRNA-miRNA-mRNA pathways could possibly be a novel strategy to suppress cardiomyocyte loss in order to protect against myocardial infarction (MI) and tailor therapies for hypertrophy. Thorough understanding of these networks in development and disease could help design better therapeutic strategies in the field of regenerative medicine.

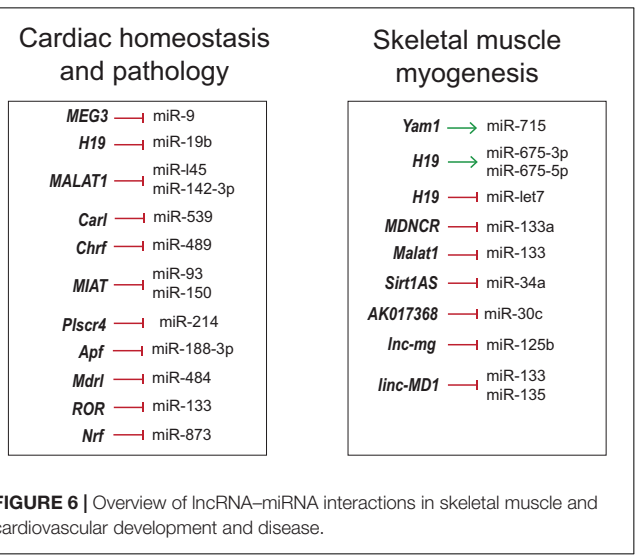

FIGURE 6 | Overview of lncRNA–miRNA interactions in skeletal muscle and cardiovascular development and disease.

lncRNA THERAPEUTICS AND CHALLENGES

There has been an increased interest in the understanding of lncRNA regulation in disease systems due to their relatively restricted expression patterns and different possible actions on cellular function(s). Given that several of these ncRNAs are dysregulated in disease conditions, modulating the levels of such lncRNAs appears to be a promising approach for therapeutics and preclinical testing. However, only a few lncRNAs have been studied in depth in relevant *ex vivo* and *in vivo* systems. Upregulation or downregulation/inhibition of lncRNA function has been the most rigorously adopted methods to understand their therapeutic potential in muscular defects and cardiovascular damage. The most commonly used gene delivery methods for RNA based therapeutics are recombinant viral systems such as adenovirus, lentivirus and adeno-associated viruses (AAVs), which are employed either to inhibit or to overexpress the mRNA, miRNA, lncRNA whole transcripts (Eulalio et al., 2012; Gomes et al., 2017; Gabisonia et al., 2019). The use of RNAi and antisense oligonucleotides (ASOs) present the most commonly used approaches for selective downregulation of potential lncRNAs. On the contrary, the upregulation of lncRNAs can be achieved by viral vectors that are very efficient for muscle delivery. Using the above mentioned strategies, there have been promising results toward miRNA targeting and protein coding transcripts in preclinical systems, which reached clinical trials. However, using the same delivery methods the therapeutic relevance of lncRNAs remain to be resolved.

The siRNA mediated pharmacological inhibition of cytoplasmic lncRNA *SMILR* in the *ex vivo* vein graft model significantly reduced the SMC proliferation to ameliorate the effects of vascular remodeling (Mahmoud et al., 2019). In another approach using nanoparticle mediated transfection of siRNA against lncRNA *Chaer* directly into mouse heart

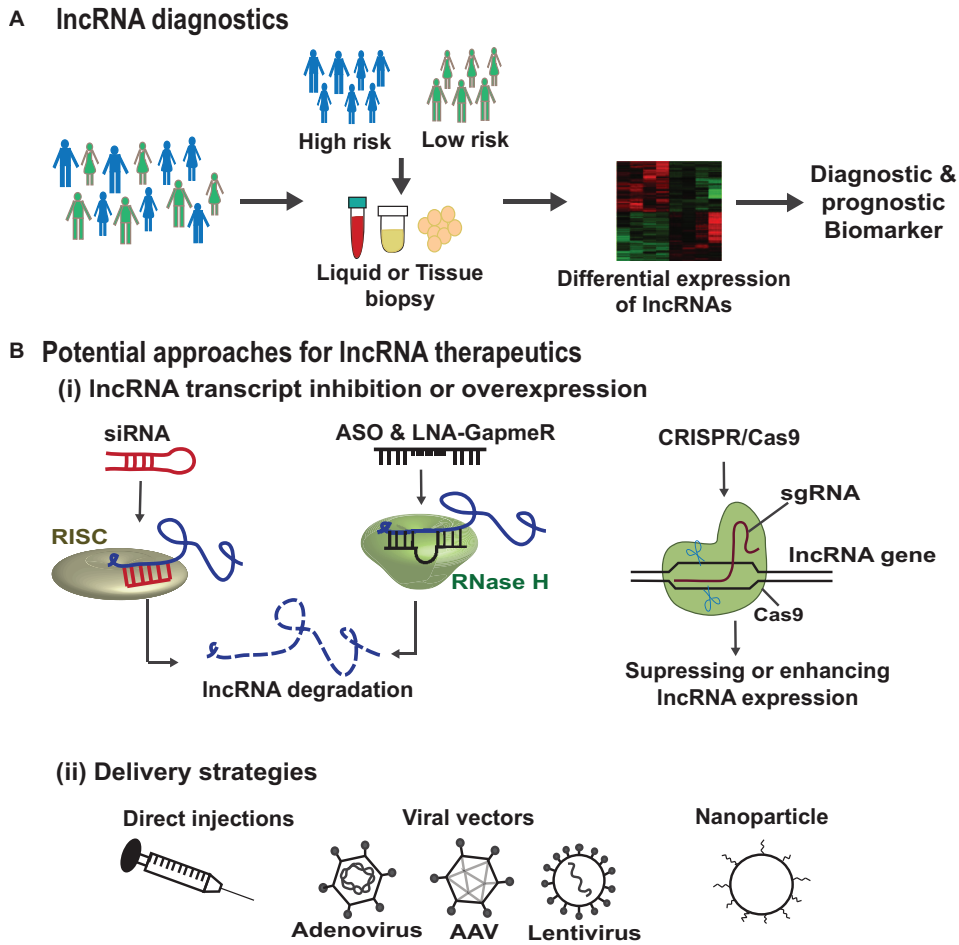

FIGURE 7 | lncRNA based diagnostics and therapeutic strategies. **(A)** Genome wide association studies (GWAS) help in the identification of lncRNA single nucleotide polymorphisms (SNPs) associated with disease susceptibility. These SNPs can alter the lncRNA expression in the body fluids or tissues which can serve as diagnostic or prognostic markers. In addition, improvements in RNA-sequencing technologies can lead to the identification of lncRNAs as biomarkers/disease targets in different diseases. **(B)** (i) Summarizes the potential approaches for lncRNA therapeutics, such as siRNAs that are associated with RNA-induced silencing complex (RISC) bind and cleave the target lncRNA. ASOs and LNA-GapmeRs bind to the target lncRNA in sequence specific manner leading to RNase H mediated lncRNA degradation. CRISPR/Cas9 gene editing tool can make deletion or insertion in the DNA sequence of the target lncRNA to either enhance or abrogate its expression. **(B)** (ii) Demonstrates the different lncRNA delivery strategies such as direct injections, viral particles, or via encapsulation in nanoparticles.

decreased the cardiac hypertrophy and fibrosis and restored cardiac function (Wang Z. et al., 2016). The inhibition level achieved by shRNAs and siRNAs are, in general, limited to the cytoplasmic lncRNA molecules. Hence, on the other hand, ASOs or GapmeRs are more suitable for nuclear lncRNAs that direct Dicer independent degradation of the target lncRNA by RNase H activity (Fluiter et al., 2009; Bennett and Swayze, 2010; Lennox and Behlke, 2016). The ASO technology has fewer off target effects than the small RNA mediated approach. One such example is GapmeR mediated inhibition of lncRNA *Chast* for the prevention and regression of cardiac hypertrophy *in vivo* (Viereck et al., 2016). No noticeable side effects of GapmeR treatment were reported. In another study, GapmeR mediated knockdown of *Malat1* in mice muscle resulted in poor blood flow recovery and diminished capillary density (Michalik et al., 2014). In addition, *in vivo* therapeutic intervention using GapmeRs targeting lncRNA *Wisper* suppressed cardiac fibrosis and improved function (Micheletti et al., 2017). So far, none of the antisense based lncRNA drugs have been tested in clinical studies but, this strategy has been proven to be effective and safe in a clinical trial targeting liver miR-122 with LNA-based antimiRs (mirvarsen) (Zumla et al., 2013). The newer generation of ASOs provide spatial control for target delivery and with further improvements, this could be translated for lncRNA inhibition in future clinical trials.

In comparison to inhibition, overexpression of cardioprotective lncRNAs appear to be much more challenging attributing to their length and locus complexity, and sometimes incomplete knowledge of the complete sequence identity and isoform structure in the current genome annotation. In this context, the major challenge is efficient transportation of these large transcripts across the membrane barriers and determining

the potential toxicity. The transgenic gene activation of the cardioprotective lncRNA *Mhrt* demonstrates the first overexpression study highlighting the translational importance of delivery of lncRNA as a drug (Han et al., 2014). In another example, *Chast* overexpression using AAV9 (adeno-associated-virus serotype 9) viral particles promoted cardiac hypertrophy in mouse heart, suggesting it to be a crucial target for cardiac hypertrophy (Viereck et al., 2016).

Advancements in RNA-sequencing technologies have led to the identification of several lncRNAs in different diseases suggesting them to be crucial targets for therapeutics. In addition, due to their distinct expression patterns they can be powerful tools for diagnostics, personalized medicine and drug development. A brief description of lncRNA-based diagnostic and therapeutic strategies is depicted in **Figure 7**. There are several key challenges in any lncRNA-focused therapy. Firstly, the lncRNAs due to their pleiotropic actions regulate multiple targets and hence may not be target-specific and can act via more than one mechanism in a diseased state. In addition, low conservation of lncRNAs across species, is another challenge, for example a human specific lncRNA lacking conservation in mouse, makes its preclinical testing inappropriate (Lu and Thum, 2019). A further challenge in lncRNA therapeutics is the fact that lncRNAs are often integrated in complexes which make them inaccessible for access. Small molecules such as aptamers may help the lncRNA binding with the interacting protein complexes or induce conformational changes in the secondary or tertiary structures of lncRNA (Lunse et al., 2010). This is mainly important, where the lncRNA expression does not attribute to the disease phenotype but, the lncRNA interaction with other molecules accounts for the disease. Hence, the secondary and tertiary structure of lncRNAs and their structure-function relationship should to be resolved before lncRNAs enter into therapeutics.

The other translational limitations of lncRNA therapeutics is the lack of efficient and safe delivery systems for a controlled and targeted release. The delivery vehicle should ensure high transfection efficiency with minimal cytotoxicity but also controlled release of the lncRNA based drug during the complete process. lncRNA levels can be manipulated using viral vectors, however, the use of viral delivery methods is restricted due to the associated issues including off-target effects, activation of host immune response and the risk of insertional mutagenesis, although clinical evidence in gene therapy to date demonstrates a favorable situation. To overcome these risks, researchers believe non-viral vectors with chemical modifications and/or nanoparticles targeted to specific cell type would be advantageous in clinical trials, however, are relatively inefficient dampening down the possibility for clear efficacy signals. Finally, the use of gene editing technique such as CRISPR/Cas9 has provided both loss- or gain-of-function of lncRNA in *in vitro* and *in vivo* studies (Aparicio-Prat et al., 2015; Leisegang et al., 2017; Ballarino et al., 2018). Although, similar delivery issues need to be addressed with the use of gene editing tools *in vivo* too.

CONCLUSION

Several lncRNAs have been identified to be involved in development and pathophysiology by regulating gene expression at DNA, RNA and protein levels. Their specific expression during differentiation and disease helps qualify them as key regulators and potential therapeutic targets. However, at present the use of lncRNAs in therapeutics is in its inception. There are still certain technical issues that need to be addressed, such as the difficulties in targeting, accurate outcome predictions and discrepancies in knockdown phenotypes in *in vitro* and *in vivo*. Further improvement in detection, silencing approaches, mechanistic understanding and development of *in vivo* models would uncover the full potential of lncRNAs as diagnostic and possibly therapeutic targets.

AUTHOR CONTRIBUTIONS

SwS collected and analyzed the data, prepared the figures, and wrote the manuscript. TD and SmS corrected and gave suggestions to improve the manuscript. RB conceived, planned, analyzed, wrote, coordinated, and finalized the manuscript. AB wrote, reviewed, coordinated, and finalized the manuscript.

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Role of TGF-β/Smad Pathway in the Transcription of Pancreas-Specific Genes During Beta Cell Differentiation

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Autoimmune destruction of pancreatic beta cells causes absolute insulin deficiency and results in type 1 diabetes mellitus (T1DM). The substitution of healthy pancreatic beta cells for damaged cells would be the ideal treatment for T1DM; thus, the generation of pancreatic beta cells from adult stem cells represents an attractive avenue for research. In this study, a cocktail of factors was used to induce the differentiation of pancreatic beta cells from mesenchymal stem cells (MSCs). The differentiation program was divided into five stages, and the roles of the cocktail factors used during each stage were systematically elucidated. Activin A was found to phosphorylate Smad2 and Smad3 in stage III, thereby activating the TGF-β/Smad pathway. Meanwhile, the endocrine-specific transcription factor, Ngn3, and the pancreas-specific miRNAs, miR-375 and miR-26a, were dramatically elevated in stage III. We next demonstrated that Smad4, an important transcription factor in the TGF-β/Smad pathway, could bind to the promoter sequences of target genes and enhance their transcription to initiate the differentiation of beta cells. Use of SB-431542, an inhibitor of the TGF-β/Smad pathway, demonstrated *in vivo* and *in vitro* that this pathway plays a critical role in the production of pancreatic beta cells and in modulating insulin secretion. Thus, the TGF-β/Smad pathway is involved in the production of beta cells from adult stem cells by enhancing the transcription of Ngn3, miR-375, and miR-26a. These findings further underline the significant promise of cell transplant therapies for type 1 diabetes mellitus.

Keywords: pancreatic beta cells, stem cells, TGF-β/Smad pathway, Ngn3, microRNAs

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INTRODUCTION

Pancreatic beta cells regulate blood glucose homeostasis through their production of insulin. Type 1 diabetes mellitus (T1DM) results from the autoimmune destruction of pancreatic beta cells. The replacement of these beta cells with healthy cells would be the ideal therapy for T1DM; thus, the production of pancreatic beta cells from adult stem cells or embryonic stem cells offers a promising avenue for research. Mesenchymal stem cells (MSCs) are attractive donor cells for cell transplantation, as they are multipotent and exert a strong immunoregulatory effect. Umbilical cord

tissue is readily obtained from discarded term placentae without any risk to the donors. Umbilical cord MSCs are typical adult MSCs but with the added advantages of being easy to culture *in vitro* and of simpler ethical access compared with other stem cells. Therefore, umbilical cord MSCs are a promising candidate for cell therapy.

Genome-encoded microRNAs (miRNAs) regulate gene expression post-transcriptionally. These non-coding small RNAs (18–25 nt) regulate gene expression through binding to the 3′-untranslated regions of specific mRNAs and inhibiting their translation. The role of miRNAs in the regulation of beta cell differentiation has been demonstrated by the generation of a mouse model with beta cell-specific ablation of Dicer1 (Plaisance et al., 2014; Bai et al., 2016), and disruption of *Dicer1* in rats with the use of a insulin promoter 2 (RIP)-Cre transgene results in changed islet morphology, reduced pancreatic beta cell numbers, and dysregulated glucose-induced insulin secretion (GSIS) (Kalis et al., 2011). Many miRNAs have been shown to be important regulators in the differentiation and function of pancreatic beta cells, including let-7 (Krek et al., 2005; Lovis et al., 2008), miR-223, miR-21 (Du Rieu et al., 2010; Bai et al., 2016), miR-200, miR-30d, miR-124a (Tang et al., 2009), miR-26 (Bai et al., 2017a), miR-24, miR-148 (Melkman-Zehavi et al., 2011), miR-204 (Roldo et al., 2006), and miR-375 (Poy et al., 2004), as well as miR-146a, miR-15a, miR-29a, miR-9, miR-16, and miR-34 (Rosero et al., 2010; Bai et al., 2017b). However, as yet, there have been no reports regarding the role of induction factors in promoting the transcription of pancreatic miRNAs during beta cell differentiation from stem cells, and the molecular mechanisms underlying this process remain unclear.

The TGF-β superfamily of secreted polypeptide growth factors plays an important role in a variety of pathophysiologic processes, including vascular remodeling, angiogenesis, and atherogenesis, as well as in regulating cellular responses such as differentiation, proliferation, growth, adhesion, migration, survival, and the specification of developmental fate. Apart from TGF-β, this superfamily also includes the activins and the BMPs (bone morphogenetic proteins). Activins are dimeric proteins composed of either two βA subunits (activin A), two βB subunits (activin B) or a βA and βB subunit (activin AB). Activin A is extensively involved in the production of beta cells from stem cells (Shi et al., 2005; Pagliuca et al., 2014; Bai et al., 2017a) but the functions of the TGF-β pathway in beta cell differentiation and pancreatic miRNA expression have not been fully investigated.

In this study, we used a segmented induction method to produce beta cells from mouse umbilical cord MSCs, and we detected the expression of pancreatic miRNAs and the activation of the TGF-β/Smad pathway by examining quantitative reverse transcription PCR (RT-qPCR) and western blotting results of each stage of beta cell production. Combining our data with those from previous reports, we found that the pancreatic miRNAs, miR-26a and miR-375, play an important role in the formation of beta cells and in their secretion of insulin (Bai et al., 2017a,b), and that the TGF-β/Smad pathway plays an important role in regulating the transcription of these pancreatic miRNAs. To elucidate the mechanisms of transcriptional regulation during the production of beta cells and to better understand the interaction of the TGF-β/Smad pathway with pancreatic miRNAs expressed during the differentiation of beta cells from mouse umbilical cord MSCs, we tested both the activation and suppression of this pathway *in vitro*. The results confirmed the critical role of the TGF-β/Smad pathway during pancreatic beta cell differentiation.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Committee on the Ethics of Animal Experiments of Jining Medical University (License ID: 2017-JZ-003). All surgery was performed under pentobarbital anesthesia, and all efforts were made to minimize suffering.

Umbilical Cord MSC Culture and Differentiation Into Beta Cells

Wharton's Jelly was selected from 16-old-day mice embryos and digested with collagenase type IV (Sigma-Aldrich, MO, United States) under sterile conditions to isolate the umbilical cord MSCs. Umbilical cord MSCs were cultured in complete medium containing L-DMEM (Gibco, Carlsbad, CA, United States), 10% FBS (Gibco), 100 mg/mL streptomycin, and 100 U/mL penicillin. Positive markers of MSCs, CD44 (1:500; ab157107, Abcam, Cambridge, MA, United States), CD90 (1:800; ab3105, Abcam), CD105 (1:500; ab107595, Abcam), and negative markers of MSCs, Ngn3 (1:500; ab216885, Abcam) and Pdx1 (1:800; ab47267, Abcam) were analyzed using flow cytometry (Bai et al., 2017b).

For the production of beta cells, we used the classical cocktail factor method, and the cocktail factors included 1 μM 5-aza-2′-deoxycytidine (5-AZA; Sigma-Aldrich), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1 mM non-essential amino acids (Gibco), 15 ng/mL activin A (Sigma-Aldrich), 10 mM *all-trans* retinoic acid (ATRA; Sigma-Aldrich), 1% B27 (Gibco), 10 ng/mL bFGF (Peprotech, Rocky Hill, TX, United States), 10 mM nicotinamide, and 1% ITS (Gibco). In brief, umbilical cord MSCs were exposed to 1 μM 5-AZA for 18 h before induction. The cells were then cultured in low-glucose DMEM containing 10% FBS, 0.1 mM β-mercaptoethanol, and 1 mM non-essential amino acids. On day 5, the medium was supplemented with 15 ng/mL activin A. On day 7, 10 mM ATRA was added. On day 9, the medium was replaced with fresh medium supplemented with 1% B27, 10 ng/mL bFGF, 10 mM nicotinamide, and 1% ITS to encourage further differentiation (Bai et al., 2017a). On day 16, the cocktail factors were used to induce MSCs to achieve terminal beta cell differentiation, and the culture media was then replaced with beta cell media (DMEM medium containing 25 mM glucose, 70 μM β-mercaptoethanol, and 4 mM L-glutamine, and supplemented with 15% FBS).

Glucose-Stimulated Insulin Secretion (GSIS)

GSIS was tested according to a previously reported procedure (Bai et al., 2016). Briefly, beta cells derived from MSCs were washed with Krebs buffer and then pre-incubated in Krebs

buffer containing 2.5 mM glucose for 2 h to remove residual insulin. The cells were washed three times using Krebs buffer, incubated again in 2.5 mM glucose-Krebs buffer for 30 min, and the supernatant was selected. Next, the cells were washed three times and incubated in 20.5 mM glucose-Krebs buffer for 30 min, after which the supernatant was selected. This experiment was repeated four times. Finally, cell clusters were incubated in Krebs buffer containing 2.5 mM glucose. Cell supernatants containing secreted insulin were analyzed with an insulin ELISA test kit (EZRMI-13K; Millipore, Billerica, MA, United States) (Bai et al., 2017b).

Flow Cytometry (FCM)

To analyze the expression of specific markers in the umbilical cord MSCs and pancreatic beta cells, these cells were analyzed using a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Brea, CA, United States). Briefly, cells were selected using 0.125% (w/v) trypsin, separated into 500 μL aliquots, and labeled with FITC- or Cy5-conjugated antibodies against CD44 (1:500; ab157107, Abcam), CD90 (1:800; ab3105, Abcam), CD105 (1:500; ab107595, Abcam), Ngn3 (1:500; ab216885, Abcam), and Pdx1 (1:800; ab47267, Abcam) as per the manufacturer's instructions (Hackstein et al., 2003). The FCM data were analyzed with CXP software (Beckman Coulter). Mean fluorescence intensity was analyzed after subtraction of the negative control signal.

Immunofluorescence Detection

The MSCs or differentiated beta cells were fixed with 4% PFA (paraformaldehyde) for 20 min at room temperature and then incubated in 0.15% Triton X-100 for 10 min at room temperature. Next, PBS containing 4% (w/v) goat serum was applied for 30 min at room temperature or 2 h at 4°C. The cells were then incubated with the primary antibody overnight at 4°C. The FITC- or Cy5-labeled secondary antibody (Bioss, Beijing, China) was applied at a concentration of 10 μg/mL. Primary antibodies included anti-Ngn 3 (1:100; ab216885, Abcam) and anti-Pdx1 (1:1000; ab47267, Abcam). Photomicrographs were taken with a Nikon TE2000 confocal microscope with an attached Nikon ZE-1-C1 digital camera system (Nikon, Tokyo, Japan).

Western Blotting

Ngn3, Pdx1, the Smads, Sox6, Bhlhe22, Mtpn, and Gapdh were analyzed using western blotting following the activation or suppression of the TGF-β/Smad pathway in umbilical cord MSCs. Cells were lysed using the Protein Extraction Reagent (Beyotime, Beijing, China) supplemented with a PI (protease inhibitor, Beyotime) to obtain whole-cell lysates. Nuclear extracts from umbilical cord MSCs were prepared as described previously using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) (Bai et al., 2019). Protein concentrations of the extracts were measured with the BCA assay (Beyotime) and equalized with extraction reagent. Extracts of equivalent total protein content were loaded and subjected to SDS-PAGE, followed by transfer onto 0.2 μm nitrocellulose membranes. Primary antibodies against Ngn3 (1:500); Pdx1 (1:1,000); p-Smad2 (1:400; 18338, Cell Signaling Technology, Danvers, MA, United States); p-Smad3 (1:500; 9520, Cell Signaling Technology); Smad2/3 (1:1,000; 8685, Cell Signaling Technology); Smad4 (1:500; 46535, Cell Signaling Technology); Sox6 (1:1,000; ab30455, Abcam); Bhlhe22 (1:2,000; ab204791, Abcam); Mtpn (1:1,000; ab241982, Abcam); Gapdh (1:5,000; ab181602, Abcam); and Histone (1:2,000; ab1791, Abcam) were used together with HRP (horseradish peroxidase)-coupled secondary antibodies (1:3,000; A0216 and A0208, Beyotime). Signals were developed using ECL (enhanced chemiluminescence) detection reagents on the nitrocellulose membranes. Gapdh and Histone were used as internal controls. Protein abundance was calculated and analyzed with ImageJ tools.

MiRNA Quantitative Reverse-Transcription PCR (RT-qPCR)

MiRNA RT-qPCR was used to analyze the expression of pancreatic miRNAs during the differentiation of beta cells from umbilical cord MSCs. Twenty-two miRNAs, known to be involved in either pancreatic development and/or the maintenance of the function of beta cells, were selected for analysis after induction. These were miR-26a, miR-375, miR-100, miR-99, miR-125b, miR-181a, miR-92, miR-30, miR-221, miR-21, miR-19b, miR-33-5p, miR-142-3p, miR-30d, miR-33-3p, miR-27, miR-142-5p, miR-429, miR-29a, miR-222, miR-200, miR-128, miR-204, miR-223, miR-146a, miR-15, miR-21, miR-34, and miR-212/132. The miRNAs were obtained from umbilical cord MSCs and induced MSCs with the miRcute miRNA Isolation Kit V1.0 (Tiangen, Beijing, China), and the isolated miRNAs were then poly(A)-tailed and reverse-transcribed with the miRcute miRNA complementary DNA (cDNA) kit v2.0 (Tiangen). Quantitative PCR of these miRNAs cDNA was performed with the SYBR Green method using the miRcute miRNA quantitative PCR Detection Kit v2.0 (Tiangen) on the Roche Light Cycler 480 system and under the following conditions: 95°C for 15 min; 5 cycles at 94°C for 20 s, 65°C for 30 s, and 72°C for 34 s; 40 cycles at 94°C for 20 s, and 60°C for 34 s. The small nuclear RNA, U6, was used as an internal control for normalization. All miRNA primers were purchased from the miRNA primer bank of Tangen Biotech (Beijing, China). Each experiment was performed in duplicate and repeated three times. The relative expression of the miRNAs was calculated with the comparative threshold cycle (CT) method as $2^{-\Delta\Delta CT}$. Changes in miRNA expression were illustrated with a heat map prepared using Mev-tm4[1] and assessed with the *t*-test. A value for $p < 0.05$ was considered statistically significant.

In situ PLA

Umbilical cord MSCs and induced MSCs were fixed with pre-chilled 4% PFA for 15 min. The cells were washed three times with PBS and then permeabilized with 0.15% (v/v) Triton X-100 for 10 min at room temperature. The cells were blocked for 0.5 h at room temperature or for 2 h at 4°C with 4% (w/v) bovine serum albumin (BSA; Gibco) containing 0.1% (v/v) Tween 20, and incubated with the antibody pairs (Smad 3 and Smad 4, or Smad 2 and Smad 4) for 1 h at 37°C. Duolink *In situ* PLA (Sigma-Aldrich) was performed according

[1]http://mev.tm4.org/

to previous reports (Bai et al., 2019). Images were scanned with a Nikon TE2000-E inverted confocal microscope (Nikon, Yokohama, Japan).

ChIP-PCR Assessment

ChIP was executed with the ChIP assay kit (Beyotime) using the Smad 4 antibody, Smad 4, and anti-IgG antibodies (Cell Signaling Technology). The ChIP DNA was extracted with the DNA Purification Kit (Beyotime) and the samples were then subjected to qPCR amplification with primers spanning the protein-binding sites. The primers used were for miR-26a (F: 5′-GAGGCCTGATGGAGCCGTGGGGACC-3′, R: 5′-AGAG CCACAGCAGGCGGAAAGCCAGATGCCACAGG-3′), miR-375 (F: 5′-TTTCCTAGAACTGCTGTCTTGTCCCATCGCC CACA-3′, R: 5′-AAGAACCCACCCTTTCCTCATCAGAACCAC TTTGC-3′), and Ngn3 (F: 5′-TTAGGCTTTGGATTCTTCATAG TTCTGATAGGATTTGC-3′, R: 5′-AAGTGTCTTCTGGTCCC AGGAATGGAGGCGTAAGG-3′).

EMSA (Electrophoretic Mobility Shift Assay)

Nuclei from induced MSCs were obtained according to previous reports (Bai et al., 2019) using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). The protein concentrations were tested using the bicinchoninic acid method. The probes used for EMSA are shown in **Supplementary Table S1**. Gel-shift assays were executed using the EMSA/Gel-Shift Kit (Beyotime). The supershifting antibody against Smad4 (46535, ChIP Grade) was purchased from Cell Signaling Technology.

Transplantation *in vivo*

Transplantation *in vivo* was executed as previously described (Shi et al., 2005; Bai et al., 2017a). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Jining Medical University. Streptozocin (STZ, optimal concentration of 50 mg/kg) was intraperitoneally injected each day for 5 days into 100 mice at 6 weeks of age. The Blood glucose of mice were measured from snipped tails every day after injection by GlucoTREND (Roche), the hyperglycemia (> 13.9 mM) was found after 7 days, and stabilized after 10 days. When the blood glucose levels of the mice rose above 13.9 mM, either 1×10^6 normal MSCs, induced MSCs or SB-431542-treated induced MSCs were seeded into the renal capsule of the mice. For the sham control, cells were replaced with PBS. The blood glucose in the peripheral blood was measured every 4 days after transplantation.

Statistical Analysis

All experiments were performed at least three times independently and repeated in triplicate. All data were analyzed using SPSS 16.0. All results represent the mean ± SD (standard deviation). The differences in the data were assessed with the Student's *t*-test. $P < 0.05$ were considered statistically significant.

RESULTS

Cocktail Factors Induce the Differentiation of Beta Cells From Umbilical Cord MSCs

Mice umbilical cord MSCs expanded rapidly to exhibit classical "fusiform" morphology after isolation from Wharton's Jelly. The expression of specific markers in these cells, including CD44, CD90, CD105, Ngn3, and Pdx1, was then analyzed (**Figure 1**). Based on flow cytometry (FCM), the mice umbilical cord MSCs were positive for specific markers of MSCs including CD44, CD90, and CD105 (**Figure 1A**) but negative for specific

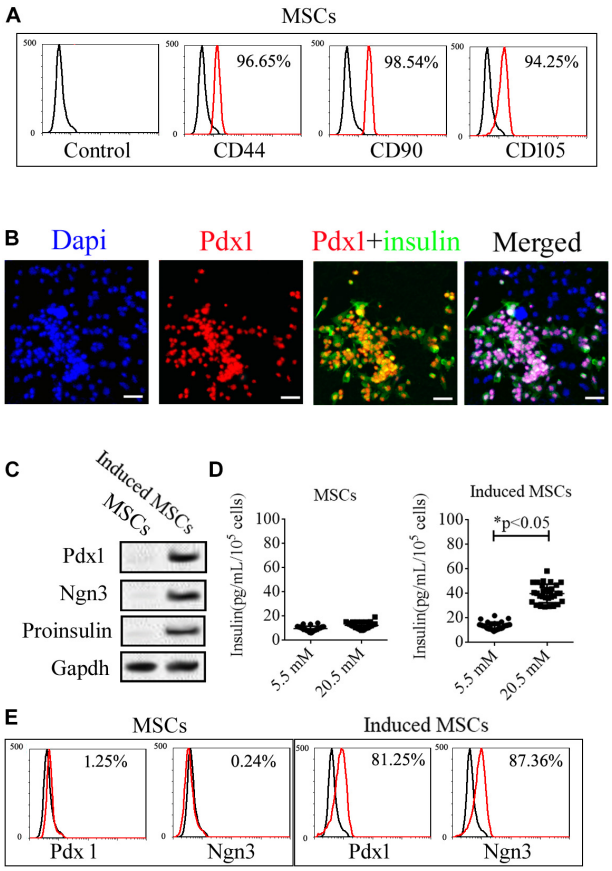

FIGURE 1 | Expression of specific markers in uninduced and induced mice umbilical cord MSCs. **(A)** The isolated MSCs were positive for the MSC surface markers, CD44, CD90, and CD105, based on flow cytometry. **(B)** Mice umbilical cord MSCs were induced to differentiate into pancreatic beta cells with the use of a cocktail of factors, following which, the expression of the islet hormones, Pdx1 and insulin, was confirmed with immunofluorescence microscopy. **(C)** Based on western blotting, the induced MSCs exhibited expression of the islet hormones, Ngn3, Pdx1, and insulin, while the uninduced MSCs showed no expression. **(D)** Glucose-induced insulin secretion (GSIS) as measured by ELISA indicated that the insulin secretion of induced MSCs but not uninduced MSCs increased when treated with increasing glucose concentrations ($n = 20$; paired two-tailed *t*-test). **(E)** Expression of the islet markers, Pdx1 and Ngn3, in the induced MSCs was confirmed by flow cytometry.

markers of pancreatic beta cells including insulin, Ngn3, and Pdx1 based on immunofluorescence assays and western blotting (**Figures 1B,C**). We then used cocktail factors to induce the differentiation of beta cells from umbilical cord MSCs. Immunofluorescence microscopy and western blotting confirmed the expression of the islet hormones, Ngn3, Pdx1, and insulin in the induced MSCs (**Figures 1B,C**), with approximately 80% of the induced MSCs showing positive for both insulin and Pdx1 and the remainder showing negative for these islet hormones (**Figure 1B**). FCM confirmed the expression of Ngn3 and Pdx1 in the induced MSCs with no expression observed in the uninduced MSCs (**Figure 1E**). Insulin release is an important function of pancreatic beta cells, and GSIS as detected by ELISA demonstrated that the induced MSCs released insulin when treated with 5.5 and 20.5 mM glucose, while the uninduced MSCs did not release insulin *in vitro* (**Figure 1D**).

The Expression of Specific Genes in the Five Stages of MSC Differentiation

Differentiation of umbilical cord MSCs into beta cells was induced by treatment with 5-AZA, β-mercaptoethanol, non-essential amino acids, ATRA, TSA, and nicotinamide. To elucidate the molecular mechanisms of beta cell differentiation from MSCs, the differentiation program was divided into five stages (**Figure 2A**). Ngn3 (Neurogenin3) is a member of the bHLH (basic helix-loop-helix) family of transcription factors involved in the development of neural stem cells in the

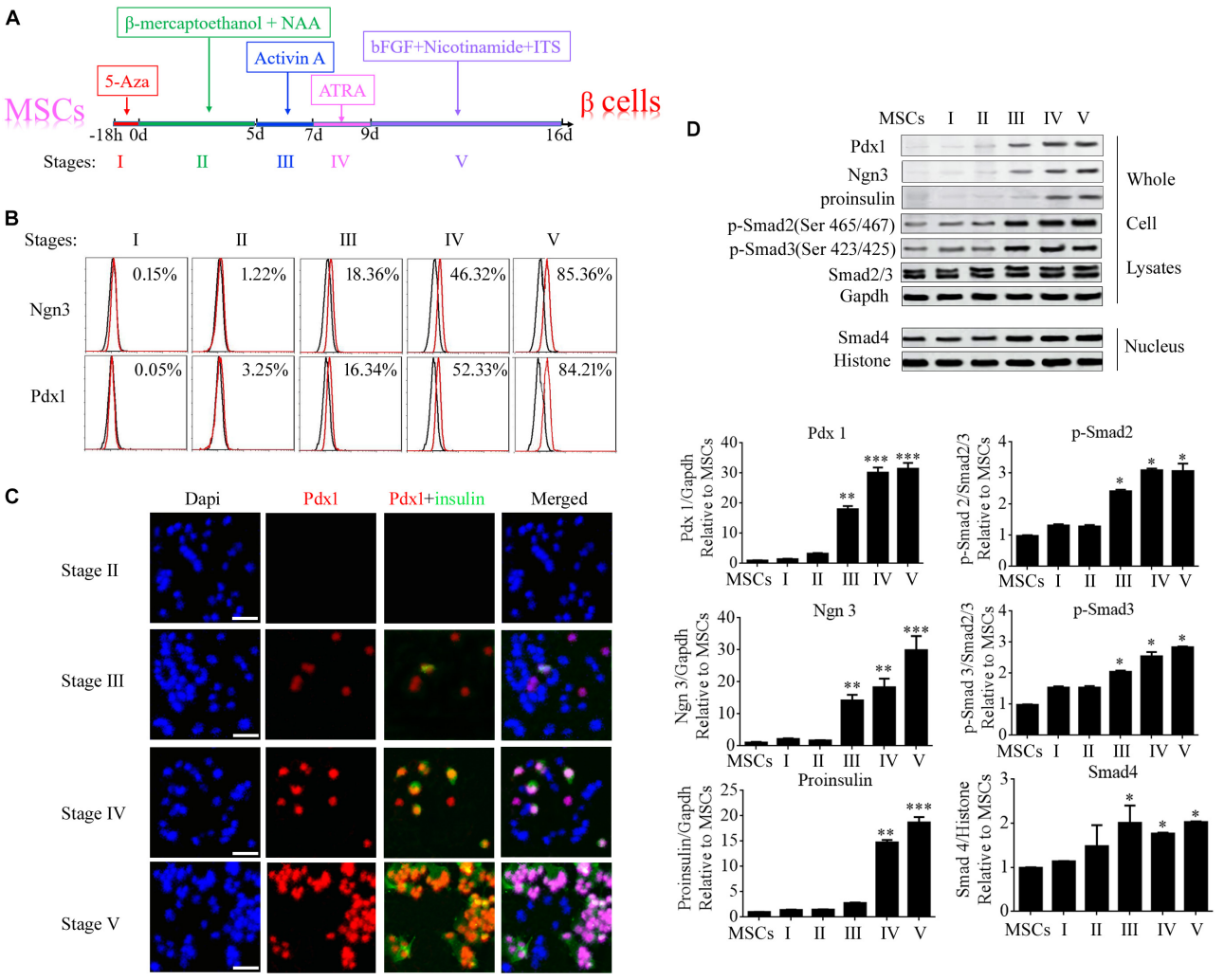

FIGURE 2 | Specific genes were significantly up-regulated during stage III of beta cell differentiation from MSCs. **(A)** Schematic diagram of the differentiation program. The program was divided into five stages according to the order in which the induction factors were added. **(B)** Flow cytometry analysis of the expression of Ngn3 and Pdx1 during each stage of differentiation. **(C)** Immunofluorescence microscopy was used to determine the expression of Pdx1 and insulin during each stage of differentiation. **(D)** Western blotting was used to analyze the expression of proteins during each stage, including the islet hormones, Ngn3 and Pdx1, and the main members of the TGF-β/Smad pathway, namely p-Smad2, p-Smad3, and Smad4. The data showed that these proteins were markedly elevated from stage III, implying that the TGF-β/Smad pathway plays an important role in beta cell differentiation. Values represent the mean ± SEM, $n = 3$. The paired two-tailed t-test was used for comparisons (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Gapdh and Histone were used as endogenous controls in the lysate and nuclear fractions, respectively.

neuroectoderm, and it is expressed in scattered cells throughout the embryonic pancreas (Gradwohl et al., 2000). During the generation of the pancreas, Ngn3 is required for the development of pancreatic endocrine cells and it is considered a marker for islet precursor cells (Habener et al., 2005). The *Pdx1* (pancreatic and duodenal homeobox 1) gene is also known as *Ipf1* (insulin promoter factor 1) or *Idx1* (islet/duodenum homeobox 1), and it is selectively expressed in islet beta cells where it binds to the promoter region of the insulin gene in the mouse. Therefore, in the present study, the expression of Ngn3 and Pdx1 was determined at each stage of the differentiation program using FCM, immunofluorescence microscopy, and western blotting. The data showed that Ngn3 and Pdx1 were significantly elevated from stage III and insulin was elevated from stage IV (**Figures 2B–D**). Activin A is a member of the Activin family, which is a major branch of the TGF-β superfamily. In stage III, activin A was added to the MSCs to continue the induction of their differentiation into beta cells. The expression of the main members of the TGF-β superfamily, Smad2/3, Smad4, phosphorylated (p-)Smad2, and p-Smad3, was also determined using western blotting. The results demonstrated that cytoplasmic levels of p-Smad2 and p-Smad3 and the nuclear level of Smad4 were significantly up-regulated from Stage III following activin A treatment (**Figure 2D**). These data implied that the TGF-β/Smad pathway plays an important role in inducing the formation of beta cells from stem cells.

Pancreatic miRNAs Also Exhibited Increased Expression From Stage III of MSC Differentiation

MicroRNAs in mammals exhibit developmental stage-specific or tissue-specific expression, implying that miRNAs play important roles in development processes. Several miRNAs regulate the development of beta cells and their secretion of insulin, including miR-375, miR-124a, and miR-26a. In the present study, we reviewed previous reports to identify the miRNAs expressed in developing or adult pancreatic tissue and/or that play a functional role in islet or insulinoma cells (**Supplementary Table S2**). To determine whether these miRNAs were up-regulated in the induced MSCs, we performed RT-qPCR at every stage of beta cell differentiation. The data were analyzed with the $2^{-\Delta\Delta CT}$ method and illustrated with a heat map (**Figure 3A**). The results indicated that specific miRNAs were dramatically elevated from stage III to V, with miR-375 and miR-26a being among those exhibiting the greatest increase in expression. The expression levels of miR-375 and miR-26a were positively correlated with those of Ngn3 and Pdx1 (determined in the previous section) for the normal MSCs and induced MSCs ($p < 0.05$). To determine whether the expression of miR-375 and miR-26a could induce the differentiation of beta cells from normal MSCs without cocktail factor treatment, we synthesized miR-375 and miR-26a mimics for co-transfection into normal MSCs, however, Pdx1 and proinsulin remained negative for expression after 16 days post-transfection (**Supplementary Figure S1**).

In our previous report (Bai et al., 2017a), miR-375 and miR-26a combined with niacinamide were shown to promote beta cell differentiation from MSCs. We, therefore, determined the protein levels of the targets of miR-375 and miR-26a, namely Sox6, Bhlhe22, and Mtpn, and found these proteins to be markedly down-regulated after cocktail factor treatment, while the opposite trend was observed when the expression of miR-375 and miR-26a was inhibited in induced MSCs (**Figure 3B**). We next investigated the role of miR-375 and miR-26a in insulin secretion in induced MSCs with the GSIS method. Our results demonstrated that when miR-375 or miR-26a were over-expressed in combination or singly in induced MSCs, the beta cells derived from these MSCs exhibited normal promotion of insulin secretion, while the single or combined expression of miR-26a and miR-375 or its inhibitors decreased insulin secretion under the treatment of 20.5 mM glucose (**Figure 3C**). These results indicated that the TGF-β/Smad pathway plays an important role in the expression of these miRNAs, but the mechanisms by which the TGF-β/Smad pathway regulate miRNA transcription to promote beta cell differentiation from MSCs remain poorly understood.

TGF-β/Smad Pathway Enhances the Transcription of miR-375, miR-26a, and Ngn3, Thereby Promoting Beta Cell Differentiation in Stage III

Activin A is an activator of the TGF-β/Smad pathway. In earlier experiments, this protein was found to significantly elevate the levels of p-Smad2 and p-Smad3 in the whole-cell lysates of induced MSCs, as well as the nuclear expression of Smad4, from differentiation stage III onward. Phosphorylated Smad2 and Smad3 form a complex with Smad4 to translocate it into the nucleus, wherein it regulates the transcription of its target genes. To determine the mechanisms causing the elevated levels of specific genes from differentiation stage III, the ALGGEN-PROMO (version 8.3) (Farre et al., 2003) and JASPAR (version 7.0) (Khan et al., 2017, 2018) tools were used to screen for potential Smad4-binding sites in the promoter regions of these genes. PROMO is a classic bioinformatics tool for the selection of putative transcription factor-binding sites in promoter sequences of target genes and it makes use of the TRANSFAC database. Transcription factor-binding sites defined in the TRANSFAC database are used to construct specific binding site weight matrices for transcription factor-binding site prediction. JASPAR is an open-access database of curated, transcription factor binding profiles stored as position frequency matrices and transcription factor flexible models for transcription factors across multiple species in six taxonomic groups. By combining these tools, we found many putative Smad4-binding motifs within the promoter regions of miR-375, miR-26a, and Ngn3 but none were identified for Pdx1 (**Figures 4A,D,G**). Ngn3 is the earliest factor that specifically regulates the development of the endocrine compartment in the embryonic pancreas, and it regulates the transcription of many beta cell genes (Habener et al., 2005; Sancho et al., 2014).

Interactions between transcript factors and nucleic acids are usually tested using EMSA. In this study, to investigate

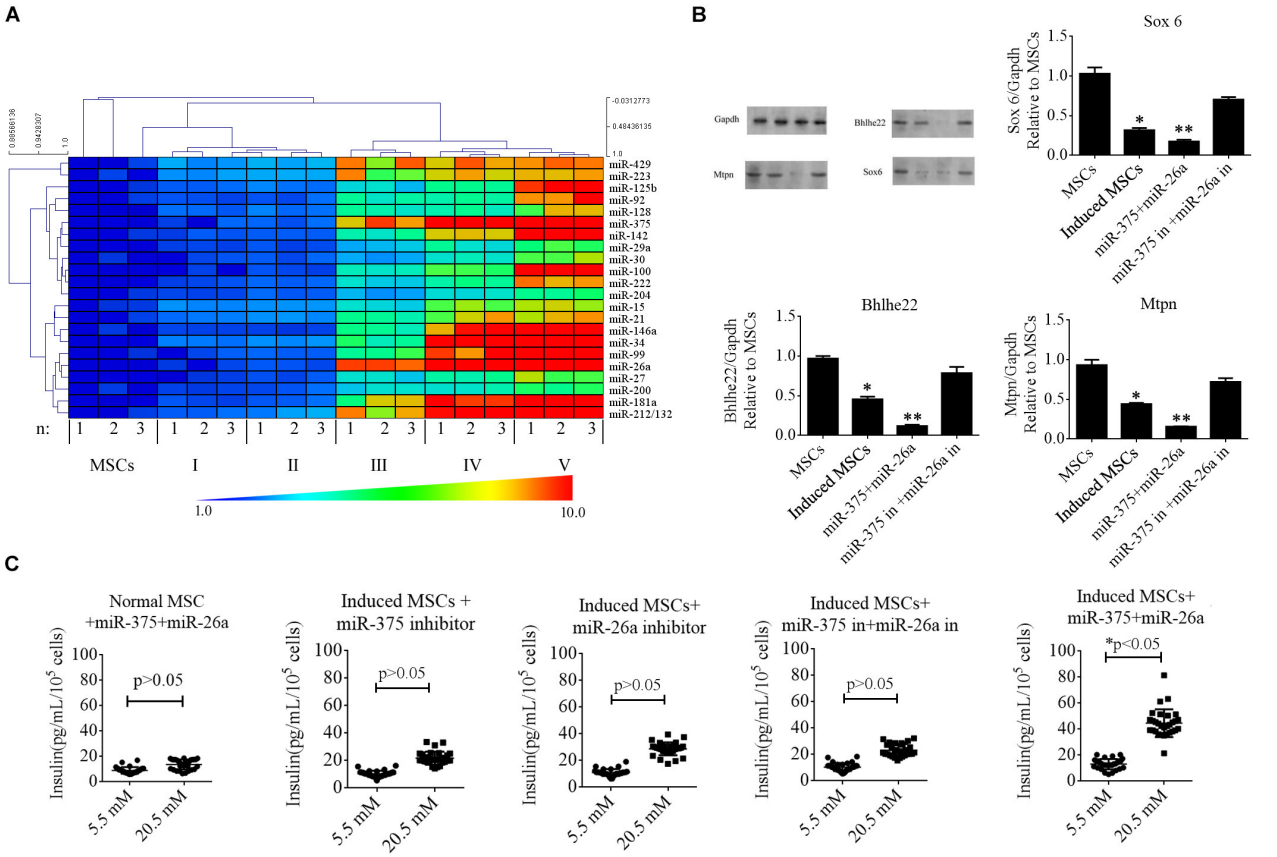

FIGURE 3 | Roles of specific miRNAs in beta cell differentiation from MSCs. **(A)** A heat map was used to represent the fold change in the expression of specific miRNAs during the five stages of differentiation of beta cells from MSCs based on RT-qPCR. MiR-375 and miR-26a were among those exhibiting the greatest increase in expression. **(B)** Western blotting analysis of targets of miR-375 and miR-26a in induced MSCs following the overexpression of miR-375 and miR-26a or that of their inhibitors (in). Over-expression of miR-375 and miR-26a inhibited the endogenous expression of Sox 6, Bhlhe22, and Mtpn, while the over-expression of miR-375 and miR-26a inhibitors enhanced the expression of these targets. Gapdh was used as an endogenous control. Values represent the mean ± SEM, $n = 3$. **(C)** Glucose-stimulated insulin secretion (GSIS) analysis of induced MSCs following the overexpression of miR-375 and miR-26a or that of their inhibitors (in). The data show that insulin secretion of all groups of induced MSCs decreased when the cells were treated with a higher glucose concentration except when miR-375 and miR-26a were over-expressed ($n = 30$; paired two-tailed t-test).

the interactions predicted by bioinformatic analysis, probes for EMSA were designed for Smad4 based on the putative binding sites within the promoter sequences of its target genes with the highest probability score for Smad4 binding. The EMSA data demonstrated that Smad4 did indeed bind to the predicted binding sites (**Figures 4B,E,H**). The specificity of protein binding to these sites was further verified by competition with a 100–200-fold molar excess of unlabeled EMSA probe. Supershifted bands were also observed following incubation with the Smad4 antibody (ChIP grade). To accurately compare the quantities of Smad4 enriched in the promoter region after induction, ChIP was combined with qPCR analysis. The input sample (whole lysate) was used as a positive control, and non-specific IgG was used as a negative control. The quantities of immunoprecipitated chromatin were dramatically changed in the induced MSCs compared to the uninduced MSCs, with a fold change in occupancy of 8.36, 7.89, and 6.61 for miR-375, miR-26a, and Ngn3, respectively (**Figures 4C,F,I**).

To further illuminate the role of the TGF-β/Smad pathway in the differentiation of beta cells from MSCs, SB-431542 (a TGF-β/Smad pathway inhibitor) was added to the cocktail of factors. The levels of p-Smad2, p-Smad3, and intranuclear Smad4, as well as the interactions of Smad2 or Smad3 with Smad4, were analyzed using Duolink PLA and western blotting. The data showed that the number of PLA-positive cells was dramatically increased under treatment with the cocktail factors compared with the uninduced MSCs, while treatment with SB-43152 produced much fewer PLA-positive cells relative to the induced MSCs alone (**Figures 5A,B**). We then performed western blotting of SB-431542-treated induced MSCs using specific antibodies against p-Smad2, p-Smad3, and intranuclear Smad4. The results showed that SB-431542 down-regulated p-Smad2 and p-Smad3, which led to decreased levels of intranuclear Smad4 (**Figures 5C**). To test for beta cell differentiation after SB-431542 treatment, the percentage of Pdx1- and Ngn3-positive cells, the expression levels of miR-375 and miR-26a, and the level of insulin

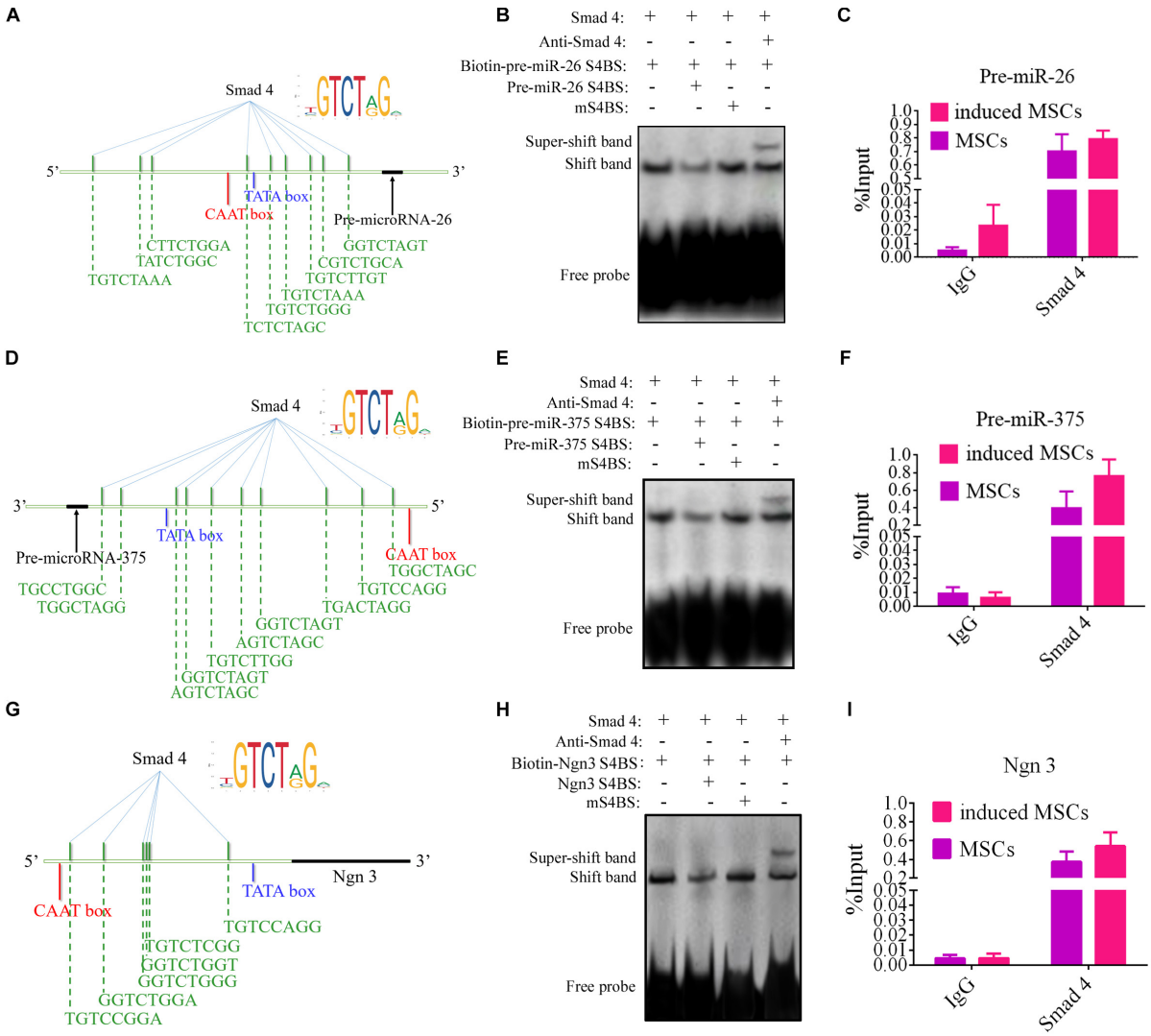

FIGURE 4 | EMSA and ChIP-qPCR assay of the physical binding of Smad4 to the promoter regions of miR-375, miR-26a, and Ngn3. **(A**,**D**,**G)** Diagrams indicating the predicted binding sites for Smad4 within the promoters of miR-375, miR-26a, and Ngn3. **(B**,**E**,**H)** EMSA of the physical binding of Smad4 to the promoter regions of miR-375, miR-26a, and Ngn3. The promoter Smad4-binding sites with the highest scores based on bioinformatic predictions were selected for EMSA probe design. Following induction, cell nuclear extracts were prepared and incubated with the specific biotinylated EMSA probe for Smad4. Non-biotinylated EMSA probes were used as competitor probes. Smad4 was observed to bind to each of the three predicted binding sites incorporated into the EMSA probes. Furthermore, incubation with specific antibodies against Smad4 clearly resulted in supershifted bands. Probes containing mutated binding sites (mS4BS) were unable to compete for binding. S4BS, Smad4-binding site. **(C**,**F**,**I)** The amounts of Smad4 enriched within the promoter regions of miR-375, miR-26a, and Ngn3 in uninduced and induced MSCs were quantified by ChIP-coupled real-time qPCR. The percentages of input were calculated according to the threshold cycle values (CT). Non-specific IgG was used as the negative control. The amounts of immunoprecipitated chromatin were significantly higher for the induced MSCs than the uninduced MSCs, with a fold change in occupancy of 8.36, 7.89, and 6.61 for miR-375, miR-26a, and Ngn3, respectively. Values represent the mean ± SEM, $n = 3$.

secretion after treatment were verified using FCM, qPCR, and the GSIS method, respectively. The results showed that all three measures were dramatically decreased after SB-431542 treatment (**Figures 5D–F**). These data confirmed that the TGF-β/Smad pathway plays an important role in beta cell differentiation.

Transplantation *in vivo*

To confirm the *in vivo* function of beta cells differentiated from MSCs, we transplanted MSCs, induced MSCs, and SB-431542-treated induced MSCs under the kidney capsule of STZ-treated diabetic mice ($n = 10$). After transplantation, the sham group and MSC-transplanted mice all died within 16 and 20 days due to persistent metabolic acidosis, respectively. The survival probability of induced MSC-transplanted mice was approximately three times higher than that of SB-431542-treated induced MSC-transplanted mice (**Figure 6C**). Twenty days after transplantation, the blood glucose of the induced MSC-transplanted mice decreased to a normal level (less than 13.9 mM). In contrast, the blood glucose of the SB-431542-treated induced MSC-transplanted mice remained above

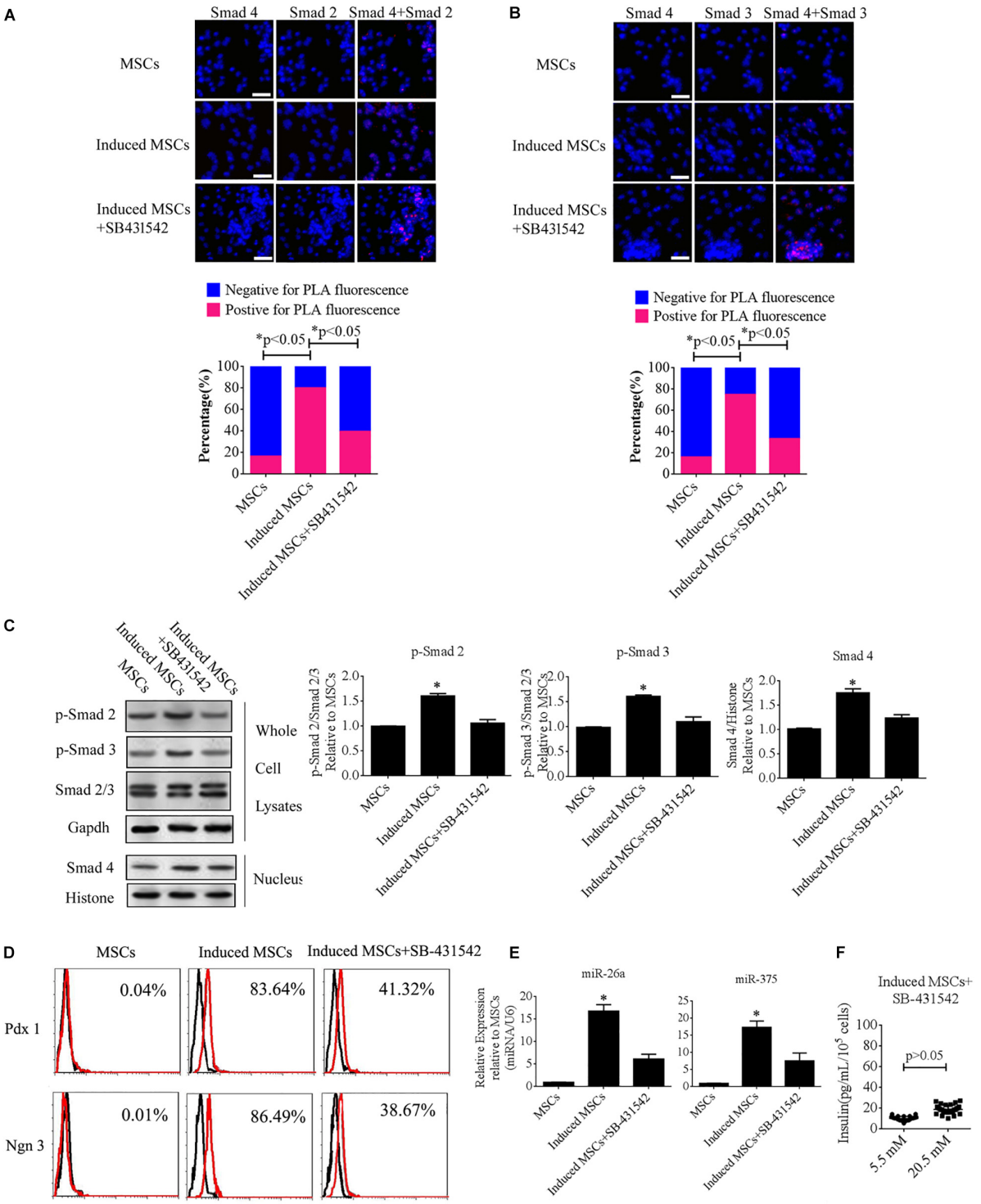

FIGURE 5 | SB-431542 inhibits beta cell differentiation from MSCs by blocking the TGF-β/Smad pathway. **(A,B)** Duolink PLA fluorescence assay of the interactions of p-Smad2 or p-Smad3 with Smad4 in MSCs following different treatments. Colocalization of p-Smad2 or p-Smad3 with Smad4 around and within nuclear foci is shown in the upper panel, which corroborated the interaction of these proteins in uninduced and induced MSCs (scale bar = 50 μm). The percentages of PLA-positive cells following the different treatments are shown in the bottom panel. The number of positive cells was significantly elevated after treatment with the cocktail of factors compared with uninduced MSCs or SB-431542-treated induced MSCs. **(C)** Western blotting showed that SB-431542 down-regulated p-Smad2 and p-Smad3, which led to decreased levels of intranuclear Smad4. **(D)** Flow cytometry analysis of Pdx1 and Ngn3 expression showed that the percentage of Pdx1- and Ngn3-positive induced MSCs was markedly decreased after SB-431542 treatment. **(E)** RT-qPCR demonstrated that SB-431542 prevented activation of the TGF-β/Smad pathway leading to reduced expression of miR-375 and miR-26a. **(F)** Glucose-stimulated insulin secretion (GSIS) of induced MSCs after SB-431542 treatment indicated that insulin secretion under conditions of 20.5 mM glucose increased to a much smaller degree than that in cells not treated with the inhibitor (n = 30; paired two-tailed t-test).

13.9 mM (**Figure 6D**). At 40 days post-transplantation, the mice were sacrificed and the engrafted kidneys underwent histologic analysis. Hematoxylin and eosin staining revealed the presence of pancreatic beta cells adjacent to the mice kidney in the induced MSC-transplanted mice and SB-431542-treated induced MSC-transplanted mice, while none were observed in the MSC-transplanted mice (**Figure 6A**). IHC showed the presence of pancreas islet-like structures adjacent to the mouse kidney (**Figures 6B**). These data revealed that the induced MSCs, when transplanted into the renal capsule, could improve blood glucose control and functionally rescue the STZ-treated diabetic mice, while SB-431542 treatment of the induced MSCs did not have this beneficial effect.

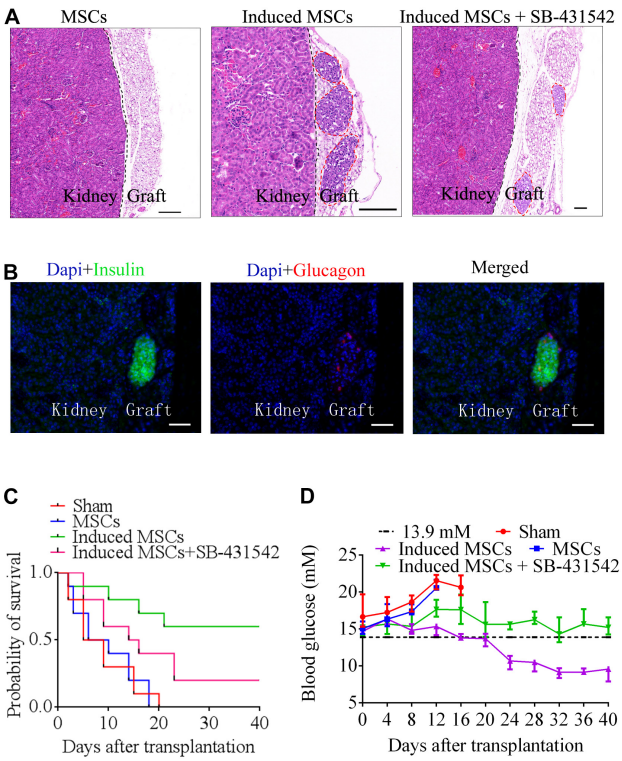

FIGURE 6 | Beta cells differentiated from MSCs were transplanted below the renal capsule of STZ-treated diabetic mice. **(A)** Hematoxylin and eosin staining of a graft at day 40 after transplantation. Higher numbers of islet-like structures were observed in induced MSCs than in SB-431542-treated induced MSCs (red circles). The MSC-transplanted group did not exhibit any of these structures in the graft 18 days after transplantation. **(B)** Immunofluorescence images of a graft at day 40 after transplantation, stained for insulin and glucagon to confirm the presence of engrafted islets (scale bar = 50 μm). **(C)** Survival curves of diabetic mice following transplantation. Induced MSC-transplanted diabetic mice (n = 10) survived the longest with a survival probability reaching 60%, while the survival probability of SB-431542-treated induced MSC-transplanted diabetic mice (n = 10) only reached 20%, and that of the sham control and MSC-transplanted groups (n = 10) was 0%. **(D)** Blood glucose analysis of the various transplantation groups showed that the induced MSC-transplanted diabetic mice recovered to normal glucose levels (<13.9 mM) after 20 days. The other groups retained high levels of blood glucose (>13.9 mM) over the 40 days observation period.

Taken together, our data revealed that activin A activates the TGF-β/Smad pathway through phosphorylation of Smad2 and Smad3 during beta cell differentiation from MSCs, and that phosphorylated Smad2 and Smad3 then interacts with Smad4 to form a transcription complex that enters the nucleus. This complex combines with the promotor sequences of the endocrine-specific transcription factor, Ngn3, and the pancreas-specific miRNAs, miR-375, and miR-26a, to enhance their transcription in stage III and thereby initiate MSC differentiation into beta cells (**Figure 7**).

DISCUSSION

The TGF-β superfamily is a component of a critical pathway involved in cell-to-cell signaling during a multitude of processes, including cell proliferation in somatic tissues, specification of cell fate during embryogenesis, cell differentiation, and cell death. Apart from TGF-β, the superfamily also includes the activins and the bone morphogenetic proteins (BMPs). In particular, activin A phosphorylates Smad2 and Smad3 to activate the TGF-β/Smad pathway, and this activin has been extensively used in the production of beta cells from stem cells (Shi et al., 2005; Pagliuca et al., 2014; Bai et al., 2017a) as it is important for early definitive endoderm development. Activin A binds to cell surface receptors to induce the expression of specific targets, including mix11 and goosecoid, thereby regulating the differentiation of pancreatic beta cells. In addition, activin A also promotes insulin secretion to maintain its function in cultured pancreatic islets (Florio et al., 2000; Li et al., 2004; Shi et al., 2005). However, there have been no reports to date regarding the mechanism by which activin A directly regulates the expression of pancreatic genes.

The endocrine-specific transcription factor, Ngn3, plays an important role in the production of beta cells from other

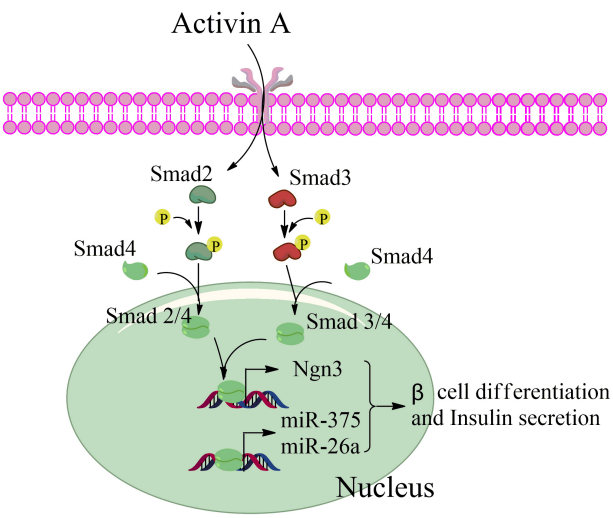

FIGURE 7 | Schematic of the factors promoting pancreatic beta differentiation from mesenchymal stem cells.

pancreatic cells through cell reprogramming, including exocrine cells (Zhou et al., 2008) and ductal cells (Sancho et al., 2014). In pancreatic exocrine cells in mice, Ngn3 works in concert with two other transcription factors, Pdx1 and Mafa, to reprogram this differentiation to form cells that are indistinguishable from endogenous islet beta cells in size, shape, and ultrastructure. The ubiquitin ligase, Fbxw7, normally destabilizes Ngn3; genetic deletion of *Fbxw7* in pancreatic ductal cells, a type of stem cell in the pancreas, stimulates the direct conversion of these cells into endocrine β-cells (Sancho et al., 2014; Seifert and Xiong, 2014). In the present study, activin A was added into the differentiation program of beta cells in stage III, which activated the TGF-β/Smad pathway. Our results revealed that activin A enhanced the transcription of *Ngn3* through Smad4 binding of the promoter region of *Ngn3*. Therefore, activin A plays an important role in the transcription of endocrine-specific genes during beta cell differentiation from MSCs.

MicroRNAs play an important role in the regulation of gene expression at the post-transcriptional level (Bartel, 2004; Tang et al., 2007, 2009; Bai et al., 2017b). Many studies have investigated the expression and function of miRNAs in the formation of pancreatic beta cells from stem cells, but studies on the transcription of specific miRNAs during the formation of pancreatic beta cells from stem cells are limited. MiR-375 is a pancreas-specific miRNA that acts in endocrine tissues and is highly expressed in the pancreas (Poy et al., 2004; Avnit-Sagi et al., 2009; Bai et al., 2017a). Knockout of miR-375 disrupts islet morphogenesis and reduces endocrine cell differentiation. MiR-375 can induce the formation of pancreatic beta cells from adult or embryonic stem cells. Furthermore, miR-375 can also directly induce the differentiation of pancreatic beta cells from induced pluripotent stem cells (Poy et al., 2009; Bai et al., 2017a), however, the mechanism underlying this process remains unclear (Lahmy et al., 2014). MiR-26a was shown to be highly enriched in pancreatic beta cells in the current study, and it is known to be upregulated in response to glucose-stimulated repression of Sox6 and Bhlhe22 expression (Melkman-Zehavi et al., 2011). Furthermore, miR-26a can promote pancreatic cell differentiation both *in vitro* and *in vivo* by targeting the TET family of proteins (Fu et al., 2013). In our previous research, miR-375 and miR-26a were found to be dramatically elevated following the differentiation of pancreatic beta cells from MSCs (Bai et al., 2017a). The functions of miR-375 and miR-26a during this process lie not only in the promotion of differentiation but also in their effect on insulin secretion under glucose stimulation. However, the upstream mechanisms by which cocktail factors elevate the expression of miR-375 and miR-26a to promote the differentiation of pancreatic beta cells remain to be elucidated. In the present study, to investigate the expression of pancreatic-specific miRNAs during the differentiation of pancreatic beta cells from MSCs, we divided the differentiation program into five stages and tested the expression of specific miRNAs at each stage using RT-qPCR. The data showed that these miRNAs, particularly miR-375 and miR-26a, exhibited increased expression from stage III. We further demonstrated that the promoter regions of miR-375 and miR-26a were bound by activin A-activated Smad4, which enhanced their transcription and thereby promoted the differentiation of pancreatic beta cells from MSCs and increased their insulin secretion.

CONCLUSION

In conclusion, we systematically elucidated the important role of activin A in inducing the differentiation of pancreatic beta cells from MSCs. Activin A phosphorylates Smad2 and Smad3 to activate the TGF-β/Smad pathway, thereby enhancing the transcription of the endocrine-specific transcription factor, Ngn3, which, in turn, promotes the differentiation of pancreatic beta cells. Additionally, miR-26a and miR-375, important miRNAs in the formation of beta cells and in modulating their secretion of insulin, are up-regulated by Smad4. *In vivo* and *in vitro* addition of an inhibitor of the TGF-β/Smad pathway, SB-431542, during the differentiation of the beta cells further demonstrated the critical role of the TGF-β/Smad pathway in the differentiation of beta cells and in insulin secretion. Our study, which particularly focused on the transcription of specific genes involved in beta cell formation from stem cells, may assist in the future development of effective cell transplant therapies for the treatment of type I diabetes mellitus.

DATA AVAILABILITY STATEMENT

The raw data used to support the conclusions of this article will be made available by the corresponding authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Jining Medical University.

AUTHOR CONTRIBUTIONS

YG performed the cell differentiation, western blotting, and FCM, and drafted the manuscript. CB, SD, XZ, and RZ prepared the cell cultures. CB performed the RNAi assays. XL and CB analyzed the data and reviewed the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00351/full#supplementary-material

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CORRECTION
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Corrigendum: Role of TGF-β/Smad Pathway in the Transcription of Pancreas-Specific Genes During Beta Cell Differentiation

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A Corrigendum on

Role of TGF-β/Smad Pathway in the Transcription of Pancreas-Specific Genes During Beta Cell Differentiation

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In the original article, there was a mistake in **Figure 3B** as published. The immunoblot bands of Mtpn was lost. The corrected **Figure 3B** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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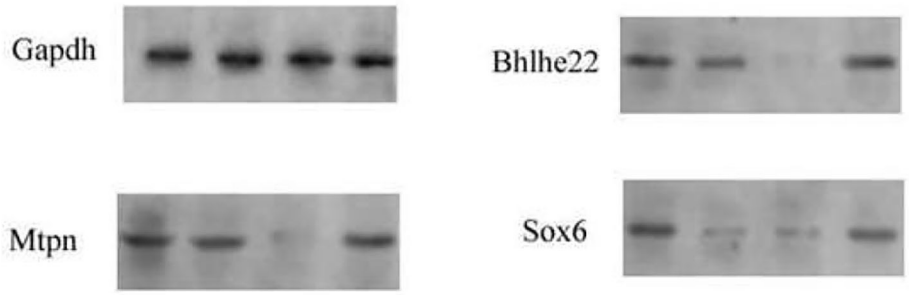

FIGURE 3 | Western blotting analysis of targets of miR-375 and miR-26a in induced MSCs following the overexpression of miR-375 and miR-26a or that of their inhibitors (in).

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REVIEW
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IGF2BP3 From Physiology to Cancer: Novel Discoveries, Unsolved Issues, and Future Perspectives

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RNA network control is a key aspect of proper cellular homeostasis. In this context, RNA-binding proteins (RBPs) play a major role as regulators of the RNA life cycle due to their capability to bind to RNA sequences and precisely direct nuclear export, translation/degradation rates, and the intracellular localization of their target transcripts. Alterations in RBP expression or functions result in aberrant RNA translation and may drive the emergence and progression of several pathological conditions, including cancer. Among the RBPs, insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3) is of particular interest in tumorigenesis and tumor progression. This review highlights the molecular mechanisms underlying the oncogenic functions of IGF2BP3, summarizes the therapeutic potential related to its inhibition and notes the fundamental issues that remain unanswered. To fully exploit IGF2BP3 for tumor diagnosis and therapy, it is crucial to dissect the mechanisms governing IGF2BP3 re-expression and to elucidate the complex interactions between IGF2BP3 and its target mRNAs as normal cells become tumor cells.

Keywords: RNA-binding protein, IGF2BP3, embryonic development, cancer, biomarker

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INTRODUCTION

RNA-binding proteins (RBPs), along with microRNAs (miRNAs; **Box 1**) and long non-coding RNAs (lncRNAs; **Box 1**), dictate the entire RNA life cycle from alternative splicing to nuclear export, transcript storage, stabilization, subcellular localization and degradation (for a review, please consider Coppin et al., 2018), thus representing major cotranscriptional and/or posttranscriptional regulators of gene expression. In humans, 1393 RBPs, which account for 7.5% of the proteome, have been recently identified (Hentze et al., 2018). Each contemporary RBP binds to hundreds of RNAs, including both coding and non-coding RNAs, and affects their expression and translation, thus playing a wide regulatory role in practically all physiological processes. Accordingly, the deregulation of RBPs frequently occurs in pathological conditions, particularly cancer (Coppin et al., 2018). Recent next-generation sequencing analyses in tumor specimens have demonstrated that genes encoding RBPs have significantly higher overexpression than non-RBP-coding genes (Neelamraju et al., 2018). In addition, evidence has consistently shown that RBPs are strongly implicated in the regulation of most cancer hallmarks, such as cell proliferation, resistance to cell death, stemness, cell dissemination, and immune system evasion, and may act as promising biomarkers of tumor progression (Pereira et al., 2017).

At least 16 families of RBPs are deregulated in cancer (Pereira et al., 2017). Of those, the highly conserved family of insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs), which includes the paralogs IGF2BP1, IGF2BP2, and IGF2BP3, primarily play oncogenic roles in cancer. Over the past few years, studies have increasingly documented the contribution of IGF2BPs to

BOX 1 | Glossary.

Chromatin immunoprecipitation (ChIP): method for the identification of transcription factor DNA target, based on DNA/proteins crosslinking, immunoprecipitation of the transcription factor of interest, DNA extraction, and qRT-PCR or sequencing

circularRNAs (circRNAs): large class of non-coding RNAs deriving from a non-canonical alternative splicing called "backsplicing" and characterized by a covalent link between the 3′ and 5′ ends

K-homology (KH) domains: RNA-binding domain of ∼70 amino acids which forms a three-stranded β-sheet packed against three α-helices, which recognizes both DNA and RNA throught a conserved GXXG loop

Locasomes: large, motile RNP granules containing untranslated mRNAs and acting as cytoplasmic repository for transcripts

Long non-coding RNAs (lncRNAs): non-coding transcripts larger than 200 nucleotides regulating gene expression

microRNAs (miRNAs): class of short non-coding RNAs (19-25 nucletides) regulating posttranscriptional silencing of target transcripts

N^6-methyladenosine modification: epigenetic RNA modification influencing mRNA fate including stability, splicing, and translation rate

Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP): method for mapping RBP/RNA interaction, based on incorporation of photoreactive nucleosides in newly transcribed RNAs followed by UV crosslinking between transcripts and RBPs, immunoprecipitation of the RBP of interest and RNA extraction. Upon reverse transcription, sequencing analysis is performed

Processing bodies (P-bodies): cytoplasmic RNP granules composed by mRNAs and proteins with a role in translation repression and mRNA decay

Ribonucleoprotein (RNP) granules: cytoplasmic protein/RNA assemblies acting as posttranscriptional regulators of gene expression

RNA immunoprecipitation (RIP): method for RBP RNA target identification, based on immunoprecipitation of the RBP of interest followed by RNA extraction, reverse transcription and qRT-PCR or sequencing analysis

RNA-induced silencing complex (RISC): cytoplasmic complex incorporating miRNAs for the recognition and degradation of complementary mRNAs

RNA-recognition motifs: RNA-binding domain of ∼ 80–90 amino acids which folds in two α-helices packed against a four-stranded anti-parallel β-sheet, involved in RNA recognition

Stress granules: cytoplasmic RNP granules, composed by mRNA, proteins and 40S ribosome subunits, that are induced under stress conditions and where transcripts are stabilized and translation is silenced

Untranslated region (UTR): sequences of mature mRNA, located upstream (5′-UTR) or downstream (3′-UTR) from the coding region, holding post-transcriptional regulatory elements that affect gene expression

fundamental processes in cancer biology, and their overexpression has been widely associated with adverse patient outcomes in many different tumors. This family was named IGF2BPs because, originally, the three members were identified as posttranscriptional regulators of the fetal growth factor IGF2 (Nielsen et al., 1999). Structurally, IGF2BP1, IGF2BP2, and IGF2BP3 share a 59% amino acid sequence identity, which reaches 73% between IGF2BP1 and IGF2BP3. These family members are characterized by a peculiar structure composed of the following six RNA-binding domains: two RNA recognition motifs (RRMs; **Box 1**) in the N-terminal region and four K-homology (KH; **Box 1**) domains in the C-terminal region arranged in three pairs of didomains (RRM1 + 2, KH1 + 2, and KH3 + 4) and separated by flexible linkers (Jia et al., 2018). Overall, all four KH domains contribute to RNA-binding, ribonucleoprotein (RNP) granules formation (**Box 1**), and cellular localization (Wachter et al., 2013). In general, IGF2BPs bind to their target RNAs at the 5′-UTR, 3′-UTR or coding regions (**Box 1**) by recognizing specific RNA motifs, such as the first identified CAUH (H = A, U, C) (Hafner et al., 2010). In addition, posttranscriptional modifications of target RNAs, such as the N^6-methyladenosine modification (**Box 1**), render modified RNAs more attractive for IGF2BP binding (Huang et al., 2018b). Approximately 55–70% of the recognized target RNAs are shared among the three proteins (Huang et al., 2018b). Accordingly, IGF2BPs can form homodimers and heterodimers on target RNAs, partially explaining the observed overlap among the recognized targets (Nielsen et al., 2004; Hammerle et al., 2013). Physiologically, the IGF2BPs are expressed during embryogenesis but are absent in adult tissues, except for IGF2BP2, which is mainly involved in metabolic processes and is maintained in most normal tissues (Nielsen et al., 1999; Dai et al., 2015). IGF2BPs are mainly localized in the cytoplasm (Nielsen et al., 1999), but some evidence also demonstrates their presence in the nucleus, where they directly bind to target RNAs after transcription and shuttle them between the nucleus and cytoplasm. The nuclear role of IGF2BPs is further demonstrated by the identification of nuclear export signals within the RNA-binding KH2 and KH4 domains (Nielsen et al., 2003; Oleynikov and Singer, 2003; Rivera Vargas et al., 2014). However, the exact mechanisms governing the nuclear localization of IGF2BPs still need elucidation. For a more general introduction to the phylogenetic origin, gene/mRNA/protein structure and expression pattern of the IGF2BPs in normal or pathological tissues, readers are referred to several excellent reviews (Bell et al., 2013; Lederer et al., 2014; Degrauwe et al., 2016b; Cao et al., 2018).

This review focuses on IGF2BP3 and its role in human cancer, highlighting the contradictions and discrepancies related to its still poorly understood mechanisms of action and the potential of this protein as diagnostic, prognostic and therapeutic biomarker.

IGF2BP3

The *IGF2BP3* gene (also known as *IMP3*, *KOC*, *CT98*, *KOC1*, and *VICKZ3*) is located on chromosome 7p15.3 in humans (Monk et al., 2002) and encodes a 69 kDa protein; this gene was first identified by Mueller-Pillasch et al. (1997) to be overexpressed in pancreatic cancer. IGF2BP3 expression was

subsequently observed in mouse embryos (Mueller-Pillasch et al., 1999; Nielsen et al., 2002), but the physiological effects elicited by IGF2BP3 in these tissues are still elusive mostly due to the lack of available knockout *in vivo* models. Insight regarding the putative IGF2BP3 peculiar functions in normal embryonic development is based on studies investigating its ortholog Vg1-RBP in *Xenopus laevis*, which shares an 84% amino acid identity with human IGF2BP3. The loss of Vg1-RBP causes an abnormal head morphology, the lack of a lens and dorsal fin, a curved neural tube, the absence of the roof plate in the neural tube (Yaniv et al., 2003), impaired gut morphogenesis, the loss of pancreatic organogenesis (Spagnoli and Brivanlou, 2006) and the lack of meiotic maturation (Git et al., 2009). These data, which insinuate that IGF2BP3 plays a general role in neural development and organogenesis, are overall consistent with evidence concerning the spatial distribution of IGF2BP3 expression during advanced stages of gestation (E11.5–E12.5) in embryonic mice, indicating that this RBP is present in neural cells, the intestine, thymus, pancreas, and kidney epithelial germ layers (Mueller-Pillasch et al., 1999; Mori et al., 2001; Hansen et al., 2004). Evidence in humans is even more limited but confirms the major role of IGF2BP3 as an embryonic regulator. Accordingly, fetal hematopoietic progenitors, including megakaryocytes, express IGF2BP3 at higher levels than their adult counterparts, and IGF2BP3 contributes to the maintenance of the molecular and phenotypic features of fetal-type cells (Elagib et al., 2017). Accumulating data indicate that IGF2BP3 is also present in mature tissues (Hammer et al., 2005; Burdelski et al., 2018). In adult mice, IGF2BP3 is measurable in the lungs, spleen, muscles, gut, pancreas, kidneys, brain, ovaries and testes (Hammer et al., 2005; Bell et al., 2013). In human adult tissues, IGF2BP3 is detectable in the placenta, lymph nodes, tonsils, and testes (**Figure 1**), confirming an intriguing but still unclear association between IGF2BP3 and reproductive organs. Sporadic evidence regarding the role of IGF2BP3 role in human adult tissues shows that IGF2BP3 drives normal placental development through the correct migration of trophoblast cells into the maternal decidua in both *in vivo* and *ex vivo* models (Haouzi et al., 2011; Li et al., 2014).

Sexual dimorphism has been barely investigated for this RBP. IGF2BP3 mRNA expression in the mouse gonads appeared higher in testes than in ovaries (Hammer et al., 2005). A direct comparison between IGF2BP3 expression and sex was performed in the brains of zebrafish, but no differential expression was found in male versus female individuals (Arslan-Ergul and Adams, 2014).

Transgenic overexpression of IGF2BP3 was performed in mice to shed light on the effects of re-expression of this protein in adult tissues. Interestingly, transgenic mice displayed extensive remodeling of the exocrine pancreas, leading the pancreas to resemble embryonic tissues, with increased acinar cell proliferation, a reduction in the acinar cell compartment, and the appearance of interstitial cells with a dual differentiation capacity (Wagner et al., 2003). Overall, these features corresponded to acinar-to-ductal metaplasia, which represents a major origin of the pancreatic preneoplastic lesions that eventually develop into pancreatic ductal adenocarcinoma, in both humans and in mice (Chuvin et al., 2017). More recently, Palanichamy et al. (2016) created an *in vivo* model of IGF2BP3-enforced expression in a murine hematopoietic system and observed increased hematopoietic stem and progenitor cell proliferation, skewed hematopoietic development to the B cell/myeloid lineage, atypical B cell infiltration into the thymic medulla, and increased myeloid cells in the spleen, features similar to those seen early in leukemogenesis. Beyond indicating the capability of IGF2BP3 to recapitulate a fetal-like phenotype, these evidences suggest a putative role of IGF2BP3 in tumorigenesis since the *de novo* expression of RBP in adult tissues apparently provides a favorable context for the emergence of neoplastic lesions. Accordingly, IGF2BP3 is detectable in some premalignant human lesions, including dysplasia in Barrett esophagus (Gadara et al., 2017), pancreatic intraductal neoplasia (Wang et al., 2015), and atypical endometriosis (Vercellini et al., 2013); in addition, many tumor types upregulate IGF2BP3 compared to normal tissue counterparts (**Figure 2**).

REGULATION OF IGF2BP3 EXPRESSION IN CANCER

Very limited information regarding the molecular regulatory mechanisms responsible for human IGF2BP3 expression is available. The mechanisms include genomic alterations, epigenetic and transcriptional control, and post-translational modifications/interactions, summarized in a schematic in **Figure 3**.

Mutations in RBP coding genes are rare. Germline mutations affecting the coding regions of RBPs occur in less than 1% of all proteins, while only 15% of RBPs across solid tumors are mutated in the protein sequence (Sebestyen et al., 2016; Pereira et al., 2017). Accordingly, to date, mutations in the *IGF2BP3* gene have not been described, and gene amplification has been observed in less than 20% of lung adenocarcinoma, pancreatic, and bladder cancers (Panebianco et al., 2017). Furthermore, 5% of thyroid tumors and 25% of pancreatic cancers hold a specific balanced chromosomal translocation between the IGF2BP3 chromosomal locus on 7p15.3 and the actively transcribed THADA locus on 2p21, which results in the strong overexpression of IGF2BP3 (Panebianco et al., 2017).

Other mechanisms include DNA methylation and acetylation processes. Demethylated CpG islands characterize the *IGF2BP3* promoter in intrahepatic cholangiocarcinoma cases, which were in stark contrast to normal liver tissues that were heavily methylated (Gao et al., 2014). More recently, a large-scale sequencing analysis of datasets of 15 cancer types from The Cancer Genome Atlas (TCGA) confirmed these data, showing an inverse correlation between the DNA methylation status of the *IGF2BP3* promoter and *IGF2BP3* mRNA expression (Panebianco et al., 2017). Consistently, the treatment of murine osteosarcoma cells with a DNA methyltransferase inhibitor or histone deacetylase inhibitors resulted in a significant upregulation of IGF2BP3 expression (Ueki et al., 2012).

In addition, the increased transcriptional activation of the *IGF2BP3* promoter has been attributed to the binding

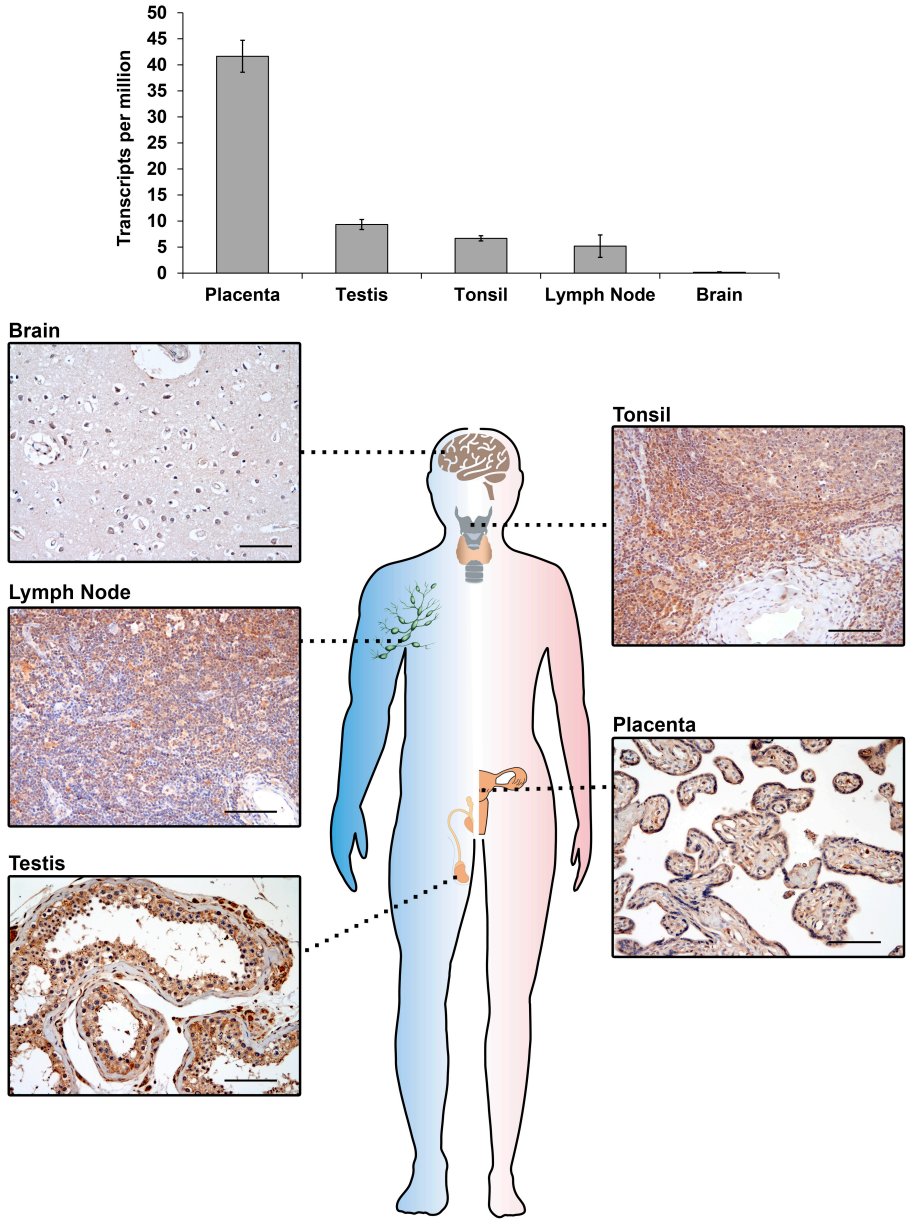

FIGURE 1 | IGF2BP3 mRNA **(top)** and protein **(bottom)** expression detected by RNA-seq or immunohistochemical analyses in normal human tissue samples. RNA-seq data are courtesy of the Human Protein Atlas, www.proteinatlas.org (Uhlen et al., 2015). For immunohistochemistry, an anti-IGF2BP3 primary antibody (Santa Cruz, cat.# sc-47893; dilution 1/50) was utilized. A scale bar of 100 μm is shown.

of aberrantly expressed transcription factors. Chromatin immunoprecipitation (ChIP) assays (**Box 1**) confirmed the direct binding of the transcription factors Nanog and NF-κB to the *IGF2BP3* promoter, thus sustaining its expression in tumor cells and favoring the stemness and migration properties, respectively (Chen et al., 2013; Bhargava et al., 2017). In triple-negative breast cancer cells, EGFR signaling regulates *IGF2BP3* transcription since the *IGF2BP3* promoter activity decreased after MEK1/2 signaling inhibition downstream of EGFR (Samanta et al., 2012).

At posttranscriptional level, several miRNAs regulate IGF2BP3 in different tumor types. In particular, *IGF2BP3* expression is inhibited by the let-7 family of miRNAs (Mayr et al., 2007; Fawzy et al., 2016; Kugel et al., 2016; JnBaptiste et al., 2017; Lin et al., 2017), miRNA-34a (Zhou et al., 2017), miRNA-129-1 (Kouhkan et al., 2016), miRNA-375-3p (Cen et al., 2018), miRNA-654 (Jin et al., 2018), miRNA-9-5p (Canella et al., 2015), and miRNA-200a (Kim et al., 2018).

In addition to regulating the expression of IGF2BP3, intracellular signaling mechanisms may impact its function. IGF2BP3 activity can be influenced by mTOR, which is a major downstream effector of the phosphoinositide 3-kinase (PI3K) (Fruman et al., 2017) and/or mitogen-activated protein

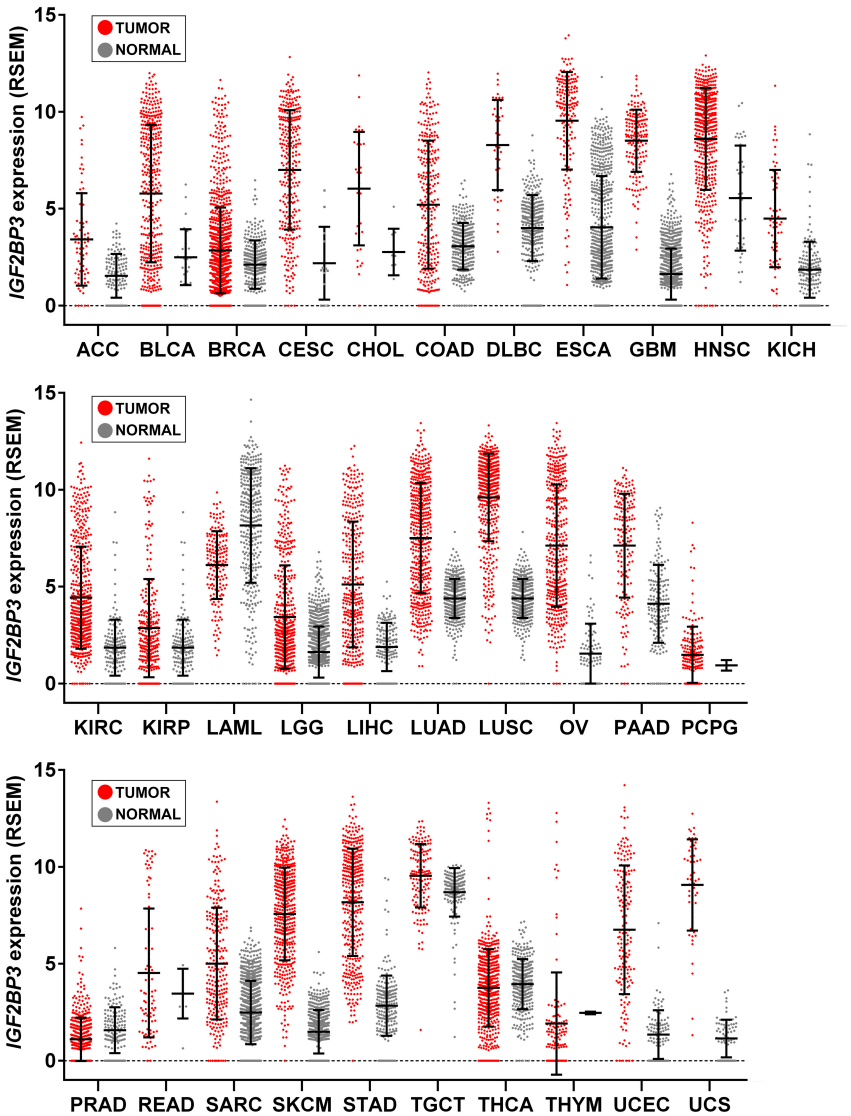

FIGURE 2 | *IGF2BP3* gene expression across human tissue and cancer types. Scatter plots showing *IGF2BP3* levels from The Cancer Genome Atlas (TCGA), Genotype-Tissue Expression (GTEx), and Target projects obtained from the UCSC Xena browser (Goldman et al., 2019). Data are RSEM normalized. Mean ± standard deviation is shown. LAML, Acute Myeloid Leukemia; ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; LGG, Brain Lower Grade Glioma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THYM, Thymoma; THCA, Thyroid carcinoma; UCS, Uterine Carcinosarcoma; UCEC, Uterine Corpus Endometrial Carcinoma.

kinase (MAPK) pathway (Liu et al., 2018). In humans, the Ser183 residue, which is located between the RRM2 and KH1 domains of IGF2BP3, has been indicated as a phosphorylation site of mTORC2. It has been suggested that IGF2BP3 undergoes phosphorylation during translation and, importantly, that the phosphorylated status enhances IGF2BP3 binding to the 3′-UTR of *IGF2*, leading to translation initiation of *IGF2* mRNA and increased IGF2 expression (Dai et al., 2013). Therefore, a positive feedback loop may exist between the IGF/PI3K/MAPK/mTOR pathway and IGF2BP3 expression in cancer cells. In *X. laevis*, similar studies were conducted to investigate the ortholog Vg1-RBP. These studies demonstrated that Vg1-RBP is phosphorylated by the MAPK mediator Erk2 at residue S402, which is located in the linker between the KH1 + 2 and KH3 + 4 didomains and represents a crucial modification for the release of its mRNA target Vg1 during meiotic maturation (Mueller-Pillasch et al., 1999; Git et al., 2009).

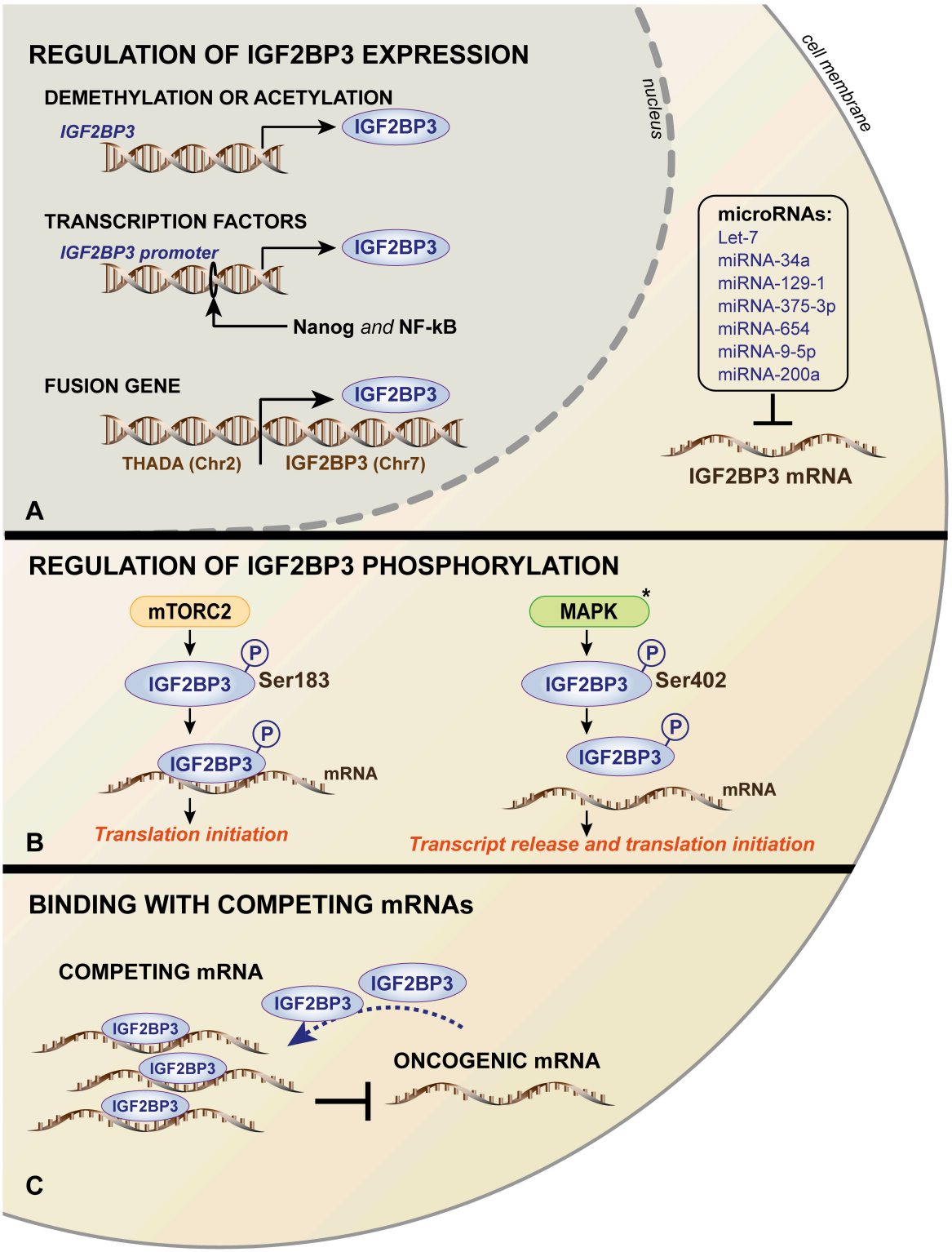

FIGURE 3 | Schematic representation of the mechanisms governing IGF2BP3 expression **(A)** and functions **(B,C)**. **(A)** In the nucleus, IGF2BP3 transcription is regulated by (i) DNA methylation or the acetylation of the *IGF2BP3* gene; (ii) activation of the *IGF2BP3* promoter by transcription factors, such as Nanog and NF-kB; and (iii) occurrence of chromosomal translocation. In the cytoplasm, *IGF2BP3* mRNA is regulated by several microRNAs. IGF2BP3 functions are controlled by **(B)** mTORC2- or MAPK-mediated phosphorylation, which influences the translation of its target mRNAs, and **(C)** competing, non-oncogenic mRNAs that may prevent the interaction between IGF2BP3 and its oncogenic transcript targets. The black asterisk (*) indicates that the process was described in the ortholog Vg1-RBP in *Xenopus Laevis*.

Finally, an interaction with endogenous competing RNAs, including mRNAs or lncRNAs, was reported as an alternative mechanism regulating RBPs activity (Kim et al., 2016). Consistently, as observed in Ewing sarcoma cells, the functions of IGF2BP3 can be limited by the mRNA expression of *ABCF1*, which is a partner transcript of IGF2BP3. *ABCF1* mRNA can associate with IGF2BP3 and limit its interaction with oncogenic target transcripts, thus acting as a sponge to repress the oncogenic function of IGF2BP3 (Mancarella et al., 2018b).

MOLECULAR MECHANISMS OF ACTION OF IGF2BP3

Specificities of IGF2BP3 With Respect to the Other IGF2BPs

IGF2BPs share many common features; however, the three proteins are not functionally redundant because they do not recognize RNAs with the same affinity or recognize the same RNAs. Accordingly, 30–50% of the target RNAs are specifically regulated by each family member (Chao et al., 2010; Huang et al., 2018b). These specificities mainly rely on the different RNA-binding properties displayed by each IGF2BP. For instance, IGF2BPs bind to sites within the 3′-UTR more frequently than they bind to sites within the 5′-UTR (Huang et al., 2018b). Nevertheless, IGF2BP3 binds to coding regions with a higher frequency than either IGF2BP1 or IGF2BP2 (Conway et al., 2016). In addition, a recent analysis of RNA recognition by multidomain IGF2BP proteins indicated that while IGF2BP1 associates with the CGGAC RNA motif, IGF2BP3 recognizes two related GGC-core elements (GGCA and CGGC), further supporting the existence of differences during the recognition of RNA (Schneider et al., 2019). The data described in the literature suggest that multiple RNA motifs, including CACA, UACA, AACA (Conway et al., 2016), GCAC (Palanichamy et al., 2016), and GGAC (Huang et al., 2018b), are recognized by the IGF2BP family, but the extent to which these sequences are specific to each RBP is still unclear. These differences are possibly due to distinct paralog-specific biochemical properties of the RNA-binding domains. While all four KH domains were identified as relevant for RNA binding, recent evidence demonstrates a crucial contribution of both the RRMs (Jia et al., 2018) and the KH domains (Wachter et al., 2013) to IGF2BP3, adding an additional element of diversity separating this RBP from its paralogs.

Another major difference among the paralogs relies on their mechanism of action on target RNAs. In the cytoplasm, IGF2BP1 and IGF2BP3 form large (200–700 nm optical diameter), motile ribonucleoprotein (RNP) granules named locasomes (**Box 1**), which are located beneath the plasma membrane in the perinuclear region or the lamellipodia of the leading edge depending on the cell type and cell confluence (Nielsen et al., 2002; Weidensdorfer et al., 2009). These granules represent a unique entity, that is distinct from processing bodies (P-bodies) and stress granules (**Box 1**; Jonson et al., 2007; El-Naggar and Sorensen, 2018; Luo et al., 2018). Locasomes lack 60S ribosomal units; elongation factors, such as eIF4E and eIF4G (Jonson et al., 2007; Weidensdorfer et al., 2009); and the RNA-induced silencing complex (RISC; **Box 1**; Jonson et al., 2014), indicating that these granules serve as a protected cytoplasmic repository for IGF2BP target transcripts. However, differences still exist between IGF2BP1 and IGF2BP3 because IGF2BP3 was also observed to recruit RISC to locasomes (Ennajdaoui et al., 2016), further adding another level of complexity and heterogeneity to the action of these closely related molecules. The mechanisms of action of IGF2BP2 are less defined but may putatively differ since this protein was found to interact with P-bodies (Degrauwe et al., 2016a,b), which are cytoplasmic RNP granules mainly involved in mRNA decay.

Mechanistic Events

RNA immunoprecipitation and sequencing (RIP-seq; **Box 1**) and photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP; **Box 1**) approaches indicate that ∼1000 to ∼4000 transcripts are bound by IGF2BP3 in humans (Jonson et al., 2014; Ennajdaoui et al., 2016; Palanichamy et al., 2016; Huang et al., 2018b). Among these, IGF2BP3 regulates RNA stability, RNA degradation, RNA localization (Bell et al., 2013), and miRNA biogenesis, but the exact molecular processes governing these functions have only begun to be elucidated. The current knowledge regarding IGF2BP3 action in cancer cells is summarized in **Figure 4**.

A major mechanism of IGF2BP3 activity is based on its complex interaction with the miRNA machinery (Degrauwe et al., 2016b; Trabucchi and Mategot, 2019). Both RBPs and miRNAs converge on the 3′-UTR of mRNAs, and the juxtaposition of their binding contributes to the combinatorial mechanisms of posttranscriptional gene regulation with a relevant impact on cellular fate and behavior. IGF2BP3 promotes mRNA stability/degradation by interacting with miRNAs through different processes as follows: (1) IGF2BP3 may protect target mRNAs from miRNA-dependent degradation by segregating transcripts into cytoplasmic RNP granules that do not contain RISC (Jonson et al., 2014); (2) IGF2BP3 may modulate the association between target transcripts and RISC (Ennajdaoui et al., 2016); (3) IGF2BP3 may compete with miRNAs for common binding sites on the 3′-UTRs of target transcripts (Ennajdaoui et al., 2016); and (4) IGF2BP3 may affect miRNA biogenesis, thus indirectly affecting the fate of miRNA targets (Wang et al., 2019).

The best described example of IGF2BP3 activity is its opposing effect on let-7 miRNA action. IGF2BP3 has been shown to segregate *HMGA2* and *LIN28B* transcripts and other let-7 targets into RISC-free RNP granules (locasomes), thereby protecting them from let-7-dependent silencing and providing generalized protection from miRNAs, including the activity of miR-181a/b (Jonson et al., 2014; Degrauwe et al., 2016b). Therefore, RISC-free locasomes represent a cytoplasmic shelter ("safe house") in which oncogenes are protected from degradation. However, much still needs to be learned: the mechanism by which RISC is excluded from these granules; the specificity of the

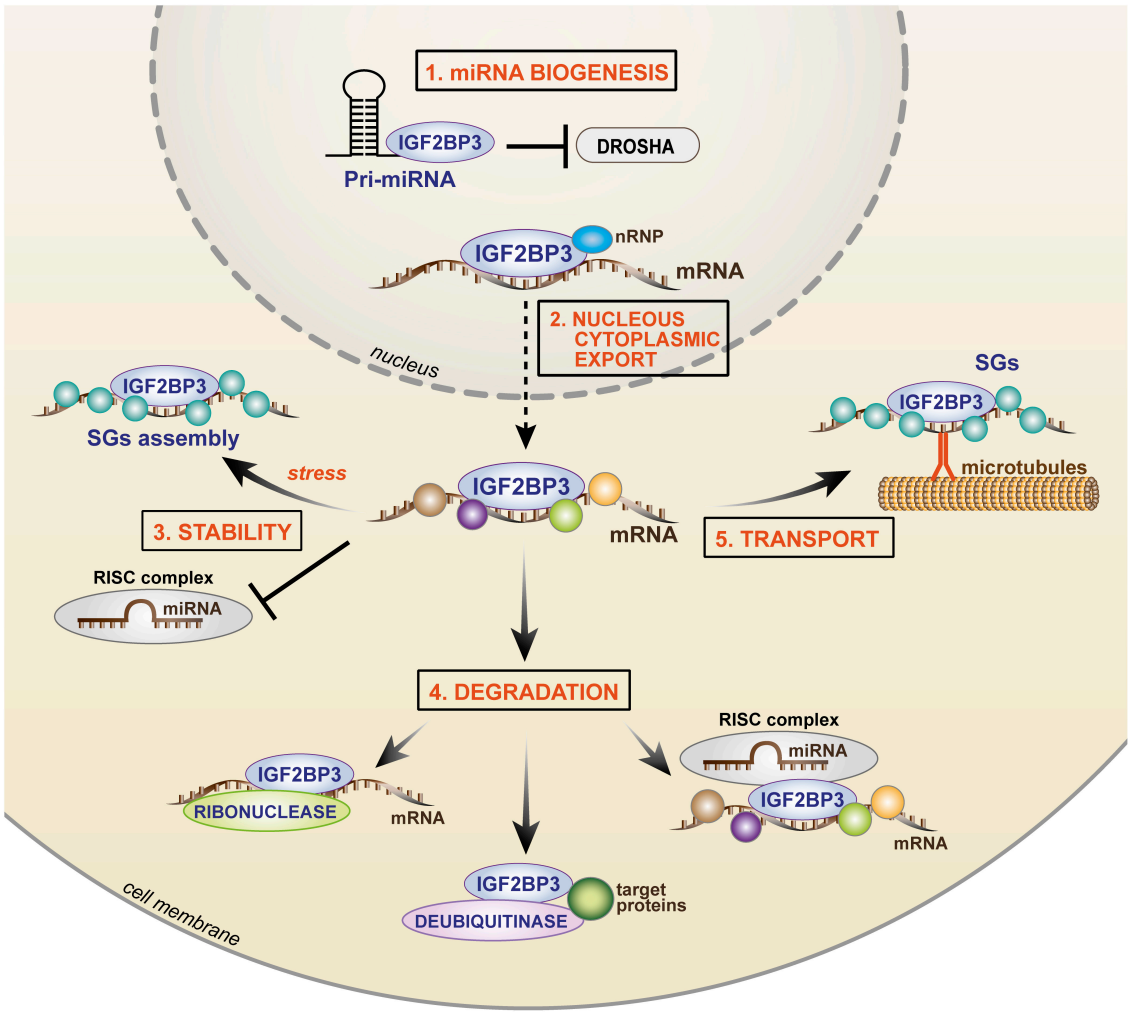

FIGURE 4 | Cartoon depicting the mechanisms of IGF2BP3 activity. In the nucleus, **(1)** IGF2BP3 drives microRNA biogenesis, preventing the binding of Drosha to the pri-miRNA and **(2)** IGF2BP3 interacts with nRNP and it binds target transcripts favoring nuclear export. In the cytoplasm, IGF2BP3 acts within ribonucleoprotein granules as depicted, thus controlling **(3)** stability, **(4)** degradation, or **(5)** transport of its target mRNAs. For stability, IGF2BP3 can prevent the activity of RISC or recruit SG proteins under stress conditions. For degradation, IGF2BP3 can recruit RISC or directly interact with enzymes, such as ribonuclease or deubiquitinase, thus inducing microRNA-dependent and microRNA-independent target degradation. For transport, IGF2BP3 is assembled in SGs with its target mRNAs and drives their localization along microtubules toward areas of active translation. nRNP, nuclear ribonucleoprotein; SGs, stress granules; RISC, RNA-induced silencing complex.

IGF2BP3 locasome composition; mechanisms underlying the interaction between IGF2BP3 and RISC. A study conducted by Ennajdaoui et al. (2016) revealed the bimodal capability of IGF2BP3 to regulate mRNA fate because, on one side, the RBP is able to compete with miRNAs for common binding sites on target transcripts to avoid binding to the RISC complex; on the other side, IGF2BP3 can promote the association between mRNAs and RISC, thus favoring mRNA degradation.

Overall, IGF2BP3 influences the expression of malignancy-associated RNAs by modulating their interactions with miRNAs through multiple and complex mechanisms, including the recently identified effect on miRNA maturation (Wang et al., 2019). During this process, IGF2BP3 competes with the ribonuclease Drosha to bind to pri-miRNAs in the nucleus, thus avoiding miRNA maturation and indirectly favoring the stability of miRNA transcript targets.

Beyond the interaction with miRNAs, evidence from the literature indicates that the mechanism of action of IGF2BP3 also relies on its direct interaction with protein partners. Studies of immunoprecipitation followed by mass spectrometry demonstrated specific functional interactions between IGF2BP3 and (1) enzymes (helicases, deubiquitinases or ribonucleases); (2) nuclear ribonucleoproteins; and (3) stress granule-associated proteins. Particularly, IGF2BP3 can directly interact with the ribonuclease XRN2 (Mizutani et al., 2016), or the deubiquitinase ubiquitin-specific peptidase 10 (USP10) (Zhao et al., 2017), causing *EIF4EBP* mRNA

or p53 protein degradation, respectively. How IGF2BP3 recruits its protein partners onto transcript targets still needs to be elucidated. On the other hand, interaction with the nuclear ribonucleoprotein (nRNP) HNRNPM was found to be crucial for the specific localization of IGF2BP3 within the nucleus and, indirectly, for the stability of IGF2BP3 transcript targets (Rivera Vargas et al., 2014). Much can still be uncovered regarding the functional effects of the interaction between IGF2BP3 and its protein partners. Indeed, multiple partners interacting with IGF2BP3 have been identified, including stress granule-associated proteins (G3BP1 and G3BP2) (Zhao et al., 2017). IGF2BP3 acts within RNP granules in a dynamic process that likely occurs through the polymerization of low-complexity sequences present in RBPs (Kato and Nakamura, 2012; Hentze et al., 2018), leading to the aggregation and recruitment of hundreds of RBP molecules and 10–30 mRNA transcripts into these granules (Jonson et al., 2007; Huang et al., 2018a). Stress granules represent cytoplasmic protein/RNA aggregates in which mRNAs are stored during stress conditions, such as nutrient deprivation, hypoxia, and oxidative stress. IGF2BP1 and IGF2BP2 have been previously reported to participate in stress granule formation in mammalian cells under oxidative stress conditions (Tourriere et al., 2003; Kedersha et al., 2016; for a review, see Protter and Parker, 2016). Similarly, IGF2BP3 may interact with G3BPs or TIAR stress granule-associated proteins, determining the formation of stress granules, under specific conditions, including stress exposure or, interestingly, mRNA transport (Taniuchi et al., 2014a,b; Huang et al., 2018b). In HeLa cells exposed to heat shock, the mRNA stability of the IGF2BP3 target *MYC* was significantly higher in cells with forced IGF2BP3 expression than that in control cells, demonstrating the protective effect of IGF2BP3 on its targets during stress (Huang et al., 2018b). In contrast, in pancreatic cancer cells, IGF2BP3 is assembled in stress granules for the transportation of its target RNAs along microtubules toward cell protrusions, thus favoring the local translation of cell migration-related transcripts (Taniuchi et al., 2014a,b). Therefore, IGF2BP3-mediated mRNA storage within stress granules represents a further mechanism underlying the enhancement of mRNA stability and the safe transport of RNAs within subcellular compartments.

Notably, while most of the studies reported in the literature investigate the regulation of coding RNA, IGF2BP3 also interacts with non-coding RNAs, including miRNAs (Samanta et al., 2018), lncRNAs (Li et al., 2018) and the new class of circular RNA (circRNA; **Box 1**; Schneider et al., 2016). In particular, IGF2BP3 can exert its effects by destabilizing miR145-5p, thus favoring the function of breast cancer stem cells (CSCs), or by stabilizing the lncRNA LINC01138, thus sustaining the proliferation and invasion abilities of hepatocellular carcinoma cells. Interestingly, at least 34 IGF2BP3-associated circRNAs, including the circRNAs CDYL, NFATC3, and ANKRD17, have been recently identified; however, the functional effects of these interactions are still unknown.

EFFECTS OF IGF2BP3 ON TUMOR PROGRESSION

IGF2BP3 has been implicated in various aspects of human tumor progression regulating cell growth, migration, and the response to drugs. These effects largely depend on the cellular context and presence of target transcripts. Some examples are provided in relation to the different cellular processes, but it is necessary to acknowledge that functional connections strictly require dedicated studies. This section of the review highlights the multiplicity of the mechanisms and targets that have been described in different tumors thus far, but mounting evidence indicates a complex scenario that may change dynamically as tumor cells become more aggressive and/or interact with tumor microenvironment components. To render this sweeping information, which includes different effects in different cellular contexts, more clear for the readers, a summary of to date reported IGF2BP3 targets in cancer is reported in **Box 2**.

BOX 2 | List of IGF2BP3 targets.

ABCF1	CD164	LINC01138
ABCG2	c-myc	LIN28
ARF6	COX2	MMP9
ARHGEF4	EIF4EBP2	PDPN
CCND1	HMGA2	Slug
CCND3	hsa-miR-145-5p	TP53
CCNG1	hsa-miR-3614	ULBP2
CDK6	IGF1R	
CD44	IGF2	

At the experimental level, IGF2BP3 sustains cancer cell growth and proliferation while putatively inhibiting apoptosis. As stated above, a well-established mechanism of action of IGF2BP3 is based on its protection from Let-7 miRNA-mediated decay. In this landscape, IGF2BP3 sustains the expression of *HMGA2* (Jonson et al., 2014), a DNA-binding protein that cooperates with the transcription machinery to alter the chromatin structure (De Martino et al., 2019), thus enhancing fibrosarcoma cells proliferation *in vitro*. In addition to HMGA2, Let-7 miRNAs directly repress a pantheon of well-known oncogenes, such as RAS, MYC, LIN28, and IGF1R, and cell cycle factors, such as cyclin D1 and cyclin D2 (Chirshev et al., 2019), most of which are indeed described as targets of IGF2BP3 (Jonson et al., 2014; Rivera Vargas et al., 2014; Palanichamy et al., 2016; Mancarella et al., 2018a) with a key role in cell proliferation. It can be speculated that through protection against Let-7 miRNA, IGF2BP3 favors the stability and translation of (1) *IGF1R* mRNA, thereby affecting the constitutive activation of its intracellular pathway and the *in vitro* growth of hepatocellular carcinoma or Ewing sarcoma cells (Fawzy et al., 2016; Mancarella et al., 2018a); (2) *MYC* and *CDK6* transcripts, thereby promoting the proliferation of hematopoietic stem and progenitor cells in mice (Palanichamy et al., 2016). In addition, by upregulating LIN28, which also enhances the expression of IGF2, histone H2a, cyclin A, cyclin B, and CDK4 (Balzeau et al., 2017), IGF2BP3 can establish complex, positive-feedback loops that further facilitate

tumor cell growth and malignancy. Overall, a direct interaction between IGF2BP3 and IGF2, with a consequent promotion of cell proliferation *in vitro*, has been demonstrated in leukemia (Liao et al., 2005), thyroid cancer (Panebianco et al., 2017), and glioma (Suvasini et al., 2011). In contrast to evidence in normal embryonic tissues, IGF2BP3 promotes *IGF2* mRNA translation in cancer cells by binding its 3′-UTR, leading to the increased activation of IGF signaling. In addition, IGF2BP3 contributes to stabilizing *COX-2* mRNA, favoring the translation of this crucial mediator of inflammation and antiapoptotic signals in leukemia cells (Ko et al., 2016). However, in the latter cases, the exact mechanism of action elicited by IGF2BP3 on these targets has not been investigated. It can only be speculated that binding the 3′-UTRs, IGF2BP3 protects these targets from miRNA-mediated decay.

IGF2BP3 has also been found to repress RNAs and miRNAs. This effect has been described in lung and cervical cancer cells in the case of *EIF4E-BP2*, which encodes a negative regulator of eukaryotic translation initiation factor 4E (eIF4E) (Mizutani et al., 2016), and miR145-5p in breast cancer (Samanta et al., 2018). The repression of EIF4E-BP2 through the IGF2BP3-mediated recruitment of ribonucleases within RNPs promotes the proliferation of cancer cells (Mizutani et al., 2016); the IGF2BP3-induced destabilization of miR145-5p favors the expression of WNT5B, which activates TAZ, a transcriptional coactivator of Hippo signaling necessary for the function of breast cancer CSCs (Samanta et al., 2018). A putative role of IGF2BP3 in self-renewal and tumor initiation, i.e., two properties associated with CSCs, has been suggested in several tumors through different mechanisms. As mentioned, IGF2BP3 regulates the expression of HMGA2 and LIN28, allowing the symmetrical division of CSCs thus sustaining their stemness-like phenotypes (Jonson et al., 2014; Puca et al., 2014; Balzeau et al., 2017). IGF2BP3 recruits the deubiquitinase USP10 thereby attenuating p53 protein stability and increasing tumorigenicity of lung cancer cells *in vivo* (Zhao et al., 2017). In addition, IGF2BP3 sustains *SNAI* (*Slug*) mRNA translation in breast cancer cells (Samanta et al., 2016), putatively preventing its miRNA-mediated decay; in turn, SNAI favors the transcription of the stem cell factor SOX2. Consistent with a putative role in the maintenance of cellular stemness, IGF2BP3 expression is higher in triple-negative breast CSCs (Samanta et al., 2016) and hepatocellular carcinoma tumor-initiating stem-like cells (Chen et al., 2013) than in the entire population of tumor cells. In hepatocellular carcinoma, the IGF2BP3/AKT/mTOR pathway inactivates TGF-β signaling to maintain the expression of pluripotency genes along with the tumorigenesis and chemoresistance of CD133(+) stem cells (Chen et al., 2013). Altogether, these observations highlight a putative role of IGF2BP3 in promoting or preserving tumor cell subpopulations with stem cell features, thereby contributing to tumor establishment and progression.

In addition, IGF2BP3 promotes cell migration. Compared to the phenotype of IGF2BP3-null cells, IGF2BP3-expressing tumor cells display a marked motility-prone phenotype with an adherent shape, cellular extensions, lamellipodia, frequent cell–cell adhesion contacts (Vikesaa et al., 2006) and an increased capability to form metastases *in vivo* (Zhao et al., 2017; Mancarella et al., 2018b). Accordingly, different mediators of cell migration/invasion and cell adhesion have been reported as IGF2BP3 mRNA targets in different tumor types. Besides favoring cell proliferation, IGF1R and HMGA2 also regulate cell migration (Sheen et al., 2015; Mancarella et al., 2018a). Other described mediators of IGF2BP3-induced cellular motility include the type IV collagenase MMP9, which drives the degradation of the basement membrane and promotes the release of growth factors from the extracellular matrix, and the cell surface receptor of sialomucin, i.e., CD164 (endolyn), which is involved in cell adhesion (Hafner et al., 2010; Samanta et al., 2012). These mediators were identified as IGF2BP3 targets by PAR-CLIP studies and were subsequently validated in triple-negative breast cancer cells; however, the exact mechanisms of IGF2BP3-mediated regulation are still unknown. Furthermore, IGF2BP3 has been shown to bind to the 3′-UTR and sustain the expression of the hyaluronan receptor CD44 and the epithelial adhesion protein podoplanin (PDPN), which interact with actin and promote invadopodia formation (Vikesaa et al., 2006; Hwang et al., 2012).

However, large-scale genomic approaches identified an IGF2BP3-RNA interaction network of 164 transcripts associated with cellular migration, cell adhesion, actin cytoskeleton remodeling, and invadopodia formation; these transcripts include mRNAs previously identified by other authors, thereby indicating the existence of a complex scenario (Ennajdaoui et al., 2016).

Local translation of RNAs is required for cell migration (Mofatteh and Bullock, 2017). In pancreatic cancer cells, IGF2BP3 and IGF2BP3-bound transcripts, including *ARF6* and *ARGHEF4*, accumulate in membrane protrusions (Taniuchi et al., 2014a). This accumulation is due to the activity of the motor kinesin protein KIF20A (Taniuchi et al., 2014b), which transports IGF2BP3 and its target transcripts toward cell protrusions along microtubules, leading to the local translation of mRNA into proteins that favor the formation of membrane protrusions and cell motility (Taniuchi et al., 2014a).

Other experimental evidence indicates that IGF2BP3 regulates the response to anticancer treatments. By regulating Lin28, HMGA2, CD44, IGF2, and IGF1R, IGF2BP3 increases cell survival and resistance to conventional and targeted drugs in several tumors. In particular, by affecting IGF2 and/or IGF1R expression, IGF2BP3 has been shown to modulate sensitivity to anti-IGF1R agents (Liu et al., 2017; Panebianco et al., 2017; Mancarella et al., 2018a) and MAPK/PI3K inhibitors (Suvasini et al., 2011). Moreover, IGF signaling modulation is thought to be responsible for the association between IGF2BP3 expression and radio-resistance in chronic myeloid leukemia and squamous cell esophageal cancer (Liao et al., 2011; Yoshino et al., 2014). In ovarian cancer, the overexpression of IGF2BP3 and LIN28 has been associated with cisplatin resistance, which was attributed to the downregulation of hCTR1 (a transmembrane protein that imports cisplatin into mammalian cells), and was found to be responsible for a poor outcome (Hsu et al., 2015). In triple-negative breast cancer cells, an IGF2BP3 depletion increased cell sensitivity to doxorubicin and mitoxantrone (Samanta et al., 2013). This effect was due to the IGF2BP3-mediated stabilization

of *ABCG2* mRNA, which is an ATP-binding cassette (ABC) transporter and a major effector of drug resistance (for a review, see Robey et al., 2018).

Limited but interesting evidence indicates that IGF2BP3 affects the interaction with the tumor microenvironment. Cancer cells must face harsh microenvironmental conditions, including hypoxia, nutrient-deprivation, space constraints, oxidative stress and the immune response, to remain viable. In the immune response, interactions between tumor and immune cells represent a major determinant of cancer behavior. Schmiedel et al. (2016) demonstrated that IGF2BP3 favors the immune escape of cancer cells by inhibiting the cytotoxic effect mediated by natural killer cells through the promotion of mRNA decay of the stress-induced ligand ULBP2. In the interaction between tumor cells and the tumor microenvironment, secreted molecules represent crucial mediators of local and systemic cellular communication. Interestingly, evidence insinuating that tumor cells are able to release IGF2BP3 in the extracellular compartment is based on recent findings demonstrating the presence of IGF2BP3 in the serum of cancer patients (Szarvas et al., 2014; Tschirdewahn et al., 2019). However, the molecular mechanisms underlying this evidence are still unknown. In particular, it is still not clear how IGF2BP3 is released and whether circulating IGF2BP3 can still elicit functional malignant effects.

At the clinical level, as also reviewed by Lederer et al. (2014), IGF2BP3 is expressed *de novo* in a variety of tumor types unlike normal tissues (**Figure 2**). **Table 1** summarizes those tumor types that, to the best of our knowledge, display an higher expression of IGF2BP3 compared to normal counterpart and where IGF2BP3 has been suggested as a diagnostic and/or prognostic biomarker. Of those, the IGF2BP3-positive tumors generally display high metastatic behavior and poor outcome as well as increased tumor size, advanced tumor stage and lymph node metastasis.

This is not surprising since, as stated above, IGF2BP3 increases cell proliferation while blocking apoptosis and favoring stemness, migration and drug resistance. A direct correlation has been reported between the immunohistochemical evaluation of IGF2BP3 expression and increased staining of the proliferation index ki67 in malignant peritoneal mesothelioma (Hui et al., 2018), neuroendocrine tumors of the lung, in which IGF2BP3 was also directly correlated with the stem cell marker Nanog (Del Gobbo et al., 2014), and triple-negative breast carcinoma, in which IGF2BP3 expression was also significantly associated with a poor response to neoadjuvant chemotherapy (Walter et al., 2009; Ohashi et al., 2017). The combined evaluation of IGF2BP3 and the proapoptotic protein BCL2 was found to be particularly effective for diagnosis in squamous cell carcinoma (Richey et al., 2018). In hepatocellular carcinoma, the copresence of IGF2BP3 and its target CD44 is correlated with advanced tumor stage/grade and metastasis (Wachter et al., 2012; Hu et al., 2014), while in Ewing sarcoma patients, contemporary high expression of IGF2BP3 and low expression of its counteracting partner ABCF1 is correlated with a particularly poor outcome (Mancarella et al., 2018b). In addition, the circulating IGF2BP3 protein levels, recently detected in serum from prostate cancer and renal cell carcinoma patients, were found to be associated with a significantly higher risk of cancer-specific death or relapse (Szarvas et al., 2014; Tschirdewahn et al., 2019). These findings demonstrate that the IGF2BP3-induced phenotypic effects observed *in vitro* can be recapitulated in clinical specimens and that it is possible to exploit this knowledge in the clinical settings for monitoring tumor progression.

POTENTIAL RELEVANCE OF IGF2BP3 IN THERAPY

Based on its absence in normal tissues, with very few exceptions, IGF2BP3 represents a putative valuable and specific target for cancer therapy.

To date, no direct inhibitor of IGF2BP3 activity has been developed. However, the "druggability" of RBPs has been recently demonstrated for Musashi1 or HuR. In particular, the small molecule luteolin was observed to interfere with the RNA-binding capacities of Musashi1 by blocking its RNA-binding pocket (Yi et al., 2018). Similarly, multiple compounds, including the recently described dihydrotanshinone-I that directly blocks the RNA-binding domains of HuR, prevent its association with target RNAs (Lal et al., 2017). In these two cases, the blockades of RBP activity correlated with reduced *in vitro* proliferation, viability, and migration or decreased xenograft tumor growth, respectively, demonstrating the putative effectiveness of these approaches. Nevertheless, a deeper biochemical comprehension of the IGF2BP3 RNA-binding properties is needed for the successful development of direct inhibitors of its functions.

In contrast, consistent preclinical studies have provided pharmacological options to block IGF2BP3 expression. The use of an isocorydine derivative (d-ICD), i.e., an alkaloid monomer purified from *Papaveraceae* sp. plants, has been demonstrated to inhibit IGF2BP3 expression and reduce the growth of hepatocellular carcinoma cells (Li et al., 2015). In addition, inhibitors of bromodomain and extraterminal domain (BET) proteins, such as JQ1 or iBET, have been found to downregulate IGF2BP3 expression and its targets in Ewing sarcoma and B cell acute lymphoblastic leukemia, consequently attenuating tumor growth (Palanichamy et al., 2016; Elagib et al., 2017; Mancarella et al., 2018b). Considering that clinical trials using BET inhibitors have been performed in hematological and solid tumors with an observed manageable toxicity (Amorim et al., 2016; Berthon et al., 2016), these agents may represent a concrete treatment option for patients with high levels of IGF2BP3 (Mancarella and Scotlandi, 2018; Mancarella et al., 2018b).

IGF2BP3 has also been postulated as a potential vaccine candidate. Studies investigating lung cancer have shown that IGF2BP3 is immunogenic as assessed by the presence of an antibody against recombinant IGF2BP3 in lung pleural effusions (Wang et al., 2003), and immunogenic peptides derived from IGF2BP3 induce tumor-reactive and human leukocyte antigen (HLA)-A2 (A*02:01)-restricted cytotoxic T lymphocytes (CTL) (Tomita et al., 2011). More recently, cancer vaccination using the IGF2BP3 508-516 peptide along with the LY6K 177-186 and CDCA1 56-64 peptides was tested in a phase II open-label, non-randomized clinical trial in head and neck squamous cell

TABLE 1 | IGF2BP3 participation in human tumors.

Cancer types	Diagnosis	Prognosis	References
Solid tumors			
Skin			
Squamous cell carcinoma	✓		Richey et al. (2018)
Melanoma		✓	Sheen et al. (2016)
Lung			
Lung adenocarcinoma		✓	Yan et al. (2016)
Non-small cell lung cancer		✓	Shi et al. (2017)
Neuroendocrine tumor of lung		✓	Del Gobbo et al. (2014)
Malignant peritoneal mesothelioma		✓	Hui et al. (2018)
Breast			
Triple-negative breast carcinoma		✓	Ohashi et al. (2017)
Pancreatic and Gastrointestinal tract			
Intraductal papillary mucinous neoplasm of pancreas	✓		Senoo et al. (2018)
Pancreatic ductal adenocarcinoma	✓	✓	Johnson et al. (2016); Aksoy-Altinboga et al. (2018)
Gastroenteropancreatic neuroendocrine neoplasia		✓	Er et al. (2017)
Colorectal cancer	✓	✓	Wei et al. (2017); Xu et al. (2019)
Esophageal adenocarcinoma		✓	Plum et al. (2018)
Gastric cancer		✓	Lee et al. (2017)
Prostate			
Prostate cancer		✓	Szarvas et al. (2014)
Cervix and uterus			
Ovarian serous carcinoma		✓	Mohanty et al. (2019)
Adenocarcinoma *in situ* of the uterine cervix	✓		Li et al. (2007a)
Endometrial serous carcinoma	✓		Li et al. (2007b)
Bladder and kidney			
Urothelial carcinoma		✓	Yang et al. (2019)
Renal cell carcinoma		✓	Tschirdewahn et al. (2019)
Liver			
Hepatocellular carcinoma		✓	Hu et al. (2014)
Intrahepatic cholangiocarcinoma		✓	Gao et al. (2014)
Head and neck			
Oral squamous cell carcinoma		✓	Tarsitano et al. (2016)
Bone and soft tissues			
Ewing sarcoma		✓	Mancarella et al. (2018b)
Leiomyosarcoma	✓	✓	Cornejo et al. (2012); Yasutake et al. (2018)
Brain			
Astrocytoma		✓	Barton et al. (2013)
Glioma		✓	Del Gobbo et al. (2015)
Neuroblastoma		✓	Chen et al. (2011)
Hematological tumors			
Leukemia			
B-acute lymphoblastic leukemia	✓		Palanichamy et al. (2016)
Myeloma			
Multiple myeloma			Canella et al. (2015)
Lymphoma			
Mantle cell lymphoma			Hartmann et al. (2012)
Hodgkin lymphoma	✓		Tang et al. (2013)

cancer patients, indicating that a vaccination-induced immune response was positively correlated with a better prognosis (Yoshitake et al., 2015).

Considering the recent evidence concerning the epigenetic regulation of IGF2BP3 or posttranslational modification mediated by the PI3K/Akt or MAPK pathways, it is plausible that agents capable to influence the activity of epigenetic factors, such as inhibitors of DNA methyltransferases or histone deacetylase inhibitors, and targeted therapies that block specific intracellular signaling pathways may affect IGF2BP3 activity. However, more

detailed preclinical studies are required before these drugs can be considered a concrete possibility.

CRITICAL ISSUES AND PERSPECTIVES

Although the molecule has been largely described to impact tumor initiation and progression, there is lack of knowledge regarding relevant issues. In particular, the following issues require further investigation:

1. The physiological role of IGF2BP3 still needs to be clearly elucidated.
2. Although there is clear support showing that IGF2BP3 plays a direct role in tumorigenesis and cancer progression, the mechanisms by which IGF2BP3 elicits its effects are incompletely understood. Importantly, the molecular mechanisms underlying the IGF2BP3-mediated regulation of non-coding RNAs still need elucidation.
3. The impact of IGF2BP3 on tumor predisposition is still obscure.
4. The precise discrimination of the specific properties of IGF2BP3 in relation to the other IGF2BP members is difficult due to the high level of homology but is necessary to fully exploit the clinical potential of these molecules.
5. The identification of high-quality and highly paralog-specific antibodies is mandatory for their proper use in the clinic as tissue and/or circulating biomarkers. In addition, adequate tools to study the intracellular modifications of IGF2BP3 are required.
6. The presence of IGF2BP3 in the plasma offers novel interesting clues. More studies are required to test the clinical value of IGF2BP3 as a circulating biomarker of risk and response.
7. A deeper understanding of the posttranslational modification and phosphorylation of IGF2BP3 is highly desirable as it may open new avenues for therapy.
8. The interaction between IGF2BP3 and the tumor microenvironment is still poorly described.
9. The role of IGF2BP3 in cell metabolism is still unknown. Although IGF2, IGF1R, and LIN28 have been described as target of this RBP, the overall impact of IGF2BP3 on glycose metabolism and insulin-induced signaling has not been assessed.

CONCLUDING REMARKS

IGF2BP3 represents an intriguing posttranscriptional factor in tumor malignancy. Important advancements have been achieved over the last years concerning our understanding of the oncogenic processes driven by RBPs, revealing that the relevance of these regulators in tumorigenesis and cancer progression has been largely underscored. Regarding IGF2BP3, the information obtained to date indicates a complex scenario in which this molecule acts through multiple and highly cell type-dependent contexts. The molecule is able to influence the expression of all RNA species, thus driving key malignant processes in cancer cells. In addition, interactions between IGF2BP3 and the tumor-microenvironment have been identified, highlighting a novel putative function in the interplay between tumor and normal cells. Experimental and clinical findings indicate that the evaluation of IGF2BP3 expression and its targets may concur to address the clinical need of new biomarkers for the risk-based stratification of patients at diagnosis and may offer innovative treatment opportunities. However, the clinical use of this molecule is still far from being a concrete possibility due to the many molecular and technical issues that remain unsolved.

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

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Argonaute Proteins: From Structure to Function in Development and Pathological Cell Fate Determination

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The highly conserved Argonaute protein family members play a central role in the regulation of gene expression networks, orchestrating the establishment and the maintenance of cell identity throughout the entire life cycle, as well as in several human disorders, including cancers. Four functional Argonaute proteins (AGO1–4), with high structure similarity, have been described in humans and mice. Interestingly, only AGO2 is robustly expressed during human and mouse early development, in contrast to the other AGOs. Consequently, AGO2 is indispensable for early development *in vivo* and *in vitro*. Here, we review the roles of Argonaute proteins during early development by focusing on the interplay between specific domains of the protein and their function. Moreover, we report recent works highlighting the importance of AGO posttranslational modifications in cancer.

Keywords: argonaute proteins, expression, structure, posttranslational modifications, development, cancer

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INTRODUCTION

Historically, the Argonaute (AGO) protein family has been discovered in a plant mutagenesis screen, performed to identify new genes involved in *Arabidopsis thaliana* development (Bohmert et al., 1998). This first report already highlighted the conservation of the Argonaute gene family in multicellular organisms suggesting its important functions. It was later demonstrated that AGOs are conserved throughout all domains of life (Swarts et al., 2014). Eukaryotic AGOs are involved in many cellular processes and act as mediators of gene silencing (Bartel, 2018). In mammals, AGOs have been mainly described for their cytoplasmic role in small RNA (smRNA) biogenesis, as key components of the RNA-induced silencing complex (RISC) (Bodak et al., 2017a).

Two types of ∼22 nt smRNAs can be loaded into AGOs to induce translational inhibition or exonucleolytic messenger RNA (mRNA) decay of specific transcripts: small interfering RNAs (siRNAs) and microRNAs (miRNAs). Both species are processed in the cytoplasm by DICER, leading to the release of double-stranded RNA (dsRNA) duplexes, which will be loaded into the RISC complex to achieve its RNA interference (RNAi) functions [for reviews (Bodak et al., 2017a; Treiber et al., 2019)].

Furthermore, the regulatory role of smRNAs expands beyond the posttranscriptional regulation mediated by miRNAs. In fact, smRNAs with AGOs as their effector proteins have been described to be involved in transcriptional gene silencing or activation (Malecová and Morris, 2010), alternative splicing (Alló et al., 2009; Harel-Bellan et al., 2013), antiviral defense (Maillard et al., 2013), genome integrity control (Svoboda et al., 2004; Kanellopoulou et al., 2005; Bodak et al., 2017b), DNA repair (Hawley et al., 2017), and epigenetic modification of the chromatin (Li, 2014). Although the expression of new smRNA species such as small nucleolar RNA (sno-RNA)- and

transfer RNA (tRNA)-derived fragments has been recently described to be altered in the context of cancer, their functions remain largely unexplored (Martens-Uzunova et al., 2013; Schorn et al., 2017; Kuscu et al., 2018). However, recent evidence shows that the functions and biogenesis of these new smRNA species are tightly connected to the RNAi pathway, functioning both in the cytoplasm and the nucleus (Huang and Li, 2014; Sarshad et al., 2018).

In this review, we highlight novel findings on the structures of AGO proteins since the description of the human AGO2 (Schirle and Macrae, 2012) and link these with their roles in mammalian early development and carcinogenesis.

STRUCTURE AND DOMAINS OF THE ARGONAUTE PROTEINS

Structures of prokaryotic and mammalian Argonaute proteins have been extensively studied in the past decades and have given revealing insights into the mechanism of translational inhibition by miRNAs. In this part, we only focus on the structural data of the human AGO proteins, which are highly conserved and share ∼85% of sequence identity (https://myhits.isb-sib.ch/cgi-bin/profile_search?data=5485215623128241).

Four Argonaute proteins (AGO1–4) are expressed in humans. AGO2 is described best and has long been thought to be the only Argonaute protein member having mRNA slicing activity, due to its unique structural characteristics (Liu et al., 2004; Meister et al., 2004). Nevertheless, AGO3 has recently been shown to slice target RNAs, however, only when loaded with certain miRNAs (Park et al., 2017). In these cases compared to AGO2, the slicing activity depended strongly on the pairing of the postseed region of the guide RNA as well as on the 5′ and 3′ flanking regions of the target RNA (Park et al., 2017).

The four human Argonaute proteins are structurally very similar but nevertheless contain few non-conserved amino acids in their functional domains. The AGO2 full-length protein structure was resolved first and was largely studied (Elkayam et al., 2012; Schirle and Macrae, 2012; **Figure 1A**). Since then, structural data on all the others, AGO1, 3, 4 full proteins have become available (Faehnle et al., 2013; Nakanishi et al., 2013; Park et al., 2017, 2019). These studies have revealed four conserved domains: the N-terminal domain (N), the PIWI/Argonaute/Zwille (PAZ) domain, the MID domain, and the P-element-induced whimpy tested (PIWI) domain. The PAZ domain, which is required for anchoring the 3′ end of guide RNAs, and the MID domain, which binds the 5′ phosphate of guide RNAs (Lingel et al., 2003; Song et al., 2003, 2004; Ma et al., 2004; Yan et al., 2004; Boland et al., 2010, 2011; Frank et al., 2010), are very similar between the four AGOs (**Figure 1A**).

The N-terminal domain, however, differs between AGO1–4. In AGO2, the N-terminal domain comprises two motifs (residues 44–48 and 134–166), which are required for its full catalytic activity. Upon mutation of these motifs, AGO2 fails to initiate RISC activation and mRNA cleavage. During protein folding, these residues are located in the vicinity of the PIWI domain and hence are required for correct guide-target positioning (Hauptmann et al., 2013; Schürmann et al., 2013; **Figure 1A**). On the other hand, AGO1 harbors only one of the N-terminal motifs, required for full catalytic activity (Faehnle et al., 2013; Hauptmann et al., 2013, 2014; **Figure 1A**), whereas AGO3 and AGO4 possess none, which was thought to render them catalytically inactive (Faehnle et al., 2013; Hauptmann et al., 2013, 2014; Nakanishi et al., 2013; Schürmann et al., 2013; Park et al., 2019). In addition, AGO3 has a specific insertion (3SI) in the N-terminal domain, which leads to a wider and imperfect nucleic-acid binding channel compared to AGO2 (Park et al., 2017).

The PIWI domain is similar to an RNAse H domain, harboring the catalytic triad DDH, which is critical for the slicing activity of AGO2 (Parker et al., 2004, 2005; Song et al., 2004; Ma et al., 2005; Rivas et al., 2005; Yuan et al., 2005). This work has later been challenged by Nakanishi et al. (2012), who demonstrated that not only is a catalytic triad but also a catalytic tetrad (DEDH) is essential for the AGO2 slicing activity (**Figure 1A**). Indeed, mutation of the glutamate in this catalytic tetrad abolishes the ability of the protein to induce RNAi (Nakanishi et al., 2012). AGO3, like AGO2, has a fully functional PIWI domain with a DEDH. The slicing activity of this domain has been proven by domain swap experiments, showing that AGO3 PIWI domain introduced in an AGO2–AGO3 PIWI chimeric protein can be catalytically active (Hauptmann et al., 2013; Schürmann et al., 2013). AGO1 comprises also several domain changes, the first one being a residue change in the catalytic tetrad of the PIWI domain (**Figure 1A**). Second, two proline residues at position 670 and 675 in the unique structural element, called cluster 2 (CL2) [also known as conserved segment (CS7)] can bend the protein, which sterically hinders the positioning of the guide/target complex (Nakanishi et al., 2013). In the same conserved segment, another mutated residue, L674, has been shown to decrease the slicing efficiency of AGO1 (Faehnle et al., 2013). Similar to AGO1, AGO4 lacks key catalytic residues and has an additional AGO4-specific insertion (4SI) in the PIWI domain, together with the cluster 2, already observed in AGO1. Only swapping of these domains with their AGO2 counterparts has enabled AGO4 to be catalytically active. Therefore, the native AGO4 is thought to be slicing incompetent (Nakanishi et al., 2013; Hauptmann et al., 2014; Park et al., 2019; **Figure 1A**). In addition, in the recently published AGO4 structure, the so-called LAKEs were observed, which are an accumulation of water molecules below the nucleic acid binding channel. This formation is conserved in all human AGOs. LAKE formation aids to establish the RISC assembly and is important for smRNA duplex loading (Park et al., 2019).

Finally, AGO1 has also been detected as a candidate for programed translational readthrough, a process generating longer isoforms by continuing translation beyond the stop codon (Eswarappa et al., 2014). Two recent studies demonstrated the presence of this translational readthrough product of AGO1 in cells, termed AGO1x (Ghosh et al., 2019; Singh et al., 2019). AGO1x is a protein isoform, which is 33 amino acids longer than AGO1 (**Figure 1A**). Initially, it was shown in HeLa cells that AGO1x can interact with miRNAs and their mRNA targets; however, no interaction with GW182 has

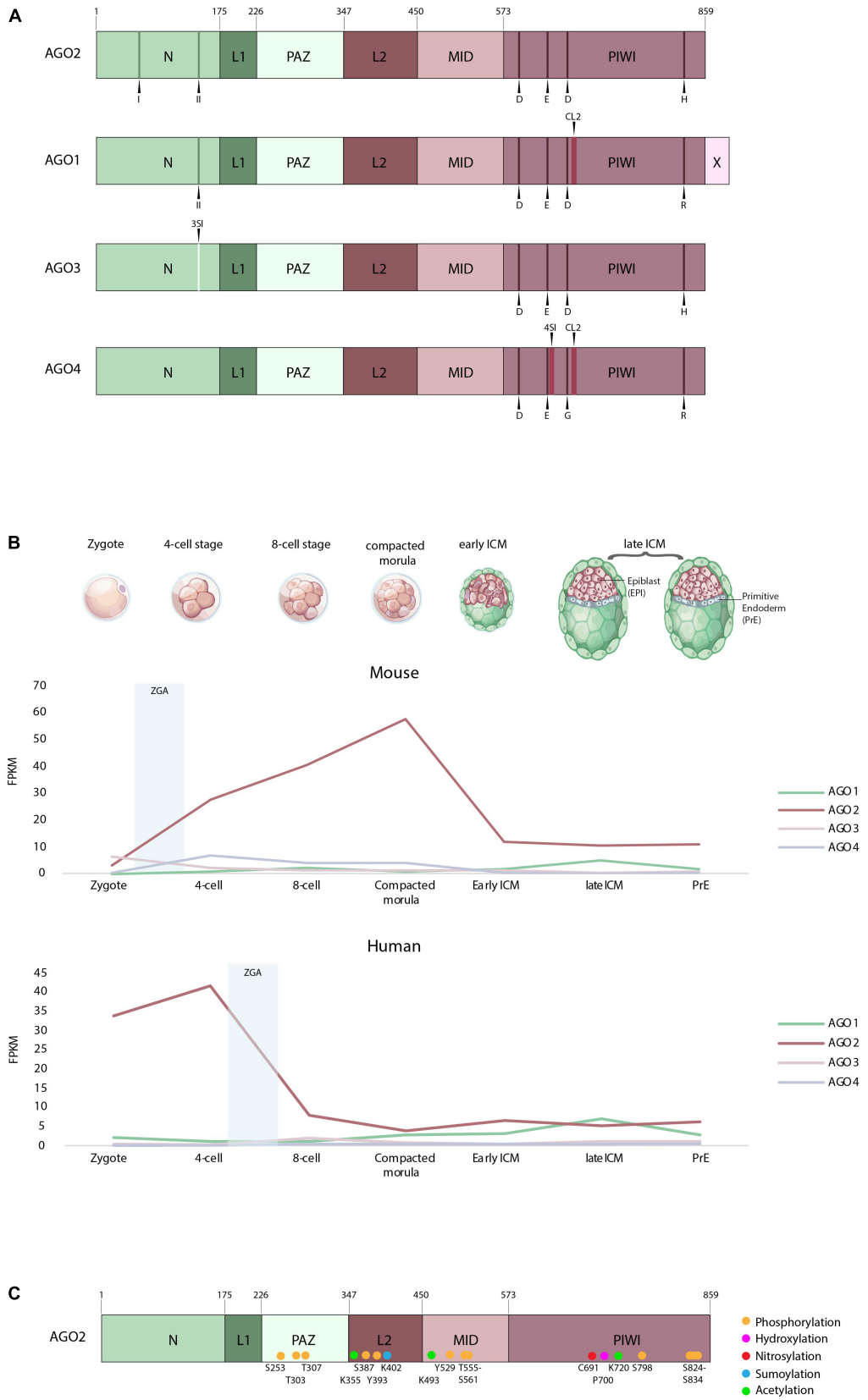

FIGURE 1 | Continued

FIGURE 1 | (A) Domain organization of AGO1–4 (adapted from Elkayam et al., 2012). Indicated are the two N-terminal motifs labeled I and II and the residues of the catalytic tetrad in the PIWI domain D, E, D, H, R, and G. Also highlighted in AGO1 and AGO4 is cluster 2 (CL2), the AGO3-specific insertion (3SI) and the AGO4-specific insertion (4SI). N, N-terminus; L1, linker domain 1; PAZ, PIWI/Argonaute/Zwille domain; L2, linker domain 2; MID, MID domain; PIWI, P-element-induced whimpy testes domain; X, AGO1x additional 33 aa; D, aspartate; E, glutamate; H, histidine; R, arginine; G, glycine. **(B)** Expression of human and mouse AGO1–4 in the zygote, four-cell, eight-cell, compacted morula, early inner cell mass (ICM) and late ICM, according to single-cell expression data from Boroviak et al. (2018). ZGA, first major wave of zygotic gene activation. **(C)** Posttranslational modifications of the human Argonaute 2 protein.

been observed. GW182 proteins normally interact with the Argonautes, mediating translational repression (Eulalio et al., 2009). Since AGO1x is incapable of interacting with GW182, it cannot induce translational repression. It is therefore thought that AGO1x competes with the canonical miRNA pathway and thereby leads to reduced posttranscriptional repression of target mRNAs (Singh et al., 2019). Second, in breast cancer cells, AGO1x has been shown to prevent the accumulation of dsRNAs and thereby suppresses the interferon response in these cells, a function independent of the canonical miRNA pathway (Ghosh et al., 2019).

EXPRESSION OF THE ARGONAUTE PROTEINS IN EARLY DEVELOPMENT

Although structurally very similar, the expression of AGOs can differ greatly during early development. We focus in this part on the difference in expression of mammalian AGO1–4 during early embryonic development. The mouse and human AGOs are highly conserved with almost identical protein sequences [99% for AGO2, 3, and 4 and 100% for AGO1 between mouse and human (https://myhits.isb-sib.ch/cgi-bin/profile_search?data=5485215623128241)].

The expression of the four AGOs during mouse early development was originally monitored using reverse transcription followed by PCR approaches and revealed the expression of the four transcripts in oocytes and at early stages of development (Lykke-Andersen et al., 2008). Nowadays, newer technologies allow to determine the expression of certain transcripts on a single-cell level (Stuart and Satija, 2019). A recent study, using this single-cell sequencing technology at different stages of preimplantation development in humans and mice allowed us to look into the detailed expression of the Argonautes throughout preimplantation development (Boroviak et al., 2018). Two cell fate decision events occur during preimplantation development (Niakan and Eggan, 2013). At the blastocyst stage, two populations of cells are segregating to create two distinct lineages: the trophectoderm, an extraembryonic tissue at the origin of the placenta, and the inner cell mass (ICM), the future epiblast at the origin of the three germ layers of the embryo. This first cell fate choice takes place 3 days postfertilization (dpf) in mice and 5 dpf in humans. The second cell fate specification event allows the segregation of the ICM and another extraembryonic layer: the primitive endoderm at the origin of the yolk sac, which appears 4 and 7 dpf in mice and humans, respectively (Niakan and Eggan, 2013).

For both species, the monitored expression of *Ago*1, 3, and 4 mRNAs during preimplantation stages is low compared to *Ago*2 transcripts (**Figure 1B**). However, it is to note that, in both species, *Ago2* represents still <1% of all detected transcripts (Boroviak et al., 2018). In mouse, *Ago*1, 3, and 4 mRNAs are lowly expressed from the zygote to the early ICM and the primitive endoderm, compared to *Ago*2 (**Figure 1B**). Interestingly, at the late blastocyst stage, a decrease in *Ago*2 is observed, in parallel with an increase in *Ago*1, suggesting a possible novel role for AGO1 just before implantation (**Figure 1B**). The expression profiles of the AGOs seem very different in human early embryos. *AGO1* mRNAs increase continuously from the eight-cell stage to reach comparable levels of *AGO2* expression, or even slightly higher at the late ICM stage (**Figure 1B**). In both species, however, *AGO2* is the most expressed Argonaute mRNA during early preimplantation development, in embryonic and primitive endoderm lineages. However, maximal expression occurs at different stages in human and mouse. Human *AGO2* (*hAGO2*) peaks at the four-cell stage, whereas mouse *Ago*2 (*mAgo2*) peaks at the morula stage (**Figure 1B**). As AGOs are actually required in early stages to degrade maternally deposited transcripts (Lykke-Andersen et al., 2008), the question arises whether the expression of *AGO2* coincides with zygotic gene activation (ZGA). In humans, the first major wave of ZGA occurs at the 4- to 8-cell stages followed by a second one at the 8- to 16-cell stages [for review, see Jukam et al. (2017)]. This, however, does not anymore correlate with the expression of h*AGO2* transcripts, which decreases after the four-cell stage. Moreover, it raises the question whether most of the *hAGO2* in early development is actually maternally deposited. On the contrary, the first major wave of ZGA in mice is already detectable at the two-cell stage, followed by a second wave at the four- to eight-cell stages [for review, see Jukam et al. (2017)]. This correlates with the increasing expression of m*Ago2*, which reaches its peak at the morula stage. Interestingly, recent studies in mice preimplantation development have demonstrated that mRNA levels do not always correlate with the protein levels (Gao et al., 2017). Moreover, protein expression often lags behind the mRNA expression in the process of ZGA. Therefore, we do not know whether the mRNA expression levels of the Argonautes discussed above reflect the protein levels within each cell (Gao et al., 2017).

Posttranslational Modifications of Argonaute Proteins and Their Functions

Posttranslational modifications (PTMs) of proteins mediate a huge range of signaling events within a cell and are therefore critical for distinct processes such as developmental timing (Seet et al., 2006). In fact, in *Caenorhabditis elegans,* phosphorylation of the ALG-1 protein, an ortholog of the human Argonaute proteins, is required for miRNA-mediated gene silencing and the proper animal development (Quévillon Huberdeau et al., 2017).

TABLE 1 | Posttranslational modifications of the human Argonaute 2 protein (Jee and Lai, 2014; Gebert and MacRae, 2019).

Posttranslational modifications	Conserved between hAGO1-4	Molecular functions	Cellular system identified	References
P700 Hydroxylation	Yes	• Increases AGO2 stability • Increases RISC function	HEK-293T, HeLa S3, U2OS, MEF and PASMCs	Qi et al., 2008; Wu et al., 2011
C691 S-Nitrosylation	Yes	• Disrupts interaction with GW182 and consequently miRNA mediated repression	HEK-293 and HeLa	Seth et al., 2019
K402 Sumoylation	Only in AGO1	• Destabilizes AGO2 • Increases siRNA activity	HeLa, N2a, MEFs, HT1080	Sahin et al., 2014; Josa-Prado et al., 2015
Ubiquitylation (sites unknown)	Only in AGO2 investigated	• Decreases AGO2 stability • Represses miRNA activity	HEK-293, EC, MEFs, $CD4^+$ T, MDA-MB-231	Adams et al., 2009; Rybak et al., 2009; Bronevetsky et al., 2013; Smibert et al., 2013
Poly(ADP-ribose)ylated (sites unknown)	AGO1-4 modified but sites unknown	• Inhibits slicing activity • Reduces RNAi activity	HeLa S3, HEK-293	Leung et al., 2011; Seo et al., 2013
K720, K493, K355 Acetylation	Only in AGO2 investigated	• Recruitment of AGO2 to miR-19b1 precursor	HEK-293T, A549, lung cancer tissue arrays, mouse xenografted tumor model	Zhang et al., 2019
Phosphorylation				
S387	Not conserved in AGO3 (others Yes)	• Increases translational repression • Decreases cleavage activity • Reduces sorting into exosomes	HeLa, HEK-293T, HEK-293, DLD1 colon cancer lines, MEFs, U2OS, H1299	Zeng et al., 2008; Rüdel et al., 2011; Horman et al., 2013; Lopez-Orozco et al., 2015; McKenzie et al., 2016; Bridge et al., 2017; Quévillon Huberdeau et al., 2017
Y393	Not conserved in AGO3 (others yes)	• Decreases maturation of AGO2-mediated miRNA under hypoxia • Inhibits loading of miRNA	HEK-293, HEK-293T, HeLa, MDA-MB-231, IMR90	Rüdel et al., 2011; Shen et al., 2013; Yang et al., 2014
Y529	Yes	• Disrupts interaction with mRNA targets and cleavage	HEK-293, HeLa, LPS-activated RAW 264.7, primary macrophages	Rüdel et al., 2011; Mazumder et al., 2013; Lopez-Orozco et al., 2015
S798	Yes	• AGO2 loses its association with P-bodies and stress granules	HeLa	Lopez-Orozco et al., 2015
S253, T303, T307	Yes for S253 and T307, T303 not conserved in AGO4	• Unknown	HEK-293	Rüdel et al., 2011
T555-S561 cluster	Yes	• Impaired localization to P-bodies and silencing	HEK-293T, HeLa	Quévillon Huberdeau et al., 2017
S824-S834 cluster	Yes	• Affects mRNA target association	HEK-293T, HeLa, HCT116	Golden et al., 2017; Quévillon Huberdeau et al., 2017

Animals expressing a phosphomutant ALG-1 display developmental defects and die at the adult stage, exemplifying the importance of posttranslational modification of Argonaute proteins during development (Quévillon Huberdeau et al., 2017).

AGO2 has been shown to be highly posttranslationally modified, which affects its protein stability and miRNA activity (**Figure 1C**) (Meister, 2013). Most of the posttranslational modifications have been observed in human cancer cells, yet their amino acids are conserved between mice and humans. The regulation of PTM of AGO proteins was recently related to the activity of well-characterized oncogenes, underlining the relevance of AGO-dependent pathways deregulation in cancer development. However, their importance in early development has not been assessed in mammals. In **Table 1**, we highlight the reported PTM sites of AGO2 and their molecular consequences.

As previously described, Argonaute proteins, through the formation of a RISC complex, enable miRNAs to downregulate partially complementary target mRNAs, making them relevant in normal physiology and disease. PTMs of AGOs can impact several features of RISC-mediated silencing. For example, a rapid cycle of AGO2 phosphorylation and dephosphorylation of a serine/threonine cluster located on a loop on the surface of the PIWI domain is relevant for miRNA binding to target mRNAs and for miRNA-mediated gene silencing. The dissection of the upstream signaling pathways that impact on AGO2 PTM and, consequently, on its cyclic functional activity would represent a relevant advance in the understanding of AGO2 activity and might possibly provide new ways to modulate the global activity of miRNAs (Golden et al., 2017).

The phosphorylation status of AGOs is also critical for the regulation of the miRNA function in humans (Quévillon Huberdeau et al., 2017). In particular, AGOs are hyperphosphorylated at a C-terminal serine/threonine cluster upon miRNA binding and repression of the mRNA target. The negative charge of phosphates within this region impairs the mRNA/AGO interaction and favors the release of target mRNA.

The balance between the phosphorylated and dephosphorylated status of AGO may be relevant also for redirecting AGO to a new target mRNA and for modulation of its degradation (Quévillon Huberdeau et al., 2017).

Furthermore, PTMs of AGOs are involved in miRNA processing. AGO2 phosphorylation has been related to certain cancer phenotypes. In these cases, specific AGO2 phosphorylation leads to reduced interaction between DICER and AGO2 with consequent impairment of miRNA processing (Shen et al., 2013).

In addition, the acetylation of specific lysine residues of AGO2 has been reported. This represents a relevant modification for the recruitment of AGO2 to the miR-19b1 precursor, resulting in the enhancement of oncogenic miR-19b processing. Notably, in lung cancer patients, high levels of both miR-19b and AGO2 acetylation correlate with a poor prognosis (Zhang et al., 2019).

Finally, AGO2 phosphorylation also impacts its localization within the cell. Specific AGO2 phosphorylation has been reported to be essential for its localization into processing bodies (P-bodies), impinging on AGO2-dependent regulation of RNA-silencing activity (Zeng et al., 2008). Horman et al. (2013) subsequently show that AGO2 phosphorylation is critical for the interaction with GW182 protein and AGO2 localization in P-bodies. Furthermore, this modification was also shown to regulate localization of AGO2 into multivesicular endosomes resulting in the suppression of AGO2 secretion and influencing the sorting of specific miRNAs into exosomes (McKenzie et al., 2016).

In summary, PTMs affect several AGO exerted functions. In this paragraph, we have given only a few examples. A broader overview can be found in **Table 1**.

THE FUNCTIONS OF ARGONAUTE PROTEINS IN MAMMALIAN EARLY DEVELOPMENT

Several studies have examined the roles of AGOs during mouse early development. Earliest works demonstrated that the knockout (KO) of *Ago2* is lethal during early mouse development at postimplantation stages (Liu et al., 2004; Alisch et al., 2007; Morita et al., 2007; Cheloufi et al., 2010). In contrast *Ago1*, *3*, and *4* KO mice are viable (Modzelewski et al., 2012; Van Stry et al., 2012). These studies have shown that *Ago2*-deficient embryos are growth retarded and developmentally delayed. In addition, they display severe phenotypic defects, such as cardiac failure and impaired neuronal tube closure (Liu et al., 2004; Alisch et al., 2007; Cheloufi et al., 2010).

Interestingly, the phenotype of the *Ago2*-deficient mice compared to other RNAi-deficient mice is not identical. In addition to other phenotypic differences, *Dicer*- or *Drosha*-deficient mice, for example, display earlier embryonic lethality compared to *Ago2*-deficient mice, suggesting individual roles for the RNAi effector proteins in regulating embryonic development (Bernstein et al., 2003; Chong et al., 2010).

The function of AGO2 in the embryonic development has already been given ample attention with the help of several mouse models (Liu et al., 2004; Alisch et al., 2007; Morita et al., 2007; Cheloufi et al., 2010). However, detailed analyses of AGO2 in the development of the extraembryonic lineages are still missing. Recently, it has been reported that early mice lethality is often associated with placental defects (Perez-Garcia et al., 2018). Interestingly, previous histological analyses have already indicated that *Ago2*-deficient mice display extraembryonic defects. Supplementing these mice with wild-type extraembryonic tissue is able to rescue the mid-gestation death of *Ago2* KO mice (Liu et al., 2004; Cheloufi et al., 2010). These defects might explain why *Ago2*-deficient mice die at the postimplantation stage; however, this has not been assessed yet.

Ngondo et al. (2018) have recently demonstrated a novel function of AGO2 in the development of the extraembryonic endoderm, *in vitro*. Using mouse embryonic stem cells (mESCs), which are derived from the blastocyst stage, they generated *Ago2* KO mESCs, by CRISPR/Cas9 genome engineering (Wettstein et al., 2016). Upon *in vitro* differentiation, *Ago2* KO mESCs were able to form all three embryonic germ layers; however, they showed impaired differentiation toward the extraembryonic endoderm (Ngondo et al., 2018). This differentiation defect was rescued by the reintroduction of a wild-type AGO2 or a catalytic dead AGO2 in mESCs, but not by an RNA-binding-deficient AGO2 (Ngondo et al., 2018). In line with these results, *Ago2* catalytic dead mice were previously shown to be viable until a few hours after birth and subsequently died of anemia (Cheloufi et al., 2010; Jee et al., 2018). In these cases, the slicing activity of AGO2 is needed to process the pre-miRNA-451 and miRNA-486-3p, two miRNAs required in the development of the erythroblasts (Cheloufi et al., 2010; Papapetrou et al., 2010; Jee et al., 2018).

Notably, the molecular mechanism by which AGO2 regulates the formation of the extraembryonic endoderm still remains elusive. Interestingly, the differentiation defect of the *Ago2* KO mESCs is comparable to what was observed previously for *Gata6* KO mESCs, a key transcription factor required for the formation of the primitive endoderm lineage *in vivo* (Capo-Chichi et al., 2005). Together, these reports indicate a function of AGO2 not only in the development of the embryo proper but also in the extraembryonic lineages.

Mouse embryonic stem cells are a very informative *in vitro* culture system to mimic mouse early development at the blastocyst stage. Nevertheless, a stable *in vitro* system mimicking the earliest stages of development is still missing. Most of the studies focusing on the first cell fate decisions in early mouse development were performed by imaging wild-type or mutant embryos and relied on specific antibodies for the protein of interest, which were not available for a long time for the mouse AGO proteins. Furthermore, single-cell bulk analysis requires a lot of material, which is hard to obtain from early embryos. However, a powerful tool to study the earliest cell fate decision, the two-cell stage-like (2C) ESCs, has been discovered a few years ago (Macfarlan et al., 2012). Two-cell like cells are totipotent and therefore can still differentiate into the extraembryonic as well as embryonic lineages (Baker and Pera, 2018). MESCs have been shown to present a heterogeneous

population, where a small subpopulation (<1%) cycles in and out of a two-cell stage (Macfarlan et al., 2012; Morgani et al., 2013; Ishiuchi et al., 2015). The totipotent two-cell stage subpopulation might provide a powerful way to study the impact of AGO2 on the early stages of mouse development, not only for the epiblast but also for the trophoblast lineage, where the impact of AGO2 loss has not been assessed yet. So far, we still do not know whether AGO2 is the only Argonaute protein well expressed in this lineage and whether it impacts trophoblast differentiation.

Since AGO2-deficient mice only die at the postimplantation stage, the question is raised, whether AGO2 is dispensable for preimplantation development or, whether maternally supplied AGO2 regulates these early stages. One important *in vivo* study assessed the requirement of AGO proteins before the blastocyst stage (Lykke-Andersen et al., 2008). Using injection of dsRNAs against maternally supplied Argonautes, they demonstrated that only AGO2 is essential for the development of mouse oocytes to the two-cell stage. Nevertheless, the molecular mechanism by which AGO2 regulates this early transition is still unknown. Strikingly, the loss of another RNAi effector protein in oocytes, DGCR8, displays a very different phenotype compared to the loss of AGO2. *Dgcr8* KO oocytes are able to develop beyond the 2-cell stage to blastocysts. As DGCR8 is only involved in the processing of canonical miRNAs, this suggests that canonical miRNAs might be dispensable for early development (Suh et al., 2010). This is in line with the previous assumptions that miRNA function is lost in oocytes. One reason for the loss of miRNA function in oocytes was proposed to be due to an AGO2-specific oocyte isoform (Freimer et al., 2018). However, a recent report shows that miRNA activity might not be lost in oocytes but that the miRNA/mRNA stoichiometry is impaired in oocytes due to the low abundance of miRNAs (Kataruka et al., 2019). Furthermore, *Ago2* KO oocytes seem very similar to *Dicer* KO oocytes. Both show abnormal spindle and chromosome positioning and fail to undergo the first cleavage to the two-cell stage (Murchison et al., 2007; Tang et al., 2007; Kaneda et al., 2009). Moreover, changes in gene transcripts in *Dicer* KO oocytes are claimed to be provoked by endo-siRNAs (Watanabe et al., 2008), which are the most prominently expressed smRNAs in oocytes and not preprocessed by DGCR8 (Tam et al., 2008; Watanabe et al., 2008; Suh et al., 2010). The loss of DICER and AGO2 in oocytes decreases siRNAs (Watanabe et al., 2008). It is therefore possible that the phenotype observed in *Ago2* KO oocytes is a result of the loss of endo-siRNA-induced target silencing.

Hence, the exact function of AGO2 in early development still needs to be elucidated, as it is undoubtedly the only one leading to a lethal phenotype.

CONCLUSION

In this review, we highlight various differences and similarities between the Argonaute proteins to better understand their specialized roles within the cell, especially in regard to AGO2.

With the structural information available nowadays, it is possible to pinpoint the exact residues responsible for the catalytic function or disfunction of the AGOs. This has clarified why AGO2 specifically was thought for a long time to be the only slicer molecule of this family.

Interestingly, however, from available sequencing data, it seems that AGO2 is the only AGO protein well expressed in early mice or human development, at least at the transcriptional level (Boroviak et al., 2018). This, when reflected on protein levels, might also explain the severe defects observed upon the loss of AGO2 in early embryos when compared to AGO1, 3, and 4. Strikingly, the *Ago2* KO phenotypes observed are not just linked to the embryonic development but also cause impairments in extraembryonic development, as studies show placental defects associated with the loss of AGO2 (Liu et al., 2004; Cheloufi et al., 2010). We argue that a deeper exploration in the early development of extraembryonic tissues is warranted in the context of AGO2 loss *in vivo* and *in vitro*.

Lastly, we present an overview of multiple to date known posttranslational modifications of AGO2. These modifications have so far been studied in several cancer models and furthermore have been linked to disease phenotypes. From such studies, we know that PTMs can impact the RISC activity as well as AGO2 stability, either positively or negatively. However, a detailed analysis of such modifications during early development is still missing. We still do not know which modifications are present in early embryos nor whether there is a switch of modification when going through different stages of embryonic or extraembryonic development. Hence, to better understand how AGO2 functions in these early stages of embryonic development, the PTMs of AGO2 must also be taken into consideration.

AUTHOR CONTRIBUTIONS

MM and CC wrote the sections related to Argonaute expression and structure in early development. MM drew the figure and established the **Table 1**. FF contributed to the writing of the section on the PTM of AGO in cancer. All authors read and approved the final version of the manuscript.

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REVIEW
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Non-coding RNAs Shaping Muscle

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In 1957, Francis Crick speculated that RNA, beyond its protein-coding capacity, could have its own function. Decade after decade, this theory was dramatically boosted by the discovery of new classes of non-coding RNAs (ncRNAs), including long ncRNAs (lncRNAs) and circular RNAs (circRNAs), which play a fundamental role in the fine spatio-temporal control of multiple layers of gene expression. Recently, many of these molecules have been identified in a plethora of different tissues, and they have emerged to be more cell-type specific than protein-coding genes. These findings shed light on how ncRNAs are involved in the precise tuning of gene regulatory mechanisms governing tissues homeostasis. In this review, we discuss the recent findings on the mechanisms used by lncRNAs and circRNAs to sustain skeletal and cardiac muscle formation, paying particular attention to the technological developments that, over the last few years, have aided their genome-wide identification and study. Together with lncRNAs and circRNAs, the emerging contribution of Piwi-interacting RNAs and transfer RNA-derived fragments to myogenesis will be also discussed, with a glimpse on the impact of their dysregulation in muscle disorders, such as myopathies, muscle atrophy, and rhabdomyosarcoma degeneration.

Keywords: myogenesis, non-coding RNAs, long non-coding RNAs, lncRNAs, circular RNAs, circRNAs, Piwi-interacting RNAs, transfer RNA-derived fragments

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INTRODUCTION

In the last 50 years, RNA has been subjected to an unprecedented revaluation that gradually shifted the perspective on how gene expression is regulated from a coding to a non-coding point of view. With the discovery of messenger RNA (mRNA) codon–transfer RNA (tRNA) anticodon interaction, tRNAs have become forerunners of non-coding functionality (Hoagland et al., 1958; Steitz and Jakes, 1975). In roughly 30 years of experiments and observations, novel classes of non-coding RNAs (ncRNAs), such as ribosomal RNAs (rRNAs) (Scherrer and Darnell, 1962), small nuclear RNAs (Weinberg and Penman, 1968), small nucleolar RNAs (Reddy and Busch, 1988), and microRNAs (miRNAs) (Lee et al., 1993; Lagos-Quintana et al., 2001; Lau et al., 2001), attracted the attention of the scientific community by showing their participation to several biological processes. A significant contribution to these discoveries was made by the advances in both sequencing technologies and data open sharing, which brought the new millennium into the "omics" revolution. In this context, the identification and the extraordinary cell-type-specific expression of new classes of ncRNAs, including long ncRNAs (lncRNAs) and circular RNAs (circRNAs), provided new clues into the understanding of tissue homeostasis (Kapranov et al., 2007; Mattick, 2011; Nagano and Fraser, 2011; Hon et al., 2017).

LncRNAs are arbitrarily defined as transcripts longer than 200 nucleotides, which regulate gene expression at transcriptional and post-transcriptional level (Rinn and Chang, 2012; Ulitsky and Bartel, 2013; Fatica and Bozzoni, 2014). The function of these transcripts is in close connection

with their specific subcellular localization, ranging from an almost exclusive nuclear (Heard and Disteche, 2006; Tripathi et al., 2010; Chen et al., 2016) or cytoplasmic (Cesana et al., 2011; van Heesch et al., 2014) enrichment, up to a more uniform and less defined distribution (Ballarino et al., 2015; Alessio et al., 2019). Together with lncRNAs, it is nowadays well-established that cells also express circRNAs. These are endogenously expressed and covalently closed single-stranded RNA species that derive from back-splicing circularization events (Jeck et al., 2013; Chen, 2016). Differently from linear lncRNA molecules, the lack of 5′- and 3′-ends confers to circRNAs greater stability and amplifies their chance to exert various biological tasks. As a common aspect, the function of both lncRNA and circRNA is determined by their ability to recognize nucleic acids by base pairing with their high versatility to interact with proteins (Guttman and Rinn, 2012; Rinn and Chang, 2012; Batista and Chang, 2013). For this reason, the post-genomic epoch has been marked by the establishment of innovative technologies, which have been extremely helpful for identifying the ncRNA interactome, thus providing crucial clues on their mechanisms of action (Ule et al., 2003; Licatalosi et al., 2008; Engreitz et al., 2014; McHugh and Guttman, 2018). The RNA antisense purification (RAP) is one of the most RNA-centric leading-edge approach able to purify and identify the interacting partners of a specific RNA (McHugh and Guttman, 2018). The protein-centric mirror technique is represented by the cross-linking and immunoprecipitation (CLIP) assay (Ule et al., 2003; Licatalosi et al., 2008). Both the systems exploit the ultraviolet (UV) cross-linking to create covalent linkages between directly interacting RNAs and proteins, purifying the molecule of interest under stringent conditions to reduce background signals. In addition, a method to systematically map RNA–RNA interactions, based on psoralen cross-linking, has been also developed (Engreitz et al., 2014).

To date, the vast majority of studies have focused on the role of miRNAs in muscle development (Ballarino et al., 2016; Wang J. et al., 2018; Colpaert and Calore, 2019). In this article, we highlight the importance of some less studied classes of ncRNAs, such as lncRNAs and circRNAs, focusing on their function in skeletal and cardiac muscles biology. We discuss paradigmatic examples that support their nuclear and cytoplasmic activities and report the latest findings of lncRNAs and circRNAs containing functional open reading frames (ORFs) (Chekulaeva and Rajewsky, 2019). In the final section, we also provide a broad overview on other classes of ncRNAs [i.e., Piwi-interacting RNAs (piRNAs) and tRNA fragments], which are well-known for their canonical functions but only recently emerged as functional in muscle physiology (Liapi et al., 2019).

lncRNAs IN SKELETAL AND CARDIAC MYOGENESIS

Since the establishment of the murine myoblast C_2C_{12} cell line, which allowed to reproduce *in vitro* the different stages of myogenic differentiation (Yaffe and Saxel, 1977), and the discovery of MYOD1 as the "master gene" for myogenesis (Davis et al., 1987), a wealth of knowledge has been accumulated regarding the ncRNA-mediated regulatory networks governing muscle biology. Indeed, multiple examples of nuclear (**Table 1**) and cytoplasmic (**Table 2**) non-coding transcripts involved in the acquisition of both skeletal and cardiac muscle identity have been detailed over the last decade, with an increasing degree of emphasis on large species (Neguembor et al., 2014; Rotini et al., 2018).

Nuclear-Enriched lncRNAs

Aside from the "finished" non-coding transcript, the *act* of transcription has been proposed to be functional in myogenesis. Few years ago, Anderson et al. (2016) reported that the transcription of Hand2-associated lncRNA, named Upperhand (Uph) is required to establish a permissive chromatin environment at the Hand2 enhancer locus. Indeed, blockade of Uph transcription, but not the knockdown of the mature transcript, abolished Hand2 expression and caused heart failure and embryonic lethality in mice. In a very recent paper, Ritter et al. identified a novel lncRNA locus named Handsdown (Hdn) that is fundamental for the transcriptional regulation of Hand2 gene (Ritter et al., 2019). *In situ* hybridization of both Hdn and Hand2 demonstrates that the transcripts are coexpressed in the same cells or at least in the same parts of the tissue at different time points of embryonic heart specification. Interestingly, Hdn knockout shows that the locus is essential for embryonic development and uterine implantation, while its reduced expression in the heterozygous mice is haploinsufficient for proper heart formation. Chromosome conformation capture (Hi-C and 4C) analyses demonstrate that Hdn expression is crucial for structuring the genomic region and regulating *in cis* Hand2 production in a transcript-independent, transcription-based manner.

Upon their transcription, most of the nuclear-enriched lncRNAs regulate myogenesis by recruiting chromatin-modifying complexes to nearby (*in cis*) or distant (*in trans*) genomic loci. The Polycomb repressive complex 2 (PRC2), which catalyzes the methylation of lysine 27 on histone H3, is one of the most studied chromatin-modifying complexes found to be recruited by myogenic lncRNAs. Earlier studies from 2013 described two paradigmatic examples, which include Braveheart (Klattenhoff et al., 2013) and Fendrr (Grote et al., 2013). Overall, these findings highlighted the functional importance of lncRNA-based epigenetic mechanisms in the regulation of cell fate and greatly contributed to advance our understanding of the regulatory networks driving cardiac lineage commitment. In skeletal muscle, a more recent study (Jin et al., 2018) reported the identification of SYISL (SYNPO2-intron sense-overlapping), an abundant and intron-encoded lncRNA whose expression increases with C_2C_{12} myoblasts differentiation. Mechanistically, SYISL promotes proliferation (and inhibits differentiation) by silencing the muscle-specific expression of myogenin, muscle creatine kinase, and myosin heavy chain (Myh4) through the recruitment of the PRC2 subunit enhancer of Zeste homolog 2 (EZH2), to their promoters. Similarly, Wang S. et al. (2019) described a functional interaction between EZH2 and Neat1 in myogenic cells. Nuclear Enriched Abundant Transcript 1 (Neat1) is a nuclear lncRNA essential for paraspeckles formation, stability, and integrity (Souquere et al., 2010), which

TABLE 1 | Nuclear long non-coding RNAs (lncRNAs) in skeletal and cardiac myogenesis.

lncRNA	Species	Function/mechanism	*In vivo* model phenotype	Expression	References
Upperhand (UPH)	Mouse, human	Its transcription is required to establish a permissive chromatin environment at Hand2 enhancer locus	Embryonic lethality and heart failure	Highly expressed in heart	Anderson et al., 2016
Handsdown (HDN)	Mouse, human	Its transcription is required to regulate *in cis* the production of Hand2	Embryonic lethality and defects in uterine implantation	Expressed in early developing heart	Ritter et al., 2019
Braveheart (BVHT)	Mouse	It is required for the activation of a core cardiovascular gene network by preventing SUZ12 to repress MesP1 promoter	Not available	Highly expressed in heart	Klattenhoff et al., 2013
Fendrr	Mouse, human	Binds PRC2 and TrxG/MLL to influence histone marks on lateral mesoderm specific gene promoters	Embryonic lethality	Lateral mesoderm-specific expression	Grote et al., 2013
SYISL	Mouse	Promotes cellular proliferation by inhibiting muscle-specific transcription factors through an EZH2-recruitment mechanism	Defects in muscle fiber density and muscle mass	Highly expressed in muscle	Jin et al., 2018
Neat1	Mouse, human	Sustains myoblast proliferation and blocks differentiation by recruiting EZH2 to muscle-specific promoters	Defects in muscle regeneration	Expressed in a wide range of tissues	Wang S. et al., 2019
SRA	Mouse, human	It is required for proper cell differentiation by coactivating MyoD together with the RNA elicase p68/p72	Not available	Expressed in a wide range of tissues	Caretti et al., 2006; Hubé et al., 2011
CE	Mouse	Acts as enhancer RNA to increase RNA POL II occupancy at MyoD locus	Not available	Expressed in myogenic lineage	Mousavi et al., 2013
DRR	Mouse	Acts as enhancer RNA to activate MyoD downstream myogenic genes	Not available	Expressed in myogenic lineage	Mousavi et al., 2013
RAM	Mouse, human	Promotes the activation of the myogenic program by binding to MyoD and supporting the assembly of MyoD-Baf60c-Brg complex	Defects in muscle regeneration	Skeletal muscle-specific	Yu et al., 2017
Dum	Mouse, human	Promotes myoblasts differentiation by recruiting Dnmts to regulate Dppa2 expression	Defects in muscle regeneration	Highly expressed in muscle	Wang et al., 2015
YY1	Mouse, human	Activates gene expression *in trans* by interacting with YY1 and removing YY1/PRC2 complex from target promoters	Downregulation of keys myogenic genes	Highly expressed in muscle	Zhou et al., 2015
IRM	Mouse	Regulates the expression of myogenic genes by binding to MEF2D and promoting the assembly of MyoD/MEF2D	Impairment of muscle regeneration	Expressed in brain and skeletal muscle	Sui et al., 2019
Myolinc	Mouse	Promotes *in vitro* myogenesis by recruiting TDP-43 on muscle-specific targets both *in cis* and *in trans*	Defects of regeneration in muscle cells	Highly expressed in heart and skeletal muscle	Militello et al., 2018
Myoparr	Mouse, human	Induces myoblasts cell cycle withdrawal and activates myogenin transcription by interacting with MyoD coactivator Ddx17	Prevention of atrophy in denervated muscle	Skeletal muscle-specific	Hitachi et al., 2019a,b
PVT1	Mouse	Interacts to and stabilizes c-Myc impacting the activity of Bcl-2	Defects in mitochondrial respiration and morphology, apoptosis, and myofiber size	Highly expressed in skeletal muscle	Alessio et al., 2019
Myheart (Mhrt)	Mouse, human	Antagonizes the function of the transcription factor Brg1 preventing the recognition of its genomic targets	Mhrt restoration improves cardiac function in TAC-stressed hearts	Highly expressed in heart	Han et al., 2014
Chronos	Mouse	Represses Bmp7 by recruiting EZH2 on its promoter	Myofibers hypertrophy	Highly expressed in heart and skeletal muscle	Neppl et al., 2017
MEG3	Mouse, human	Controls cardiac fibrosis through the regulation of matrix metalloproteinase-2 production	Decreases cardiac fibrosis and improves diastolic performance	Highly expressed in cardiac fibroblasts during pressure-overload heart remodeling	Piccoli et al., 2017; Wu et al., 2018
Charme	Mouse, human	Acts as a chromatin architect to promote myoblasts differentiation	Cardiac remodeling phenotype at developmental onset	Skeletal muscle and heart-specific	Ballarino et al., 2018

exerts critical roles in several biological processes as well as in tumorigenesis and non-cancerous diseases (Ghafouri-Fard and Taheri, 2019; Prinz et al., 2019). During C_2C_{12} differentiation, Neat1 sustains proliferation and blocks differentiation by recruiting EZH2 to p21 (cyclin-dependent kinase inhibitor 1 A) and to muscle specific promoters (i.e., Myog, Myh4, and Tnni2). To note, Neat1 depletion *in vivo* was shown to delay muscle regeneration induced by cardiotoxin treatment.

TABLE 2 | Cytoplasmatic long non-coding RNAs (lncRNAs) in skeletal and cardiac myogenesis.

lncRNA	Species	Function/mechanism	*In vivo* model phenotype	Expression	References
LNC-31	Mouse, human	Promotes ROCK1 translation by stabilizing YB-1 protein	Not available	Expressed in a wide range of tissues	Ballarino et al., 2015; Dimartino et al., 2018
LiNC-MD1	Mouse, human	ceRNA for miR-133 and miR-135 to regulate the expression of MAML1 and MEF2C	Not available	Muscle-specific expression	Cesana et al., 2011
LNC-MG	Mouse	ceRNA for miR-125b and miR-351-5p to control insulin-like growth factor 2 protein abundance and regulate lactamase β expression	Muscle atrophy and loss of muscular endurance	Skeletal muscle enriched	Zhu et al., 2017; Du et al., 2019
AK017368	Mouse	ceRNA for miR-30c to regulate trinucleotide repeat containing-6A	Muscle hypertrophy	Enriched in lung, heart, and skeletal muscle	Liang et al., 2018
LNC-MUMA	Mouse, human	ceRNA for miR-762 to regulate MyoD abundance	Its overexpression reverses muscle atrophy	Skeletal muscle enriched	Zhang et al., 2018a
MAR1	Mouse	ceRNA for miR-487b to regulate Wnt5a protein	Its overexpression increases muscle mass and strength	Skeletal muscle-enriched	Zhang et al., 2018b
LNC-MYOD	Mouse, human	Regulates the translation of N-Ras and c-Myc by sequestering IMP2 protein	Not available	Myoblasts and early myotubes specific	Gong et al., 2015
ATROLNC-1	Mouse	Interacts to and inhibits ABIN-1 protein to increase MuRF-1 expression	Attenuates muscle wasting	Highly expressed in skeletal muscle, upregulated in atrophying muscles	Sun et al., 2018
ZFAS1	Mouse, human	Binds to and inhibits SERCA2a protein affecting the Ca^{2+} transient dynamics	Restores heart contraction parameters in MI animals	Highly expressed in different cancers and MI	Zhang et al., 2018c; Jiao et al., 2019
DACH1	Mouse, human	Binds to and inhibits SERCA2a protein affecting the Ca^{2+} transient dynamics	Heart failure	Upregulated upon heart failure	Cai B. et al., 2019
CTBP1-AS2	Mouse, rat	Binds FUS to induce a non-physiological stabilization of TLR4 mRNA	Attenuates cardiomyocytes hypertrophy	Upregulated in cancer and cardiomyocyte hypertrophy	Luo et al., 2019

Besides the recruitment of PRC2, several nuclear-enriched lncRNAs regulate the binding of transcription factors and transcriptional coactivators to specific myogenic loci. Several of these lncRNAs, such as SRA (Caretti et al., 2006; Hubé et al., 2011), the CE and DRR endogenous RNAs (eRNAs) (Mousavi et al., 2013), and Linc-RAM (Yu et al., 2017) regulate the activity of the master transcription factor MyoD. Others impinge myogenesis either through the binding of specific myogenic factors or transcription regulators (Dum, Wang et al., 2015; Linc YY1, Zhou et al., 2015). Irm, a lncRNA that regulates myogenic genes expression in C_2C_{12} cells by directly binding to MEF2D (Sui et al., 2019), belongs to the first category. A recent example from the second group includes Myolinc (AK142388), a lncRNA that promotes myoblasts fusion by the recruitment of the TAR DNA-binding protein 43 (TDP-43) *in cis*, on the neighboring Filip1 promoter, and *in trans,* on muscle-specific targets (Militello et al., 2018). As for Neat1, Myolinc knockdown causes a delayed regeneration of skeletal muscle in adult mice. Hitachi et al. recently described Myoparr, a promoter-associated lncRNA derived from the upstream region of the myogenin gene, which has a dual role in myogenesis: on the one hand, it is involved in myoblasts cell cycle withdrawal by the transactivation of myo-miRNAs expression; on the other hand, it is responsible for the activation *in cis* of myogenin transcription, which allows the entrance of myoblasts into myogenic differentiation. The latter is mediated by the interaction between Myoparr and the MyoD-coactivator Ddx17, which results in the RNA-Pol II recruitment on myogenin promoter and in transcriptional activation (Hitachi et al., 2019a). In a subsequent paper, the same authors also examined the role of Myoparr in skeletal muscle atrophy. They found that, in denervated muscles, Myoparr knockdown prevents atrophy by activating the bone morphogenetic protein signaling and by increasing the expression of GDF5, a muscle atrophy inhibitor. The existence of a Myoparr human counterpart makes the lncRNA potentially eligible as therapeutic target for neurogenic atrophy (Hitachi et al., 2019b).

Like Myoparr, it is not uncommon to find association between dysregulation of lncRNA expression and pathological conditions that induce changes in muscle mass, such as atrophy, hypertrophy, and cardiac remodeling. Another example is Pvt1, an evolutionary conserved lncRNA whose upregulation in cancer cells influences positively the stability of the oncoprotein transcription factor MYC (Tseng and Bagchi, 2015). A similar Pvt1/MYC interplay also plays a role during muscle atrophy, in which the Pvt1-mediated c-Myc stabilization impacts on the activity of Bcl-2, a crucial regulator of apoptosis and autophagy (Alessio et al., 2019). First evidence on the involvement of lncRNAs in hypertrophy came in 2014 from Chang's lab, which described a reduced hypertrophic growth and improved cardiac functions upon restoration of myosin heavy-chain-associated RNA transcripts (Mhrt) levels in transverse aortic constriction

(TAC)-stressed hearts (Han et al., 2014). Analogously to Mhrt, in skeletal muscle cells, the Gm17281 lncRNA, known as Chronos, inhibits hypertrophy through the Bmp7 signaling. Mechanistically, Chronos acts as an epigenetic repressor of Bmp7 cascade through the recruitment of EZH2 on the Bmp7 promoter (Neppl et al., 2017). In murine cardiac fibroblasts, a microarray analysis led to the identification of several lncRNAs dysregulated after pressure overload upon TAC. Among the most abundant nuclear species, Piccoli et al. found the lncRNA maternally expressed gene 3 (Meg3). In cardiac fibroblasts, Meg3 controls the production of the matrix metalloproteinase-2 and, consequently, cardiac fibrosis (Piccoli et al., 2017). Indeed, Meg3 silencing was shown to reduce diastolic dysfunction and fibrosis by preventing the pathological induction of the matrix metalloproteinase-2 protein after pressure overload. Prospectively, the inhibition of Meg3 expression might represent a conceivable strategy to prevent the development of fibrosis and cardiomyocytes hypertrophic growth observed in heart diseases. The finding that Meg3 expression is upregulated in clinical heart failure samples and controls the apoptosis of human cardiomyocites (Wu et al., 2018) makes Meg3 particularly attracting as a therapeutic target. *Charme* (chromatin architect of muscle expression) is another nuclear-enriched lncRNA (Ballarino et al., 2018), whose ablation *in vivo* leads to a pronounced phenotype of cardiac remodeling at developmental onset. In myotubes, *Charme* contributes to the chromatin proximity between important myogenic loci, thus controlling their expression at transcriptional level. This epigenetic control is in line with emerging studies indicating that lncRNAs can act as modular scaffolds to shape the formation of chromosome territories where coregulated gene expression occurs (Clemson et al., 2009; Tripathi et al., 2010; Engreitz et al., 2013; Hacisuleyman et al., 2014; Ribeiro et al., 2018). Moreover, the existence of a *Charme* ortholog transcript produced by the human syntenic locus makes this lncRNA an appealing target for cardiovascular diseases.

Cytoplasmic-Enriched lncRNAs

In the cytoplasm (**Table 2**), lncRNAs mainly act as regulators of mRNA stability and translation. These activities depend on two major lncRNA peculiarities: (i) the capacity to base pair with RNA counterparts and (ii) the ability to act as flexible scaffolds for tethering RNAs and proteins and ensure their concerted action. Paradigmatic examples include the antisense lncRNAs known as half-STAU1-binding site RNAs (1/2-sbsRNAs) that induce Staufen1-mediated (STAU-mediated) mRNA decay (Wang J. et al., 2013). A new identified mechanism by which lncRNA–mRNA–protein interplay exerts a role in skeletal myogenesis is represented by lnc-31, a lncRNA controlling the maintenance of myoblast proliferation both in murine and human myoblasts (Ballarino et al., 2015). A key determinant for lnc-31 function is the presence of a 22nt-long region, which binds the 5′ untranslated region of ROCK1 mRNA and positively controls protein synthesis. The concomitant recruitment and the consequent stabilization of the translational activator Y-box protein 1 (YBX1) by lnc-31 concur to this translational control, as demonstrated by the decrease in ROCK1 protein upon Y-box protein 1 (YBX1) knockdown in proliferating myoblasts (Dimartino et al., 2018).

Another mechanism which underlies the capacity of lncRNAs to influence mRNA stability is based on their ability to act as competing endogenous RNAs (ceRNAs) for miRNAs (Cesana et al., 2011; Kallen et al., 2013; Han et al., 2015; Yan et al., 2015) or proteins (Gong et al., 2015). Since the discovery of linc-MD1, which was one of the first miRNA sponges to be identified, additional muscle relevant ceRNAs have been described. Recent examples include lnc-mg (Zhu et al., 2017), AK017386 (Liang et al., 2018), LncMUMA (Zhang et al., 2018a), and MAR1 (Zhang et al., 2018b) lncRNAs. Lnc-mg is a 5′-capped and polyadenylated ncRNA whose ablation (i) *in vitro*, reduces the capacity of murine satellite cells (MuSCs) to differentiate and (ii) *in vivo*, results in muscular atrophy associated to reduced muscle endurance. Mechanistically, lnc-mg acts as a miRNA-125b sponge to increase the abundance of insulin-like growth factor 2, an already known miRNA-125b target (Ge et al., 2011). More recently, lnc-mg was shown to act as a molecular sponge for miR-351-5p, which functions in skeletal myogenesis by targeting lactamase β (Du et al., 2019). AK017368 is a muscle highly enriched lincRNA able, to induce proliferation and inhibit myoblasts differentiation *in vitro*. AK017368 depletion in murine muscles induce fibers hypertrophy. Liang et al. (2018) demonstrated that AK017368 acts as a sponge for miR-30c by competing with its known mRNA target Tnrc6a (trinucleotide repeat containing-6A), already involved in control of myogenic differentiation by guiding Ago protein into the nucleus to lead miRNA-mediated gene silencing (Nishi et al., 2013). Another example of ceRNA promoting myogenesis is LncMUMA, a mechanical unloading-induced muscle atrophy-related lncRNA functioning as a sponge for miR-762 to regulate *in vitro* MyoD abundances (Zhang et al., 2018a). As LncMUMA enforced expression reverses the established muscle atrophy in hindlimb suspension mice, the above studies provide a novel therapeutic targeting for treating muscle atrophy following mechanical unloading. The same authors identified muscle anabolic regulator 1 (MAR1), a lncRNA that acts as miR-487b sponge to regulate Wnt5a protein, an important regulator of myogenesis (Tajbakhsh et al., 1998) and a known target of mir-487b in other cellular systems (Xi et al., 2013). MAR1 resulted to be positively correlated with muscle differentiation and growth, both *in vitro* and *in vivo*. Moreover, muscle mass and strength were increased in MAR1 overexpressing condition, suggesting a putative therapeutic role for muscle atrophy treatment.

Among the lncRNAs that are able to modulate the function of cytoplasmic proteins, good examples are lncMyoD (Gong et al., 2015) and Atrolnc-1 (Sun et al., 2018). LncMyoD is directly activated by MyoD at the onset of differentiation and negatively regulates the translation of N-Ras and c-Myc by directly binding and sequestering the IMP2 (insulin-like growth factor 2–mRNA-binding protein 2) factor. Atrolnc-1 was recently identified to be highly expressed in atrophying muscle from mice with cachexia. The authors demonstrated that Atrolnc-1 predominantly interacts with cytoplasmic proteins,

TABLE 3 | Functional long non-coding RNA (lncRNA)-derived micropeptides in skeletal and cardiac myogenesis.

Micropeptide	Species	Function/mechanism	*In vivo* model phenotype	Expression	References
Myoregulin (MLN)	Mouse, human	Interacts with SERCA protein and impedes Ca^{2+} uptake into the sarcoplasmic reticulum	Enhances Ca^{2+} handling and improves exercise performance	Skeletal muscle-specific expression	Anderson et al., 2015
DWORF	Vertebrates	Increases SERCA activity by displacing other inhibitory micropeptides	Delays Ca^{+} clearance and muscle relaxation	Muscle-specific expression	Nelson et al., 2016
Mitoregulin (MRLN)/MOXI/ MPM	Mouse, human	Regulates mitochondrial physiology and impairs fatty acid β-oxidation by interacting with different complexes	Smaller skeletal muscle fibers, reduced capacity for exercise, compromised muscle regeneration	Muscle enriched	Makarewich et al., 2018; Stein et al., 2018; Chugunova et al., 2019; Lin et al., 2019
SPAR	Mouse, human	Interacts with the v-ATPase proton pump complex to negatively regulate mTORC1	Promotes skeletal muscle regeneration	Highly expressed in lung, heart and skeletal muscle	Matsumoto et al., 2017
Myomixer/Minion/ Myomerger	Vertebrates, invertebrates	Essential for muscle formation during embryogenesis, satellite cells fusion, and muscle regeneration	Perinatal lethality	Muscle-specific expression	Bi et al., 2017, 2018; Quinn et al., 2017; Zhang et al., 2017; Leikina et al., 2018

and, in particular, they focused on A20 binding inhibitor of nuclear factor kappa B-1 (NF-κB-1), an inhibitor of IκB degradation and NF-κB activation (Mauro et al., 2006; Hooper et al., 2014). The consequence of this cytoplasmic interaction is the inhibition of A20 binding inhibitor of NF-κB-1 function, leading to an increased activity of NF-κB that in turn increases the expression of the ubiquitin E3-ligase MuRF-1 (Rom and Reznick, 2016). Moreover, the overexpression of Atrolnc-1 in wild-type muscles causes increased MuRF-1 expression, which results in myofiber atrophy.

In the heart, cytoplasmic lncRNAs have also been proposed as regulators of the calcium reuptake occurring after cardiac muscle contraction. ZFAS1 lncRNA is markedly upregulated in both mouse and human cardiac tissues subjected to myocardial infarction (MI). While its knockdown restores heart contraction parameters to normal levels in a MI mouse model, its overexpression in wild-type mice induces a MI-like phenotype. Mechanistically, ZFAS1 directly binds the SERCA2a protein, which is responsible for calcium reuptake in the sarcoplasmic reticulum, and it is responsible for the downregulation of the protein levels, thus affecting the Ca^{2+} transient dynamics (Zhang et al., 2018c). More recently, the same group described a similar mechanism for DACH1 lncRNA. This RNA molecule is upregulated in failing hearts of mice and humans and directly interacts with SERCA2a; its overexpression is associated with an augmented ubiquitination of the protein, driving it to proteasome degradation (Cai B. et al., 2019).

As described for nuclear lncRNAs, also the dysregulation of cytoplasmic lncRNAs expression can result in cardiac hypertrophy. For instance, the murine CTBP1-AS2 lncRNA is selectively upregulated in hypertrophic hearts and in Ang-II treated neonatal rat ventricular myocytes. In the cytoplasm, CTBP1-AS2 interacts with the RNA-binding protein fused in sarcoma (FUS) forming a ribonucleoparticle that includes also TLR4 mRNA. Both CTBP1-AS2 and FUS participate in the anomalous stabilization of TLR4 mRNA, which encodes for a well-known driver of the inflammation process at the base of cardiac hypertrophy (Luo et al., 2019).

lncRNA-DERIVED MICROPEPTIDES

Recently, the functional role of lncRNAs has expanded due to their ability to generate bioactive micropeptides (**Table 3**), which have been identified mainly in muscle-related functions and cancer development (Ji et al., 2015). The identification of small ORFs that are translatable and encode short peptides remains a major challenge. Two complementary approaches are normally used to discover functional small ORFs: (i) the computational one, based on bioinformatic predictions and (ii) the experimental one, that uses transcriptional and translational data. Several computational tools have been developed to estimate the coding potential of novel identified transcripts. Among the most utilized CPC (Kong et al., 2007) and its updated version CPC2 (Kang et al., 2017), CPAT (Wang L. et al., 2013), COME (Hu et al., 2017), LncRNApred (Pian et al., 2016), PORTRAIT (Arrial et al., 2009), CONC, and others can be cited. The experimental approach is mainly based on the genome-wide translatome that has been studied by ribosome footprinting, a technique introduced by Ingolia in 2009 (Ingolia et al., 2009). The analyses of ribosome dynamics during translation allows the identification of actively translated ORFs (Bazzini et al., 2014) including the ones deriving from previously non-protein coding annotated transcripts. Recently, by applying this technique to human hearts, 169 lncRNAs and 40 circRNAs encoding for previously unknown microproteins have been identified and *in vivo* validated (van Heesch et al., 2019); among them, already known microproteins such as DWORF (Nelson et al., 2016) and SPAR (Matsumoto et al., 2017) were detected (see below). Interestingly, the identified microproteins were associated to cellular processes mainly related to mitochondria. The identification of micropeptides led to hypothesize a dual function for those lncRNAs that were previously characterized for their non-coding functions. In the van Heesch et al. paper, the authors identified 27 human and 5 murine small ORFs in lncRNAs whose non-coding function was already assessed. Among the 169 identified micropeptides deriving from lncRNAs, 16 were cardiac or skeletal muscle specific, suggesting that the list of lncRNA-derived micropeptides, important for muscle

physiology, will grow in the coming years. Among the ones that have been already characterized, myoregulin (MLN) was the first transmembrane microprotein identified; it takes origin from a previously annotated muscle-specific lncRNA (NR_038041). The small ORF, located in the third exon of the lncRNA, encodes for a 46-amino-acid long micropeptide. Olson's group showed that this single transmembrane alpha helix peptide interacts with the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and impedes Ca^{2+} uptake into the sarcoplasmic reticulum, thus influencing muscle relaxation. According to this observation, MLN showed a strong structural resemblance with the sarcolipin (SLN) and phospholamban (PLN) micropeptides, already known to regulate Ca^{2+} pump activity by directly interacting with SERCA. MLN is the most abundant micropeptide in adult skeletal muscles. For this reason, the authors suggested MLN as a dominant regulator of SERCA activity. Overexpression of MLN peptide caused a reduction in Ca^{2+} uptake while MLN-null mice significantly enhanced Ca^{2+} handling and improved exercise performance (Anderson et al., 2015). A couple of years later, Zhou's lab described a different nuclear non-coding function for the same transcript (NR_038041) that was named Linc-RAM (see above) (Yu et al., 2017). More recently, the same lab extended this work by showing that the fibroblast growth factor FGF2 mediates self-renewal and differentiation of satellite cells by inhibiting Linc-RAM expression in a MyoD-dependent manner (Zhao et al., 2018). Linc-RAM represents a clear case of a non-coding RNA with a well-characterized dual role: one in the nucleus, as non-coding transcript, and the other in the cytoplasm as protein. DWORF (Dwarf Open Reading Frame) is another example of microprotein transcribed from a previously annotated muscle-specific lncRNA. It is highly expressed in the heart and is the third smallest full-length protein annotated in the mouse genome (34 amino acids long). In their paper, Nelson et al. (2016) showed how this peptide, conserved in vertebrates, is able to increase in mouse SERCA pump activity by displacing the already cited inhibitory peptides phospholamban, sarcolipin, and MLN. Hearts from DWORF-overexpressing mice exhibited an increased activity of SERCA, indicating a higher affinity for Ca^{2+}, while slow skeletal muscles from DWORF-KO mice showed a reduced SERCA activity with a delayed Ca^{2+} clearance and muscle relaxation.

In mouse, 1500011K16Rik (LINC00116 in human) is a recently identified lncRNA enriched in heart and skeletal muscle tissues and highly coexpressed with mitochondrial genes. Two papers, published at the same time by different groups (Makarewich et al., 2018; Stein et al., 2018), demonstrated that this lncRNA contains, within its first exon, a nucleotide region that encodes for a predicted conserved single-pass transmembrane microprotein (56 amino acids long) named as mitoregulin (Mtln) because of its inner mitochondrial membranes' localization (Stein et al., 2018) or micropeptide regulator of β-oxidation (MOXI) because of its role in fatty acid β-oxidation (Makarewich et al., 2018). Stein et al. revealed its involvement in supporting mitochondrial high-molecular-weight supercomplexes assembly and/or stability. They demonstrated the high affinity between Mtln and cardiolipin, a phospholipid important for the maintenance of the integrity on the inner mitochondrial membrane. Makarewich et al. showed the ability of the MOXI micropeptide to interact with the mitochondrial trifunctional protein (MTP), an enzymatic complex that catalyzes the last three steps of long-chain fatty acids oxidation, and both groups suggested that Mtnl/MOXI-KO mice present an impaired fatty acid β-oxidation upon increased metabolic demand, probably due to inefficient mitochondrial complex (ri) organization after metabolic switches. Overexpression in HeLa cells and Mtln-KO mice observation allowed to unravel its ability to influence other mitochondrial processes such as membrane potential, Ca^{2+} retention capacity, and oxidative stress (Stein et al., 2018). Moreover, Makarewich et al. observed mitochondrial abnormalities in the KO mice. The role of this micropeptide in mitochondrial respiration was confirmed in a work that was under review when the previous two were published (Chugunova et al., 2019). In this paper, the authors demonstrated that the interaction of Mtln with NADH-dependent cytochrome b5 reductase stimulates the activity of mitochondrial respiratory chain complex I by providing a favorable lipid composition of the membrane. The role of Mtln in promoting myogenic differentiation, muscle growth, and regeneration was further studied by Lin et al., which refers to LINC00116 with the name of micropeptide in mitochondria (MPM, Lin et al., 2019).

The small regulatory polypeptide of amino acid response (SPAR) is another polypeptide translated from a lncRNA (LINC00961) that is conserved among species and highly expressed in heart, skeletal muscle, and lung (Matsumoto et al., 2017). SPAR is characterized by a conserved transmembrane domain that allows its localization at the membranes of late endosomes and lysosomes; in the lysosomal membrane, it interacts with two subunits of the v-ATPase proton pump complex to exert a negative regulation on the amino-acid-dependent mTORC1 activation. In the same paper, the authors demonstrated that loss of SPAR promotes post-injury skeletal-muscle regeneration by increasing mTORC1 activation.

It is noteworthy that all the null mice (Mtln-KO, MLN-KO, SPAR-KO, DWORF-KO) analyzed so far did not show any overt phenotype. The only exception is represented by the Myomixer micropeptide (84 amino acids long), which is generated by a previously annotated lncRNA (Gm7325); germline Myomixer null mice are characterized by perinatal lethality caused by the absence of multinucleated myofibers. This lncRNA has been identified in a loss-of-function screen aimed at the identification of essential genes involved in myoblasts fusion (Bi et al., 2017). Myomixer, also known as minion and myomerger, is essential for muscle formation during embryogenesis, satellite cells fusion, and muscle regeneration (Bi et al., 2017, 2018; Quinn et al., 2017; Zhang et al., 2017); it is embedded in the plasma membrane, and it was suggested to interact with the fusogenic protein Myomaker, thus promoting myoblasts fusion. A more recent paper, in contrast with the previous one, indicate that Myomaker does not need Myomixer to promote hemifusion and that the two proteins are involved in different steps of the fusion pathway (Leikina et al., 2018). Even if the mechanism of action remains to be elucidated, Myomixer role in promoting myoblasts fusion seems to be evolutionary conserved in vertebrates: the Zebrafish

TABLE 4 | Circular RNAs (circRNAs) in skeletal and cardiac myogenesis.

circRNA	Species	Function/mechanism	*In vivo* model phenotype	Expression	References
circLMO7	Bovine	ceRNA for miR-378a-3p to positively regulate myoblast proliferation	Not available	Highly expressed in muscle	Wei et al., 2017
circFUT10	Bovine	ceRNA for miR-133a to promote myoblast survival and differentiation by upregulating the serum response factor downstream targets	Not available	Highly expressed in muscle	Li et al., 2018a
circFGFR4	Bovine	ceRNA for miR-107 to promote myoblast differentiation by de-repressing Wnt3a	Not available	Highly expressed in muscle	Li et al., 2018b
circSNX29	Bovine	ceRNA for miR-744 to promote myoblast differentiation by derepressing Wnt5a/Ca^{2+} pathway	Not available	Highly expressed in muscle	Peng et al., 2019
circSVIL	Chicken	ceRNA for miR-203 to promotes the proliferation and differentiation of myoblasts	Not available	Highly expressed in leg muscle	Ouyang et al., 2018
circ-ZNF609	Mouse, human	Regulates myoblast proliferation and contains an open reading frame that can be translated; ceRNA for miR-194-5p to inhibit myogenic differentiation	Not available	Expressed in a wide range of tissues	Legnini et al., 2017; Rossi et al., 2019; Wang Y. et al., 2019
circMbl	*Drosophila*, human	Encodes for a protein to regulate the splicing of its own host gene	Motorial defects and peculiar wing position	Expressed in muscle and brain tissues	Pamudurti et al., 2017, 2018
circ-Ttc3	Mouse, rat	ceRNA for miR-15b-5p to increase the expression of Arl2	Deterioration of cardiac dysfunction after MI	Highly expressed in heart	Tan et al., 2017; Cai B. et al., 2019
circSlc8a1	Mouse, rat, human	ceRNA for miR-133a to regulate its targets	Attenuates cardiac hypertrophy from pressure overload	Highly expressed in heart	Werfel et al., 2016; Lim et al., 2019
circMFACR	Mouse	ceRNA for miR-652-3p to downregulate MTP18 at the translational level and favors mitochondrial fission and apoptosis	Not available	Highly expressed in heart	Wang et al., 2017
circNfix	Mouse, rat, human	Induces Ybx1 degradation by ubiquitination; ceRNA for miR-214 to promote Gsk3β expression and to repress β-catenin activity	Increases in cardiomyocyte proliferation	Highly expressed in cardiomyocytes	Huang et al., 2019

ortholog was able to induce heterologous cell fusion when overexpressed with the murine Myomaker (Bi et al., 2017).

CircRNAs IN SKELETAL AND CARDIAC MYOGENESIS

CircRNAs are originated from a non-canonical splicing reaction, called back splicing, in which a 3′ donor site is unconventionally fused to an upstream 5′ acceptor site. This mechanism generates highly stable and covalently closed molecules lacking a 5′ cap and a poly-A tail and thus inaccessible to the action of cellular exonucleases. Even though the first evidence of their existence goes back to the 1970s, circRNAs have been widely studied only after the advent of next generation sequencing technologies, being recognized as a common element of all metazoan transcriptomes (Wilusz, 2018). *De novo* discovery of circRNAs is based on the presence of exon-junction spanning reads in RNA-seq datasets; the effective existence of a newly identified circRNA then requires PCR validation with divergent primers and RNase R digestion assay to prove exonuclease resistance. This experimental pipeline has allowed the identification and annotation of hundreds of circRNAs, which are expressed and modulated during cultured myoblasts differentiation (Chen et al., 2018) or in developing and aging skeletal muscle of various mammalian species (Li et al., 2017; Wei et al., 2017) (**Table 4**). The high-throughput discovery of this large amount of circular RNAs has boosted the characterization of their biological significance and the molecular mechanisms of post-transcriptional regulation in which they are involved. A large part of circRNAs show an enrichment for miRNA binding sites in their nucleotide sequence, suggesting a role in competing endogenous RNA networks, which is also corroborated by their high stability. The research of differentially expressed circRNAs between C_2C_{12} myoblasts and myotubes has identified 581 candidates; according to *in silico* prediction, the top 30 upregulated circRNAs could be part of ceRNA networks involving 91 miRNAs and core myogenic factors like myogenin, Myocyte enhancer factor 2a, myosin heavy chain (Myh)-1, Myh7, and Myh7b (Chen et al., 2018). Similarly, many differentially modulated circRNAs have been identified during several developmental stages of the bovine *longissimus dorsi* skeletal muscle (Wei et al., 2017). Among them, circLMO7, the most downregulated circRNA between adult and embryonic muscles, was shown to positively regulate myoblast proliferation while reducing myoblast apoptosis and differentiation. The biological function of circLMO7 was correlated with its ability to act as a competing endogenous RNA for miR-378a-3p. Starting from the same dataset, the authors demonstrated that other two candidates impact the development of bovine skeletal muscle through a miRNA-binding activity. Specifically, CircFUT10 was observed to promote myoblasts survival and differentiation and to reduce cell proliferation rate by directly binding to and inhibiting miR-133a; in this way, the circRNA

upregulates the serum response factor downstream targets, resulting in a positive impact on myogenesis (Li et al., 2018a). On the other side, CircFGFR4 promotes bovine primary myoblasts differentiation by sequestering miR-107 and thus derepressing its known target Wnt3a, a key upstream factor of the Wnt/β-catenin signaling pathway (Li et al., 2018b). Recently, circSNX29 was identified as another player in the regulation of bovine skeletal muscle development (Peng et al., 2019). This molecule facilitates myoblast differentiation by sponging miR-744 to attenuate its inhibitory effect on the Wnt5a/Ca^{2+} signaling pathway.

Moreover, the analysis of circRNA expression during chicken skeletal muscle development identified circSVIL. circSVIL promotes the proliferation and differentiation of myoblasts by binding and antagonizing the function of miR-203, a post-transcriptional repressor of c-JUN and MEF2C (Ouyang et al., 2018). Furthermore, a massive high-throughput screening of both proliferating and differentiated human primary myoblasts and mouse C_2C_{12} cells revealed an abundant population of cytoplasmic circRNAs, which showed a global accumulation during differentiation (Legnini et al., 2017). Interestingly, a large part of them was found deregulated in human myoblasts derived from Duchenne muscular dystrophy patients. A subset of highly conserved, strongly modulated circRNAs was selected for a wide phenotypic screening, from which circ-ZNF609 emerged as a regulator of myoblast proliferation, while Circ-QKI and circ-BNC2 showed a positive and negative effect on myogenesis, respectively. Of them, Circ-ZNF609 contains an ORF that can be translated in a cap-independent manner due to the IRES activity of the untranslated region (Legnini et al., 2017). More recently, it has been shown that CircZNF609 is upregulated in rhabdomyosarcoma biopsies and that its knockdown induces a specific arrest of G1/S transition rhabdomyosarcoma-derived cells. The cell cycle block is the result of a strong decrease in phosphorylated Akt protein level and an alteration of the p-Rb/Rb ratio (Rossi et al., 2019). The role of this molecule in tumor progression has been validated also in breast cancer, where high levels of Circ-ZNF609 are associated with poor prognosis (Wang S. et al., 2018). Moreover, the mouse homolog of CircZNF609, named circZfp609, has been shown to inhibit myogenic differentiation by sponging miR-194-5p (Wang Y. et al., 2019).

Circ-ZNF609 is only one of the examples of a protein-coding circRNA in eukaryotes (Chekulaeva and Rajewsky, 2019). The Muscleblind locus encodes for a splicing factor with a critical function in muscle development; its sequestration from aberrant CUG repeats is causative of myotonic dystrophy (Miller et al., 2000). In *Drosophila*, this locus produces also circMbl, an abundant protein-coding circRNA that regulates splicing of its own host gene (Pamudurti et al., 2017). Interestingly, circMbl knockdown in the whole organism has a specific effect on muscle development and function, as confirmed from gene expression analysis, motorial defects, and a peculiar wing position (Pamudurti et al., 2018). In their latest work, Chen et al. identified a subset of 224 circRNAs modulated during C_2C_{12} differentiation with *bona fide* coding potential according to the number of open reading frames and N6-methyladenosine motifs, which are known to work as promoting factors for cap-independent translation (Chen et al., 2018). It was recently suggested that circular RNAs expression can be affected by splicing alterations related to myotonic dystrophy type 1 (DM1), a multisystemic disorder in which expanded CTG repeats in the DMPK gene leads to splicing abnormalities. CircRNAs expressed in DM1 skeletal muscles biopsies were identified by analyzing RNA-sequencing datasets, demonstrating the upregulation of CDYL, HIPK3, RTN4_03, and ZNF609 compared to healthy controls (Voellenkle et al., 2019). The circular fraction values were positively correlated with splicing biomarkers of disease severity, reaching higher values in more severely affected patients. Measurement of circular-to-linear ratios for these candidates resulted to be a good prediction method to discriminate DM1 patients from controls, suggesting a possible future use as biomarkers.

The miRNA-binding paradigmatic mechanism has been validated also in cardiac muscle. In a recent study, Tan et al. performed a deep RNA-sequencing from human and mouse hearts and across a differentiation time-course of human embryonic stem-cell-derived cardiomyocytes. They identified a total of 15,318 and 3,017 cardiac circRNAs within human and mouse, respectively. Among them, circ-Ttc3 resulted in one of the top highest expressed circRNAs in mouse heart (Tan et al., 2017). Cai L. et al. (2019) found that circTtc3 was markedly upregulated in the ischemic myocardium, whereas there was no significant change in linear Ttc3 RNA. Overexpression of circ-Ttc3 in cardiomyocytes counteracted hypoxia-induced ATP depletion and apoptotic death. Mechanistically, this circRNA sponges and inhibits miR-15b-5p leading to the increased expression of its target Arl2. Consistently, Arl2 knockdown partially abolished the beneficial effects of circ-Ttc3 overexpression on ATP production and apoptosis of cardiomyocytes. Thus, the Circ-Ttc3-miR-15b-Arl2 regulatory cascade may have a cardioprotective role in myocardial infarction (Cai L. et al., 2019). The same screening allowed the identification of circSlc8a1, which is the most abundant single-exon cardiac expressed. A new study demonstrated that circSlc8a1 inhibition attenuated cardiac hypertrophy, while its forced overexpression resulted in heart failure. As reported for the majority of the circRNAs identified so far, also this circular molecule act in the cytoplasm by sponging a miRNA, in particular the cardiac-enriched mir-133a. Considering that the overexpression of miR-133a attenuates cardiac hypertrophy both *in vitro* and *in vivo*, inhibiting circSlc8a1 may result as a novel therapeutic target for cardiac hypertrophy (Lim et al., 2019). An increasing number of studies have indicated that mitochondrial fission dysfunction occurs in many cardiac diseases, such as MI and heart failure. Recently, it has been suggested that circRNAs could play a role also in this context. In this regard, CircMFACR favors mitochondrial fission and apoptosis in the heart by sequestering miR-652-3p, which in turn downregulates MTP18 expression at the translational level. MTP18 is a nuclear-encoded mitochondrial membrane protein whose deficiency reduces mitochondrial fission and suppresses cardiomyocyte apoptosis and MI. Thus, the silencing of MFACR expression leads to a reduction in mitochondrial fragmentation and cell apoptosis. This study revealed that circRNAs can play

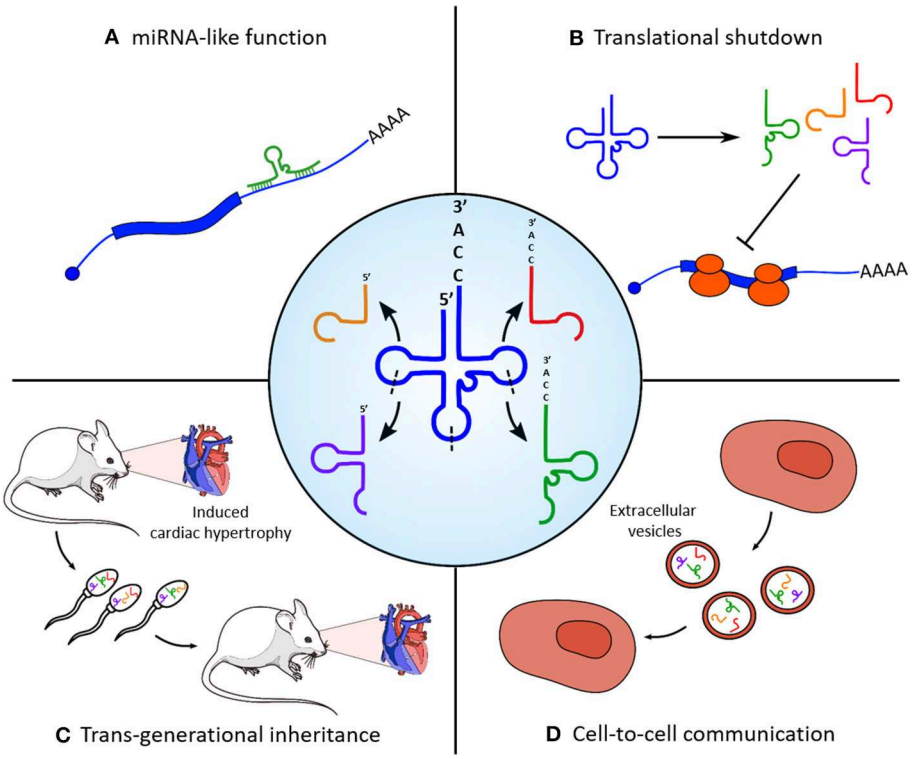

FIGURE 1 | Functional roles of tRNA-derived fragments (tRFs) in skeletal and cardiac muscle homeostasis. Central panel: tRNAs are processed into different categories of tRFs by enzymatic cleavage on specific sites (indicated with dashed lines). **(A)** tRNA fragments can target the 3′ untranslated region (UTR) of protein-coding transcripts (e.g., Kassiri et al., 2009) and regulate their stability and translation efficiency with a microRNA (miRNA)-like mechanism. **(B)** Massive cleavage of specific tRNA species in stress conditions induces a rapid translational blockade. **(C)** Accumulation of tRFs in the sperm is responsible for the non-genetic transmission of myocardial hypertrophy phenotypic traits in the offspring. **(D)** tRFs produced from myoblasts are selectively loaded in extracellular vesicles and could act as mediators of cell-to-cell communication.

an active role in the regulation of mitochondrial dynamics, cardiomyocyte apoptosis, and myocardial infarction (Wang et al., 2017). CircRNAs were found to regulate cardiac regeneration. One of the most recent example is circNfix, a superenhancer-regulated circular RNA whose depletion produces cardiomyocyte proliferation and angiogenesis. RNA pulldown and luciferase reporter assays revealed that circNfix acts both as a miR-214 sponge and as a destabilizing partner of Ybx1 *via* ubiquitin-dependent degradation (Huang et al., 2019).

piRNAs AND tRFs IN SKELETAL AND CARDIAC MYOGENESIS

The regulatory potential of the non-protein-coding transcriptome is not limited to the better characterized lncRNAs but includes also less known and more recently discovered RNA species. The piRNAs are a class of small RNAs widely conserved in all the metazoans. Following transcription and processing, the mature piRNA form associates with different members of the PIWI proteins clade, thus creating silencing molecular complexes that defend the germline genome from transposon expression by acting at both transcriptional and post-transcriptional level (Ozata et al., 2019). The common perception of the PIWI–piRNA pathway as a germline-specific feature is nowadays questioned by the evidence of expression of this molecular machinery in a variety of somatic tissues (Lee et al., 2011; Perera et al., 2019). While the functional role of somatic piRNAs is still debated in other tissues, recent works suggest that these small RNAs could regulate heart regeneration due to their effect on the Akt pathway, a fundamental network in heart physiopathology. Microarray profiling of different populations of cardiac progenitors identified a consistent set of piRNAs, which are differentially modulated between cardiospheres, cardiosphere-derived cells, and cardiac fibroblasts, thus creating a specific molecular signature of these cell populations (Vella et al., 2016). In particular, piRNAs which are specifically upregulated in cardiosphere-derived cells act as negative regulators of L1 retrotransposon transcripts, whose inhibition has been shown to decrease upon post-ischemic myocardial damage by the activation of the Akt signaling (Lucchinetti et al., 2006).

tRNAs represent another class of small RNAs which are emerging as regulators of heart and skeletal muscle physiology (**Figure 1**). They are well-known for their canonical function as adaptor molecules during protein synthesis; however, it has

been recently discovered that their controlled endonucleolitic cleavage could represent a source of small regulatory non-coding RNAs. These small molecules were first identified during miRNA library cloning experiments (Fu et al., 2005) and initially classified as random tRNA degradation misproducts; however, the conserved length and the recurrent position of cleavage sites suggested the existence of a dedicated enzymatic activity responsible for their biogenesis. tRNA fragments can be grouped in three categories (Liapi et al., 2019), based on their derivation from the 5′ or 3′ end of parental tRNA (5′/3′ tRFs), from the 3′ portion of the immature tRNA precursor (tRF1s), or from a cleavage event within the anticodon loop which splits the parental molecules in two 30–35 nucleotide long (5′ and 3′) halves. tRNA halves biogenesis is an evolutionary widespread molecular event of particular interest, since they tend to be produced as a response to several cellular stresses by the activity of the angiogenin (ANG) endonuclease. The first report of such mechanism in mammals has been made in tissues of various origin, including *ex vivo* starved mouse hearts, which showed a specific accumulation of the 5′ portion of Val-AAC tRNA (Fu et al., 2009). The accumulation of a small subset of tRNA halves compared to the complexity of parental tRNA pools corroborates the hypothesis of a controlled processing machinery and of a functional role for tRNA fragments, other than a simple mechanism of stress-induced translational shutdown. Moreover, the expression of specific tRNA fragments was found to be strongly upregulated in a rat model of induced myocardial hypertrophy (Shen et al., 2018); interestingly, transfection of a synthetic version of the two most upregulated tRFs in cultured cardiomyocites increases both hypertrophy marker expression and cell surface area. In particular, Kassiri et al. (2009) show that tRFs1, which derives from tRNA-Gly-GCC cleavage, targets the 3′ untranslated region of Timp3, a well-known cardiac hypertrophy regulatory factor in a miRNA-like manner. Remarkably, mice with induced myocardial hypertrophy had high levels of the identified tRFs also in sperm; a similar pattern of expression of these fragments was found in offspring hearts, which showed an intermediate phenotype with extended heart muscle fiber breakage and fibrosis. Taken together, these findings not only indicate a role for tRFs in the physiopathology of heart but also their involvement on the transgenerational inheritance of a non-genetic stress condition, as already reported for sperm-contained miRNAs (Rodgers et al., 2015).

Recent works have shown that small RNAs are actively loaded into extracellular vesicles (EVs) and released in almost all kind of biofluids to act as mediators of cell-to-cell communication (Tkach and Théry, 2016). Even if this phenomenon has been widely studied for miRNAs, there are also evidences of exosome-mediated secretion of other small RNA species, such as piRNAs and tRNA fragments, from different tissue sources (Chiou et al., 2018; Tosar et al., 2018). Of note, the small RNA transcriptome from both human rhabdomyosarcoma RD4 and murine C_2C_{12} myoblasts and of EVs produced by these cells has been recently analyzed (Sork et al., 2018). It emerged that small RNAs are underrepresented in EVs compared to their parental cells, suggesting a loading mechanism that is not explicable with simple diffusion. Moreover, C_2C_{12}-produced EVs present a strong enrichment of piRNAs and tRNA fragments compared to intracellular content, including the tRNA-Gly-GCC fragment cited above. Taken together, these evidence suggest that these small RNAs can be selectively included in secretory vesicles, with a potential role in long distance cell-to-cell communication events.

High-throughput small RNA sequencing from four types of wild-type and dystrophic (*mdx*) muscles and from sera allowed to discover that many piRNAs and tRFs are expressed in skeletal muscle tissue and differentially released in the circulation in dystrophic conditions. Furthermore, the high level of piR_000620 and piR_000935 detected in *mdx* serum was restored to nearly wild-type levels in response to DMD exon skipping with injected modified oligonucleotides (Coenen-Stass et al., 2018). The differential release of piRNAs has been also observed in serum vesicles isolated from heart failure patients compared to healthy donors, suggesting a possible use of these small RNAs as clinically relevant biomarkers (Yang et al., 2018).

AUTHOR CONTRIBUTIONS

JM wrote the manuscript and selected the literature. DM and FD reviewed the manuscript and prepared tables and figures. MB proposed the topic, wrote the manuscript, and reviewed the text.

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miRNAs in NK Cell-Based Immune Responses and Cancer Immunotherapy

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The incidence of certain forms of tumors has increased progressively in recent years and is expected to continue growing as life expectancy continues to increase. Tumor-infiltrating NK cells may contribute to develop an anti-tumor response. Optimized combinations of different cancer therapies, including NK cell-based approaches for targeting tumor cells, have the potential to open new avenues in cancer immunotherapy. Functional inhibitory receptors on NK cells are needed to prevent their attack on healthy cells. Nevertheless, disruption of inhibitory receptors function on NK cells increases the cytotoxic capacity of NK cells against cancer cells. MicroRNAs (miRNAs) are small non-coding RNA molecules that target mRNA and thus regulate the expression of genes involved in the development, maturation, and effector functions of NK cells. Therapeutic strategies that target the regulatory effects of miRNAs have the potential to improve the efficiency of cancer immunotherapy. Interestingly, emerging evidence points out that some miRNAs can, directly and indirectly, control the surface expression of immune checkpoints on NK cells or that of their ligands on tumor cells. This suggests a possible use of miRNAs in the context of anti-tumor therapy. This review provides the current overview of the connections between miRNAs and regulation of NK cell functions and discusses the potential of these miRNAs as innovative biomarkers/targets for cancer immunotherapy.

Keywords: human NK cells, NK cell receptors, microRNA, immune checkpoint, immunotherapy, gene expression

miRNAs AS KEY REGULATORS OF GENE EXPRESSION

About 2,000 human miRNAs are currently recognized. These are small RNAs which originate from longer precursors (Pri-miRNAs) mainly transcribed by the RNA polymerase II (Macfarlane and Murphy, 2010). These Pri-miRNAs undergo a precisely coordinated maturation process involving several steps. In the nucleus, the RNAse III Drosha, supported by the DiGeorge critical region 8 (DGCR8), converts them in short hairpin intermediates of 70–120 nucleotides-long

(Pre-miRNAs) (Gregory et al., 2004). After transport to the cytoplasm by exportin 5 (Yi et al., 2003), Pre-miRNAs are then processed by the RNAse III Dycer into mature miRNAs which are duplexes of approximately 22 nucleotides. One strand of the duplex is incorporated along with the argonaute protein in the miRNA-induced silencing complex RISC (Diederichs and Haber, 2007). These complexes promote the pairing of miRNA nucleotide sequences to their target sequences on 3′ UTR sequences of mRNAs and RISC cofactors mediate site-specific cleavage, degradation of the target mRNA, or inhibition of its translation in protein (Gu and Kay, 2010). It is well-known that miRNA can repress the expression of hundreds of different mRNAs. Furthermore, as several different miRNA target sequences may be present on the 3′ UTR of a single mRNA, complex networks of cooperative regulation by several miRNAs may affect the stability or the translation of a multitude of mRNAs (Filipowicz et al., 2008; Liu et al., 2009, 2014). As miRNA recognition sequences appear to be present on most of the protein-coding human mRNAs, the role of miRNAs as regulators of gene expression is quite relevant in mammalian development physiology and pathology (Dallaire and Simard, 2016; Peng and Croce, 2016; Ivanova et al., 2018; Johnson, 2019; Horsburgh et al., 2017). Therefore, miRNAs and in particular those present in body fluids and blood, either as free molecules or included in extracellular vesicles, are receiving increasing attention as possible disease biomarkers (Mori et al., 2019).

NK CELLS AS INNATE IMMUNE CELLS WITH A KEY ROLE IN FIGHTING VIRAL INFECTIONS AND IN THE SURVEILLANCE AGAINST MALIGNANT TRANSFORMATION

Natural Killer (NK) cells represent cytotoxic, innate lymphoid cells (ILCs) (Cortez and Colonna, 2016), and their main function is to provide the organism with a rapid immune response against infections, autologous transformed cells, and allogeneic cells (Vivier et al., 2011; Del Zotto et al., 2017; Freud et al., 2017). In fact, NK cells do not need to be primed with antigens to become fully functional and the mechanisms of non-self recognition do not rely on genomic recombination and subsequent cell clone expansion events.

Nowadays it is recognized that these cells mediate immune-surveillance also via regulatory functions by secreting cytokines, primarily interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and by interacting with other immune or adaptive immune cells (Marcenaro et al., 2006; Vivier et al., 2011; Riise et al., 2015; Pesce et al., 2017b; Bernson et al., 2019). In turn, NK cells can respond to different types of chemokines and cytokines produced by other immune cells (Marcenaro et al., 2005a,b, 2006; Moretta et al., 2006; Parodi et al., 2015; Pesce et al., 2016).

NK cells are not a homogeneous population, but there are different NK subsets that differ in phenotype, maturational step, and functions. Among circulating mature NK cells, two main subsets can be identified: regulatory NKs ($CD56^{bright}/CD16^{-}$), which are the most abundant in secondary lymphoid organs (SLO) and display the ability to secrete high amounts of pro-inflammatory cytokines, and cytotoxic NKs ($CD56^{dim}/CD16^{+}$), which represent about 90% of circulating NK cells (Farag and Caligiuri, 2006; Carrega and Ferlazzo, 2012; Del Zotto et al., 2017).

SLO have been suggested being the anatomical sites where NK cells complete their maturation process that is associated with the transition from a $CD56^{bright}$ to a $CD56^{dim}$ phenotype, acquisition of self-tolerance and lytic activity (Romagnani et al., 2007; Yu et al., 2010).

Both the killing and immune-regulative functions of NK cells depend on a balance of activating or inhibiting signals that originate from NK receptors (NKRs) (activating NKR-aNKR and inhibitory NKR -iNKR-, respectively). The iNKRs include the human leucocyte antigens (HLA) class I-specific Killer Ig-like receptors (KIRs) recognizing allotypic determinants shared by groups of classical HLA-ABC alleles (Moretta et al., 1996), the leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) that is specific for different HLA-class I molecules (Cosman et al., 1997), and the CD94/NKG2A heterodimer specific for HLA-E (Braud et al., 1998) ad well as additional non-HLA-I specific inhibitory receptors, including programmed cell death protein 1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T-cell immunoglobulin domain and mucin domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), and CD96 (Di Vito et al., 2019). The activating NKRs (aKIRs) include non-HLA-specific receptors such as NCRs (NKp30, NKp44 and NKp46), NKG2D, DNAM-1, NKp80, CD59, NTB-A, and 2B4 (Moretta et al., 2001) as well as the activating HLA class I-specific Killer Ig-like receptors and the HLA-E specific CD94/NKG2C heterodimer. NK cells can also express different Toll-like receptors (TLR), including TLR2, TLR3, TLR5, TLR7, TLR8, and TLR9 (Sivori et al., 2004; Hart et al., 2005; Tsujimoto et al., 2005; Marcenaro et al., 2008; Voo et al., 2014). These receptors, by recognizing conserved pathogen structures, induce NK cell activation (Della Chiesa et al., 2014).

During NK cell development/differentiation, CD94/NKG2A is the first HLA-I specific receptor to be expressed. It appears on the most immature $CD56^{bright}$ $CD16^{neg/dim}$ NK cell subset. After several maturation steps, $CD56^{bright}$ cells become $CD56^{dim}$ $CD16^{+}$, lose NKG2A and acquire KIR and LILRB1 receptors (Di Santo, 2006; Freud and Caligiuri, 2006; Romagnani et al., 2007). The most mature NK cells are KIR^{+} (and/or $LILRB1^{+}$), $NKG2A^{-}$ $CD16^{bright}$ and express the marker of terminal differentiation, CD57 (Moretta et al., 2004; Bjorkstrom et al., 2010; Marcenaro et al., 2017).

Under homeostatic conditions, NK cells continuously receive inhibitory signals mainly originating from the interaction between iNKRs and a large spectrum of classical and non-classical HLA-I molecules expressed on the surface of autologous cells (self-cells). Allogeneic or viral-infected or tumor cells often downregulate or lack altogether the expression of these antigens and therefore fail to be recognized as self-cells by the iNKRs. Under these conditions, the signaling from the aNKRs, engaged with ligands displayed on target

cells, prevails, and the NK-mediated killing of these non-self cells is unleashed. Notably, in tumors that maintain the expression of HLA-I molecules, the iNKRs function as immune checkpoints and block the cytotoxic activity of NK cells (Romagne et al., 2009; Vey et al., 2012; Kohrt et al., 2014).

Several strategies have been forwarded to strenghten NK cell activity against HLA-I-expressing cancer cells. For example, IL-2-based immunotherapy allows NK cells to override inhibitory signals from acute myeloid leukemia (AML) blasts (Hallner et al., 2019), and recently, immunotherapies based on the use of therapeutic monoclonal antibodies specific for iNKRs, in particular anti-pan-KIR2D (lirilumab) (Romagne et al., 2009; Kohrt et al., 2014; Vey et al., 2018) and anti-NKG2A (monalizumab), have been developed (André et al., 2018; Tinker et al., 2019; Zaghi et al., 2019). These agents efficiently disrupt the interaction between these NK cell immune checkpoints and their ligands, and will in this way enable NK cells to efficiently kill also HLA-I^{+} tumor cells (Chiossone et al., 2017; Di Vito et al., 2019).

In addition, the microenvironment of chronic infections and tumors may lead to NK cell phenotypic changes and impairment of NK cell functions (Bi and Tian, 2017). The most frequently NK cell phenotypic changes are represented by downregulation of the aNKRs expression (Costello et al., 2002; Romero et al., 2006; Carlsten et al., 2009; Pesce et al., 2015; Han et al., 2018; Poznanski and Ashkar, 2019) and/or upregulation/*de novo* expression of iNKRs (Carlsten et al., 2009; Di Vito et al., 2019; Sanchez-Correa et al., 2019). In fact, it has been unveiled that besides T lymphocytes also NK cells can express PD-1, an immune checkpoint specific for the PD-L1/2 molecules often displayed on the surface of tumor cells (Pesce et al., 2019b).

PD-1 is expressed on a subset of fully mature (KIR^{+}CD57^{+}NKG2A^{-}) NK cells from one-fourth of human cytomegalovirus (HCMV) seropositive individuals (Della Chiesa et al., 2016; Pesce et al., 2017a; Mariotti et al., 2019). Increased proportions of PD-1^{+} NK cells can be observed in patients affected by different types of tumors (Beldi-Ferchiou et al., 2016; Pesce et al., 2017a, 2019a,b; André et al., 2018). Accordingly, studies suggest a role for NK cells in immunotherapy targeting the PD-1/PD-L1 axis (Hsu et al., 2018) and this is clinically relevant for patients with tumors characterized by a T cell resistant (HLA-I^{neg}) phenotype.

Apart from the wide-spread use of checkpoint inhibitors in melanoma, lung cancer etc., agents blocking the PD-1/PD-L1 axis are currently being evaluated in clinical trials on both hematologic and solid tumors as monotherapy or in combination with other agents, including other forms of immune checkpoint blockade, such as anti-panKIR2D and anti-NKG2A antibodies in the case of HLA-I^{+} tumor cells (Moretta et al., 1996, 2001; Cosman et al., 1997; Braud et al., 1998; Sivori et al., 2004; Marcenaro et al., 2008; Di Vito et al., 2019).

In summary, NK cell activation depends on the nature of interactions between inhibitory/activating receptors on their surface and the relative ligands on target cells, and thus receptor/ligand pairs could represent key checkpoints in the regulation of anti-tumor NK cell activity and in the planning of innovative NK cell-based immunotherapy.

miRNAs AS REGULATORS OF NK CELLS SURVIVAL, DEVELOPMENT/MATURATION, AND FUNCTIONS

Numerous studies showed that miRNAs play a relevant role in the regulation of NK cell survival, development/maturation, activation, proliferation, cytotoxicity, and cytokine production both in healthy and pathological conditions (i.e., tumors/viral infections) by targeting receptors or factors involved in transcriptional expression (**Table 1**).

miRNAs Involved in NK Cell Differentiation/Development

The first evidence of the important role played by miRNAs within the immune system was provided by genetic studies showing a critical requirement for Dicer *in vivo*. Conditional deletion of Dicer in various hematopoietic lineages in mice produced defects, such as impaired cell differentiation, proliferation, and survival (Muljo et al., 2005; Cobb et al., 2006; Koralov et al., 2008; Liston et al., 2008; Fedeli et al., 2009).

Bezman et al. (2010) investigated the role of miRNAs by ablation of the miRNA biogenesis pathway, through deletion of Dicer or Dgcr8 in the mature murine peripheral NK cells. Dicer- and Dgcr8- deficient NK cells showed an increased cell death supporting the important role of miRNAs in controlling cell survival. Moreover, Dicer- and Dgcr8-deficient NK cells are able to respond efficiently through their cytokine receptors; however, function of their immunoreceptor tyrosine-based activation motif (ITAM)-containing aKIRs is impaired.

By using another molecular approach, Sullivan et al. (2012) eliminated Dicer during the earliest stages of murine NK cell development in the bone marrow to better characterize the phenotypic features derived from global loss of mature miRNA expression. These studies confirmed that the absence of miRNAs led to reduced numbers and percentages of NK cells, and a decreased *in vitro* survival/proliferation.

Studies utilizing next-generation sequencing in mouse and human reported some information about the miRNA repertoire in resting CD56^{+} CD3^{-} human or NK1.1^{+}CD3^{-} murine NK cells and upon cytokine activation (Fehniger et al., 2010; Liu et al., 2012; Wang et al., 2012). Furthermore, Ni et al. (2015) identified the miRNA profiles of human NK cells from different compartments (peripheral blood, cord blood, and uterine decidua). Very recently, our group, by analyzing peripheral blood NK cells from 10 different human healthy donors, identified a 108 miRNA signature able to discriminate CD56^{bright} from CD56^{dim} NK cell subsets independently from their surface phenotype (Pesce et al., 2018). Interestingly, we found some miRNAs (miR-146a-5p, miR-92a-3p, miR-223-3p, miR-873-5p, miR-31a-5p, hsa-miR-130a-5p, miR-181a-2-3p) with consistent differential expression in the two NK cell subsets, and with an intermediate expression in the CD56^{bright}/CD16^{dim} NK cell subset, which represents a transition phase in the NK cell maturation process of NK cells.

A key miRNA for NK cell development is miR-150. A gain of function miR-150 transgene in mouse was demonstrated to

TABLE 1 | Examples of miRNAs expressed in NK cells and involved in the modulation of several aspects of NK cell development and functions.

	miRNAs	Induced by	Inhibited by	Targets	Effects	References
miRNAs involved in NK cell differentiation/ development	miR-150¶			Myb	Promotes the development of NK cells	Bezman et al., 2011
	miR-181a/b			NLK	Promotes the development of NK cells ↑ INF-γ production	Cichocki et al., 2011
	miR-583			IL2Rγ	↓ NK cell differentiation	Yun et al., 2014
miRNAs involved in the regulation of NK cell functions	miR-27a-5p		IL-15	GzmB Prf1	↓ NK killing activity	Kim et al., 2011
	miR-30e		IFN-α	Prf1	↓ NK killing activity	Wang et al., 2012
	miR-378		IFN-α	GzmB	↓ NK killing activity	Wang et al., 2012
	miR-150		IL-15	Prf1	↓ Prf1 ↓ NK killing activity	Kim et al., 2014
	miR-362-5p¬			CYLD (neg. reg. of NF-kb)	↑ Expression of: IFN-gamma, perforin, granzyme-B, and CD107a	Ni et al., 2015
	miR-155‡	IL-2, IL15 or IL-21			↑ NK killing activity	Liu et al., 2012
	miR-155	IL-12, IL-15, IL-18		SHIP-1	↑ NK killing activity ↑ INF-γ production	Sullivan et al., 2013
	miR-99b miR-330-3p$				NK cell activation but diminished cytotoxicity	Petty et al., 2016
	miR-1245	TGFß		NKG2D	↓ NK killing activity	Espinoza et al., 2012
	miR-183	TGFß		DAP12	Destabilization of 2DS4 and NKp44 ↓ NK killing activity	Donatelli et al., 2014
	miR-218-5p		IL-2	SHMT1	↓ IFN-γ and TNF-α production ↓ Cytotoxicity	Yang et al., 2019
Pathogens-modulated miRNAs in NK cells	miR-15a†		EBV-encoded latent membrane protein (LMP1)	Myb Cyclin D1	Growth arrest	Komabayashi et al., 2014
	miR-155	IL-12 and IL-18 via STAT4		Noxa (early post MCMV); SOCS1 (late post MCMV)	↑ Antiviral immunity	Zawislak et al., 2013
	miR-29a-5p	HCV		PU.1 Prf1	↑ miR-155 ↓ Prf1 ↓ NK killing activity	Elemam et al., 2015
miRNAs in tumor-associated NK cells	miR-183^	TGFß		DAP12	Destabilization of 2DS4 and NKp44 ↓ NK killing activity	Donatelli et al., 2014
	miR-1245	TGFß		NKG2D	↓ NK killing activity	Espinoza et al., 2012
	miR-218-5p		IL-2	SHMT1	↓ IFN-γ and TNF-α production ↓ Cytotoxicity	Yang et al., 2019
	miR-150			DKC1 AKT2	↑ Apoptosis in tumor cells ↑ Tumor suppression	Watanabe et al., 2011
	miR-203		Promoter methylation in lymphoma		Tumor suppression	Chim et al., 2011
	miR-494-3p			PTEN	AKT activation	(Chen et al., 2015)
	miR-142-3p§			RICTOR	Suppression of AKT	(Chen et al., 2015)
	miR-155			SHIP1	↓ Cell survival and Cell-cycle progression	Yamanaka et al., 2009
	miR-21			PTEN; PDCD4	↓ Cell survival (anti-apoptotic)	Yamanaka et al., 2009

(Continued)

TABLE 1 | Continued

	miRNAs	Induced by	Inhibited by	Targets	Effects	References
	miR-26a/b miR-28-5 miR-30b miR-101 miR-363		c-Myc	MUM1, BLIMP1, and STMN1 in NKTL	↓ Cell growth (NK/T-cell Lymphoma)	Ng et al., 2011
	miR26a/b			BCL2	↓ Cell growth	Ng et al., 2011
	miR-363 miR-28-5				↓ Cell growth	Ng et al., 2011
	miR-101			STMN1IGF1 BCL2	↓ Cell growth	Ng et al., 2011
	miRNA-10a miRNA-342-3p			TIAM1	Low miRNA expression correlated with development of Extranodal NK/T-cell lymphoma	Huang et al., 2016
	miR-221				Poor Survival in Plasma NK/T-cell Lymphoma	Guo et al., 2010
	miR-155			BRG1	Activation of STAT3/VEGFC signaling and promotion of NKTCL viability and lymphangiogenesis	Chang et al., 2019
miRNAs involved in the regulation of NK cell immune checkpoints	miR-182[#]			NKG2D? NKG2A?	↑ Cytotoxicity via Prf1 counter intuitive effects on NKG2D and NKG2A	Abdelrahman et al., 2016; El Sobky et al., 2016
	miR-146a-5p[°]			KIR2DL1 KIR2DL2	↑ NK killing activity	Pesce et al., 2018
	miR-26b-5p miR-26a-5p miR-185-5p			KIR3DL3	NK cell activation?	Nutalai et al., 2019

[¶]*Controls iNKT cells development and apoptosis (Bezman et al., 2011; Winter and Krueger, 2019) and has negative effects on acute T-cell lymphoblastic leukemia (T-ALL) survival (Saki et al., 2015) whereas it has a protective effect on CD4$^+$ and CD8$^+$ T cells by controlling the expression of pro-apoptotic genes (Cron et al., 2019).*
[¬]*Promotes malignancy of chronic lymphocytic leukemia (CLL) (Yang et al., 2015).*
[‡]*Reported to be also involved in CD8+ T cell activation (Gracias et al., 2013) and T cell development.*
[$]*Also involved in the inhibition of TGF-β expression in CD8+ Treg cells (Rouas et al., 2019).*
[†] *Also involved in the control of chronic lymphocytic leukemia clonal expansion (Cutrona et al., 2017).*
[^]*See also involvement of miR-183C (Ichiyama et al., 2016) and miR-183-5p in Th17 cytokine production and Th17/Treg imbalance in thrombocytopenia (Hua et al., 2019), respectively.*
[§]*Also involved in CD25+ CD4 T cell proliferation by targeting the expression of GARP (Zhou et al., 2013).*
[#]*Also Promotes clonal expansion of activated T helper lymphocytes (Stittrich et al., 2010).*
[°]*Promotes growth of acute leukemia cells (Wang L. et al., 2019).*

drive the development and maturation of NK cells. In line with this, mice with a targeted deletion of miR-150 instead display cell lineage–intrinsic defect in their ability to generate mature NK cells (Bezman et al., 2011).

Additional miRNAs relevant for NK cell development and maturation are miR-181 and miR-583. Cichocki and coworkers found that miR-181 promotes NK cell development via inhibition of the Nemo like kinase (NLK) (Cichocki et al., 2011) while Yun and collaborators showed that the miR-583 targets and downregulates IL2Rγ in NK cells acting as a negative regulator of their differentiation process (Yun et al., 2014).

miRNAs Involved in the Regulation of NK Cell Functions

Accumulating evidence suggests that distinct miRNAs may play regulative roles on NK cell functions both in terms of cytotoxicity and cytokine production. In this context, miR-27a-5p (Kim et al., 2011), miR-378, miR-30e (Wang et al., 2012), and miR-150 (Kim et al., 2014), were proposed as negative regulators of NK cell killing ability. In particular, miR-378 was found to target granzyme b (Gzmb) (Wang et al., 2012), miR-30e and miR-150 have as target perforin (Prf1) (Wang et al., 2012; Kim et al., 2014) while miR-27a-5p targets both (Kim et al., 2011). By contrast, Prf1, Gzmb, IFN-γ, and CD107a in human NK cells were all upregulated after miR-362-5p overexpression. Ni and collaborators indeed found that this miRNA targets the mRNA coding for the cylindromatosis lysine 63 deubiquitase (CYLD) and suggested that miR-362-5p promotes NK cell effector functions (Ni et al., 2015).

miR-155 should also be included among the miRNAs that enhance NK cell functions. In particular, IL-2, IL-15, and IL-21 upregulate this miRNA, which in turn, enhances NK cell cytotoxicity (Liu et al., 2012). Moreover, miR-155 extensively regulates the NK cell activation threshold regulating molecules

involved in NK cell activation and their IFN-γ release by modulating the expression of the phosphatase SHIP-1, T-bet/Tim-3, or the activation of several signaling pathways, including those involving PI3K, NF-kB, and calcineurin (Trotta et al., 2012; Sullivan et al., 2013; Cheng et al., 2015). Similarly, miR-181 was also found to promote IFN-γ production in primary NK cells in response to cytokine stimulation through regulation of the Notch pathway (Cichocki et al., 2011).

In a study aimed to identify miRNAs potentially involved in the pathogenesis of chronic fatigue syndrome or myalgia encephalomyelitis in peripheral blood mononucleate cells (PBMC), 34 miRNAs were found upregulated compared to healthy controls and 2 of these (miR-99b, miR-330-3p) were confirmed having the most important deregulation in NK cells in terms of cytotoxic activity (Petty et al., 2016).

Pathogens-Modulated miRNAs in NK Cells

The host's immune responses must be strictly regulated by an sophisticated balance between positive and negative signals during the fight against pathogens.

One of the mechanisms by which pathogens can break this balance is that of interfering with the regulatory role of miRNAs.

TLRs are receptors of the innate immune system that directly recognize conserved structures of both viral and bacterial origin that are present and functional on NK cells (Della Chiesa et al., 2014). It has been recently demonstrated that several miRNAs, including miR-21, miR-146, miR-155, and let-7 family can bind to TLRs (acting also as physiological ligands for these receptors) or proteins in TLR signaling pathways. These interactions can regulate the expression and the transcriptional responses of TLRs (Bayraktar et al., 2019). In addition, some miRNAs and miRNA-containing exosomes can selectively activate innate immune effector cells, including NK cells, via the TLR1–NF-kB signaling pathway (He et al., 2013).

Enhancement of NK cell cytotoxicity with upregulation of Prf1 was described as associated with miR-182 overexpression in NK cells derived from hepatocellular carcinoma (HCC) patients (Abdelrahman et al., 2016). However, a subsequent study from the same group reported contradicting roles of this miRNA in both NK cells and in hepatocytes infected by hepatitis C virus (HCV) (El Sobky et al., 2016).

Komabayashi and collaborators demonstrated that the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) is able to downregulate the expression of the miR-15a and increase MYB and cyclin D1 in cell lines with an NK cell phenotype (Komabayashi et al., 2014), thus suggesting that miR-15a may have a role in the repression of NK cell proliferation. In line with this, Cheng and collaborators found that the down-regulation of miR-155 suppressed IFN-γ production through Tim-3 signaling and lead to HCV evading immune clearance (Cheng et al., 2015). However, the specific target of miR-155 in the context of these two studies remains unknown.

miR-155 was found to be induced by IL-2 and IL-18 via STAT4 and able to reduce the expression of NOXA and SOCS1 at distinct stages of homeostasis and activation. As NK cells of mice with a targeted miR-155 deletion displayed dramatically diminished effector activities and reduce memory cell numbers in both lymphoid and non-lymphoid tissues afterwards murine cytomegalovirus (MCMV) infection, these findings suggest that miR-155 promotes antiviral immunity (Zawislak et al., 2013). However, a study by Elemam et al. (2015) reached contrasting conclusions as they observed that HCV infection might abolish NK cell cytotoxicity via modification of PU.1 (a key transcription factor in the NK cell development), and Prf1/NKG2D expression by miR-29a-5p and miR-155 overexpression, respectively.

miRNAs in Tumor-Associated NK Cells

Several studies demonstrated that miRNAs might also act as oncogenes or tumor suppressor genes in different human cancer histotypes. Most of the endogenous miRNAs that have been characterized so far modulate NK cell antitumor activity in the tumor microenvironment (TME). TGFß released by tumor cells in the TME is a powerful inhibitor of the NK cell killing activity, and Donatelli and collaborators have shown that a specific miRNA, miR183, is induced by TGFß (Donatelli et al., 2014). They also formally proved that this miRNA downregulates the expression of the DNAX activating protein 12 kDa (DAP12) that was found critically involved in the stabilization of KIR2DS4 and NKp44 receptors on the plasma membrane and required for their signaling activities. Interestingly, loss of DAP12 was also identified as a common trait in tumor-infiltrating lymphocytes in lung cancer (Donatelli et al., 2014). TGFß has also been reported to increase post-transcriptionally the levels of mature miR-1245 which suppresses NKG2D expression, thus spoiling NKG2D-mediated immune responses and enhancing the tumor supporting properties of the TME (Espinoza et al., 2012).

Yang and collaborators reported that miR-218-5p suppresses the NK-mediated killing of lung adenocarcinoma by targeting Serine Hydroxymethyltransferase 1 (SHMT1) (Yang et al., 2019).

Several studies identified miRNAs (miR-203, miR-494-3p, miR-142-3p, miR-155, miR-21) that affect NK cell lymphoma survival and apoptosis modulating different pathways including the PTEN-AKT-mTOR pathway (Yamanaka et al., 2009; Chim et al., 2010, 2011; Ichimura et al., 2010; Chen et al., 2015).

Recent findings provided evidence on the role of some miRNAs as tumor suppressors, such as miR-150 that is involved in the pathogenesis of malignant lymphoma, by increasing the incidence of apoptosis and reducing cancer cell proliferation (Watanabe et al., 2011).

Several studies were performed using as model the NK/T cell lymphoma (NKTL), a progressive malignancy with unfavorable prognosis without a specific treatment, and most of them were pursued to identify dysregulated miRNAs that can affect targets involved in the oncogenesis of NKTL. In this context, Ng and collaborators found that miR-26a, miR-26b, miR-28-5, miR-30b, miR-101, and miR-363 were downregulated, possibly via MYC, in NKTL and NK cell lines compared to normal NK cells and that the suppressed miRNA expression allowed increased expression of genes implicated in oncogenesis (Ng et al., 2011). Furthermore, in a recent study focused on NKTL, Huang and collaborators reported data suggesting that miR-10a and miR-342-3p may be implicated in the development of NKTL through the T-lymphoma invasion and metastasis inducing factor 1 (TIAM1) pathway, which has a crucial role in the development of several

types of human cancer (Huang et al., 2016). Other miRNAs such as miR-221 and miR-155, associated with promotion of NKTL viability, have been proposed as potential molecular markers of NKTL (Guo et al., 2010; Chang et al., 2019).

Recently, growing evidence has shown that extracellular vesicles (EVs) released by NK cells transport miRNAs capable of exerting a strong anti-tumor effect in immunosuppressive TME (Fabbri, 2020). Previous studies have also demonstrated that NK cell-derived exosomes have tumor-specific accumulation with no cytotoxic activity against normal tissues (Lugini et al., 2012). Meanwhile, microenvironment acidic pH promotes the traffic of this EVs in tumor cells (Parolini et al., 2009). In addition, NK cell-derived exosomes also exhibit the benefits of being stable vesicles and maintain their biological activities. Thus, NK cell-derived exosomes can both facilitate tumor targeting and act as direct antitumor agent. These properties make them more suitable for clinical applications, thus suggesting a possible use of NK-cell derived EVs as anticancer agents as a new avenue for tumor therapy (Wang H. et al., 2019).

miRNAs Involved in the Regulation of NK Cell Immune Checkpoints

Immune checkpoints have a key role in regulating the intensity of immune responses of lymphocytes by performing inhibitory functions. The use of immune checkpoint inhibitors in immunotherapy has driven anti-cancer treatment on a novel level. Emerging evidence suggests that some miRNAs can control the expression of immune checkpoints on the surface of NK cells or that of their ligands on tumor cells. This suggests a possible use of miRNAs in the context of anti-tumor therapy.

In a recent study we proved that the miR-146a-5p is able to downregulate both KIR2DL1 and KIR2DL2, two HLA-specific inhibitory receptors belonging to KIR family (Pesce et al., 2018) (**Figure 1A**). Furthermore, *in silico* functional characterization of miR-146a-5p gene targets, identified CD94, HLA-C, HLA-E, Prf1, and several other KIRs genes as additional targets. These results are in line with the higher levels of miR-146a-5p found in $CD56^{bright}$ NK cells and with other studies suggesting that this miRNA is engaged in the regulation of NK cell maturation via the STAT1 (Xu et al., 2017) and NF-kappaB (Wang et al., 2018) signaling pathways. A different research group identified very recently three miRNAs, miR-26a-5p, miR-26b-5p, and miR-185-5p, as inhibitors of the expression of an additional KIR, the KIR3DL3, which is included in the iNKRs group but it is still poorly characterized (Nutalai et al., 2019) (**Figure 1A**). Therefore, the role of these miRNAs in NK cells development or function remains to be defined.

The study of Abdelrahman and collaborators showed that enhancement of NK cell cytotoxicity by miR-182 in human hepatocellular carcinoma and increased Prf1 expression were indirect effects likely mediated by a complex modulation of NKG2D and NKG2A levels in these cells at different stages of the disease (Abdelrahman et al., 2016) (**Figure 1B**).

Regarding miRNAs regulating PD-1 expression, miR-28 (Li et al., 2016), miR138 (Wei et al., 2016), and miR-4717 (Zhang et al., 2015) have been found to target this immune checkpoint, and to induce T cell exhaustion. It has been demonstrated that miR-4717 play a role in chronic Hepatitis B Virus (HBV) infection, where this miRNA is significantly reduced (Zhang et al., 2015). Since also NK cells may express PD-1, it cannot be excluded that these miRNAs may play an important regulatory role also in these innate cells.

Notably, some mi-RNAs target additional immune checkpoints playing a critical role for cytotoxic immune cell functions, such as miR-28 targeting TIM-3 (Li et al., 2016), expressed by both T and NK cells (Di Vito et al., 2019), and miR-16, miR-138, and miR-195 targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4), mainly expressed by T cells (Wei et al., 2016; Tao et al., 2018).

miRNAs Involved in the Regulation of the Expression of Ligands for NK Cell Receptors

Tumor immune evasion is not restricted to the upregulation of immune checkpoint proteins, but also to the dysregulation in the expression of immune checkpoint ligands, including classical and non-classical HLA-I molecules or ligands for activating NK cell receptors. In this context, it has been found that miR-9 is involved in the downmodulation of the expression of HLA-I molecules in human cancer cells, preventing the detection of cancer cells by the immune system (Gao et al., 2013) (**Figure 1C**). This suggests that tumors overexpressing this miRNA might become resistant to $CD8^{+}$ T-cell mediated killing but susceptible to NK cell-mediated attack. In addition, the tumor-suppressive miR-148 family has been found to regulate the expression of HLA-G, a ligand for different NK cell inhibitory receptors (Mandelboim et al., 1997; Seliger, 2016) (**Figure 1D**).

Recently, it has been demonstrated that some miRNAs directly target the 3′-UTR of PD-L1 mRNA and others the PD-1/PD-L1 indirectly by targeting the related signaling pathways (Wang et al., 2017; Gao et al., 2019; Omar et al., 2019). miR-15a, miR-15b and miR-16 were discovered to downregulate the PD-L1 expression in malignant pleural mesothelioma cell line (Kao et al., 2017) (**Figure 1D**). miR-34a was found to be inversely correlated with PD-L1 expression in 44 AML samples (Wang et al., 2015) (**Figure 1D**). miR-935p, miR-106b-5p, miR-138-5p, miR-142-5p, miR-193a-3p, miR-200, and miR-570 overexpression downregulate PD-L1 in tumor cell lines of different hystotypes (Chen et al., 2014; Guo et al., 2015; Cioffi et al., 2017; Jia et al., 2017; Kao et al., 2017) (**Figure 1D**). miR-152 was found to regulate PD-L1 in gastric cancer tissues (Guo et al., 2015), while miR-424 regulates the PD-L1 expression in chemo-resistant ovarian cancer patients (Xu et al., 2016) (**Figure 1D**). Notably, miR-873 decreased the stemness and resistance to chemotherapy of breast cancer cells, depending on PD-L1 and the downstream PI3K/Akt and ERK1/2 signaling, by directly inhibiting PD-L1 expression. This suggests that miR-873/PD-L1 regulatory axis may represent a new therapeutic target in breast cancer. Such data are interesting for employing miRNAs as useful diagnostic targets and valuable biomarkers for prognosis in the PD-1/PD-L1 blockade therapy. miR-155, a key component of inflammatory responses, is

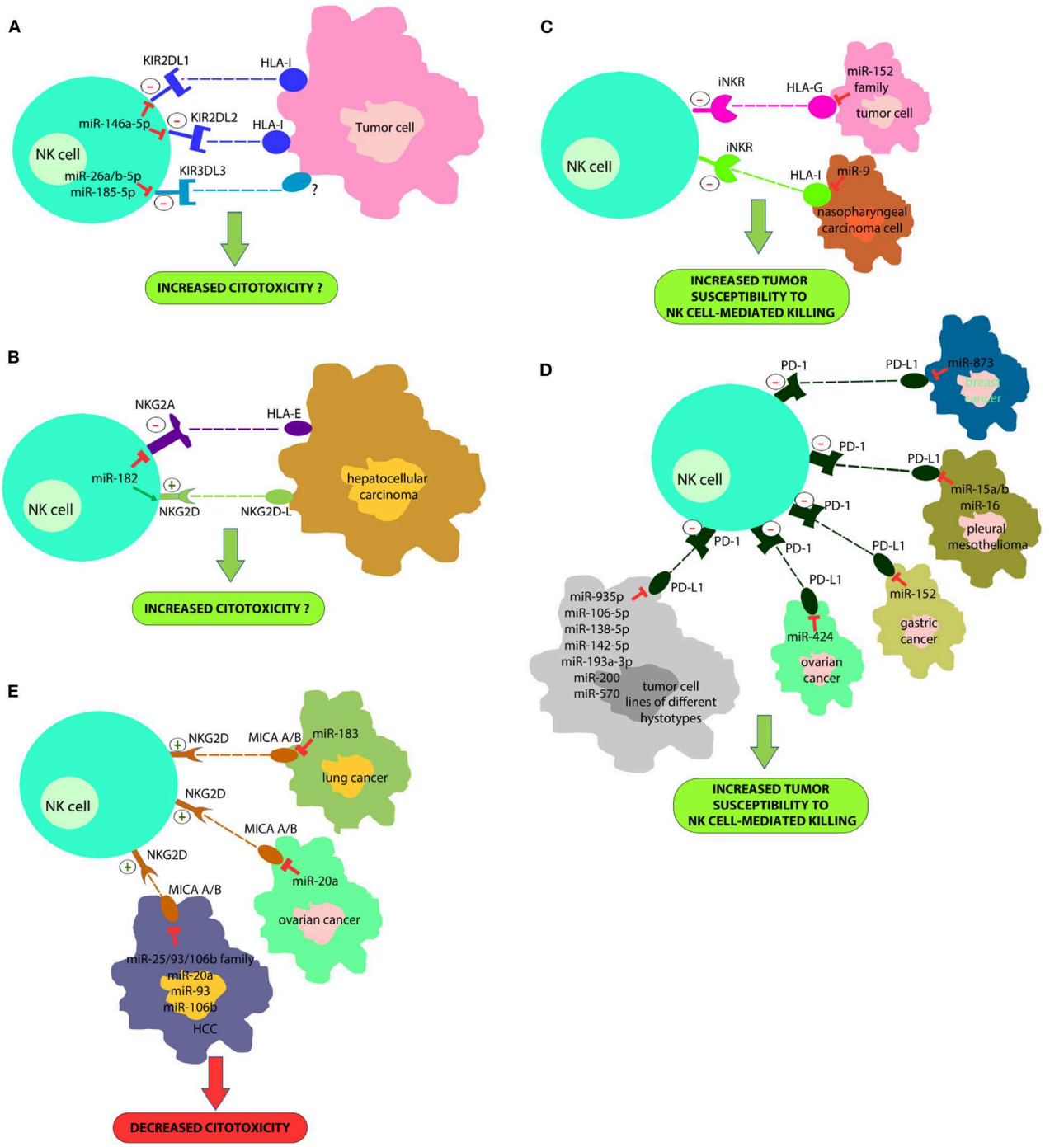

FIGURE 1 | A new strategy for improving NK cell-based immunotherapy: miRNAs can directly regulate the expression of different NK cell immune checkpoints (including KIRs, PD-1, NKG2A, and other iNKR) **(A,B)** or their ligands (HLA-I, PD-L1, HLA-E/G) **(C,D)**. In addition, some miRNAs can also regulate the expression of activating NK cell receptors (i.e., NKG2D) or their ligands (e.g., MIC A/B) **(E)**. This effect can deeply impact on NK cell ability to recognize and kill cancer cells. In particular, a downregulation of immune-checkpoints or immune-checkpoints' ligands can restore an appropriate NK cell anti-tumor activity; on the contrary, a downregulation of activating receptor expression or their ligands can affect their anti-tumor potential. This suggests innovative miRNA-based therapeutic approaches to unleash NK cell effector functions in the cancer treatment.

dysregulated in different cancer cell types. In this context, it has been recently demonstrated that the induction of miRNA-155 expression, suppresses the expression of PD-L1 in both primary lymphatic endothelial cells and fibroblasts, by exposing these cells to the TNF-a and IFN-γ proinflammatory cytokines (Yee et al., 2017).

Moreover, different miRNAs downregulate MHC class I chain-related protein A/B (MICA/B) expression (NKG2D NK-cell receptor ligands) and this represents another mechanism of immune suppression targeting NK cells cytotoxicity. These miRNAs include miR-183 that targets MIC A/B in lung cancer (Trinh et al., 2019), miR-20a that induces the same effect in ovarian cancer (Xie et al., 2014) and miR-25/93/106b family, miR-20a, miR-93 and miR-106b that act in HCC (Kishikawa et al., 2013) (**Figure 1E**).

CONCLUDING REMARKS

Recently, there has been a substantial evolution in cancer therapy, mainly oriented toward immunotherapy approaches, in substitution or in combination with classical therapy. Cancer immunotherapy represents a promising new era in cancer management due to the relatively high safety margins and selectivity, compared to the classical cancer chemotherapeutic agents.

miRNAs have come to light over the last years as key actors in epigenetic regulation and for their capacity to modulate tumor immunity by directly regulating the expression of genes involved in the activation or suppression of the immune response. In this review, we focused our attention on the current state of knowledge concerning the involvement of miRNAs in various physiologic processes of NK cells. In particular, we discussed their abilty to regulate NK immune responses and their potential implications in resistance to cancer immunotherapy, with main focus on immune checkpoints. In this context, several miRNAs have been found to modulate different immune checkpoints/ligands interaction, including the PD-1/PD-L1 axis or their upstream genes. Future studies comparing miRNAs' expression profiles in patients who respond to immune checkpoint blockade immunotherapies as compared to non-responders will help to disclose the potential role of miRNAs as non-invasive predictive biomarkers for monitoring the response and clinical outcomes to immunotherapy with immune checkpoint inhibitors.

AUTHOR CONTRIBUTIONS

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

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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.

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METHODS
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A Protocol for Transcriptome-Wide Inference of RNA Metabolic Rates in Mouse Embryonic Stem Cells

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The relative ease of mouse Embryonic Stem Cells (mESCs) culture and the potential of these cells to differentiate into any of the three primary germ layers: ectoderm, endoderm and mesoderm (pluripotency), makes them an ideal and frequently used *ex vivo* system to dissect how gene expression changes impact cell state and differentiation. These efforts are further supported by the large number of constitutive and inducible mESC mutants established with the aim of assessing the contributions of different pathways and genes to cell homeostasis and gene regulation. Gene product abundance is controlled by the modulation of the rates of RNA synthesis, processing, and degradation. The ability to determine the relative contribution of these different RNA metabolic rates to gene expression control using standard RNA-sequencing approaches, which only capture steady state abundance of transcripts, is limited. In contrast, metabolic labeling of RNA with 4-thiouridine (4sU) coupled with RNA-sequencing, allows simultaneous and reproducible inference of transcriptome wide synthesis, processing, and degradation rates. Here we describe, a detailed protocol for 4sU metabolic labeling in mESCs that requires short 4sU labeling times at low concentration and minimally impacts cellular homeostasis. This approach presents a versatile method for in-depth characterization of the gene regulatory strategies governing gene steady state abundance in mESC.

Keywords: RNA metabolic labeling, RNA metabolic rates, degradation rate, transcription rate, processing rate, MESC, 4sU, 4sU-RNA labeling

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INTRODUCTION

Gene expression control is central to ensure appropriate responses to intrinsic and extrinsic cellular stimuli and cellular homeostasis. The steady state abundance of RNA transcripts is controlled by the rates at which the gene is transcribed (transcription), processed (processing), and degraded (degradation). Understanding how these three RNA metabolic rates change in response to different cellular cues, is paramount for in-depth characterization of how gene expression regulation, in health and disease, contributes to the maintenance or changes in cell state during development and in adulthood.

Different methods allow measurement of the individual contribution of each of these RNA metabolic rates to steady state abundance. Some of the most widely used techniques are based on the use of transcription inhibition or transcription synchronization drugs, such as 1-β-D-ribofuranoside (DRB), α-Amanitin (α-Ama) or Actinomycin-D (Act-D)

(Alpert et al., 2017; Wada and Becskei, 2017). While these approaches were initially used to assess transcript-specific rates, the advent of next generation sequencing technologies now allow their transcriptome-wide implementation. The main limitation of these approaches is that transcription-inhibition induces cellular stress and can lead to a number of pleotropic effects. For example, since transcription and degradation rates have been suggested to be inherently linked (Haimovich et al., 2013), inhibiting transcription may thereby impact degradation rates and influence transcript half-live measurements. Transcriptional inhibitors have also been shown to lead to stabilization of proteins involved in gene expression control, such as p53, suggesting that secondary changes are also likely to impact the accuracy of the measurements (reviewed in Bensaude, 2011). Furthermore, it has also been suggested that decay rates of short- or long-lived transcripts cannot be accurately measured using approaches based on transcription blockage (Clark et al., 2012).

Methods based on RNA metabolic labeling using modified nucleotides, such as 5′-Bromouridine (BrU) or 4-thiouridine(4sU), overcome these limitations (Baptista and Dolken, 2018). Specifically, this class of methods allows estimation of RNA metabolic rates with minimal impact on cellular homeostasis for most transcript classes (de Pretis et al., 2015). Metabolic labeling is based on the incorporation of modified ribonucleosides into nascent RNA transcripts during cellular proliferation. Compared to BrU, 4sU is more rapidly incorporated (reviewed in Tani and Akimitsu, 2012), and is the most widely used modified ribonucleoside. Isolation of transcripts synthesized during the incubation with 4sU from preexisting transcripts can be achieved by thiol-specific biotinylation of 4sU labeled RNA followed by streptavidin-dependent enrichment. Alternatively, chemical conversion of modified ribonucleosides can also be used to distinguish newly synthesized and preexisting transcripts (Herzog et al., 2017). Finally, transcriptome-wide RNA metabolic rates can be inferred by quantification of transcript levels in both RNA fractions coupled with computational modeling (**Figure 1**). Different mathematical modeling approaches have been developed to infer RNA metabolic rates from these types of data (Rabani et al., 2014; de Pretis et al., 2015; Lugowski et al., 2018; Neumann et al., 2019).

In its simplest form, RNA metabolic labeling experimental design relies on a single labeling time, similar to what is described here. Single labeling reduces experimental cost and complexity, likely at the expense of the accuracy of the degradation rate estimates (Wada and Becskei, 2017). Alternatively, the dynamics of nucleotide incorporation can also be explored to increase rate inference accuracy using approach-to-equilibrium or pulse-chase designs (Duffy et al., 2019).

RNA metabolic labeling using 4sU (Dolken et al., 2008), has been applied for inference of RNA metabolic rates of diverse transcript classes from highly stable microRNAs (Marzi et al., 2016) to rapidly decaying transcripts, such as long non-coding RNAs, in a wide range of cell lines (Marzi et al., 2016; Mukherjee et al., 2017; Freimer et al., 2018). Despite the widespread implementation of this method, data for transcriptome-wide metabolic rates in mESC – one of the most widely used model systems for the study of cell state homeostasis and cell state transitions – is limited (Freimer et al., 2018). Given the availability of numerous constitutive and inducible mESC mutants, analysis of RNA metabolic labeling in these cells can provide a better understanding how different genes and pathways modulate gene expression. Here we present a detailed protocol based on a short pulse with low concentrations of 4sU for RNA metabolic labeling in mESCs. This approach allows non-invasive (**Supplementary Note Figures 1A–C**) quantification of metabolic rates for most transcript classes including very short-lived RNAs. Due to its negligible impact on cell state and viability, this protocol can be effectively applied to wild-type and mutant mESC lines. In addition, we provide details on quality controls, that we adapted for mESC based on previous work (Radle et al., 2013), and that allow the user to assess the labeling and RNA quality throughout the experiment.

MATERIALS AND REAGENTS

(A) Cell culture.
  (1) 10 cm tissue treated cell culture plate.
  (2) Knockout DMEM mESC growth medium (Thermo Fisher, 10829018):
    (i) 15% (v/v)Fetal Bovine Serum (Thermo Fisher, 16000044).
    (ii) 50 U/ml of Penicillin and 50 μg/ml of Streptomycin (Thermo Fisher, 15070063).
    (iii) 1 U/μl Recombinant Mouse LIF Protein (MERCK, ESG1107).
    (iv) 0.06 mM β-Mercaptoethanol (Thermo Fisher, 31350010).
    (v) 1% (v/v) 100 X Non-Essential Amino Acids (Thermo Fisher, 11140050).
  (3) Sterile 0.2% (m/v) Gelatin in H_2O.

(B) 4-Thouridine labeling and total RNA extraction.
  (1) 4-thiouridine (Sigma, T4509) dissolved in DEPC-treated H_2O (DEPC-H_2O).
    NOTE$_1$: *Keep at −20°C, protected from light (4sU is light-sensitive). Discard remaining 4sU solution after thawing.*
  (2) PBS.
  (3) Trizol reagent (Thermo Fisher, 15596026).
  (4) Chloroform (Sigma, C2432).
  (5) 15 ml phase lock-tubes (Qiagen, 129065).
  (6) 15 ml Falcon Tubes.
  (7) 1.5 ml tube (RNase and DNase free).
  (8) 5M NaCl in DEPC-H_2O.
  (9) Isopropanol.
  (10) DEPC- H_2O.
  (11) 75% (v/v) Ethanol in DEPC- H_2O.
  (12) 5 ml Serological Pipette.
  (13) Qiagen RNeasy kit (Qiagen, 74104).
  (14) RNAse Free DNAse Set (Qiagen, 79254).

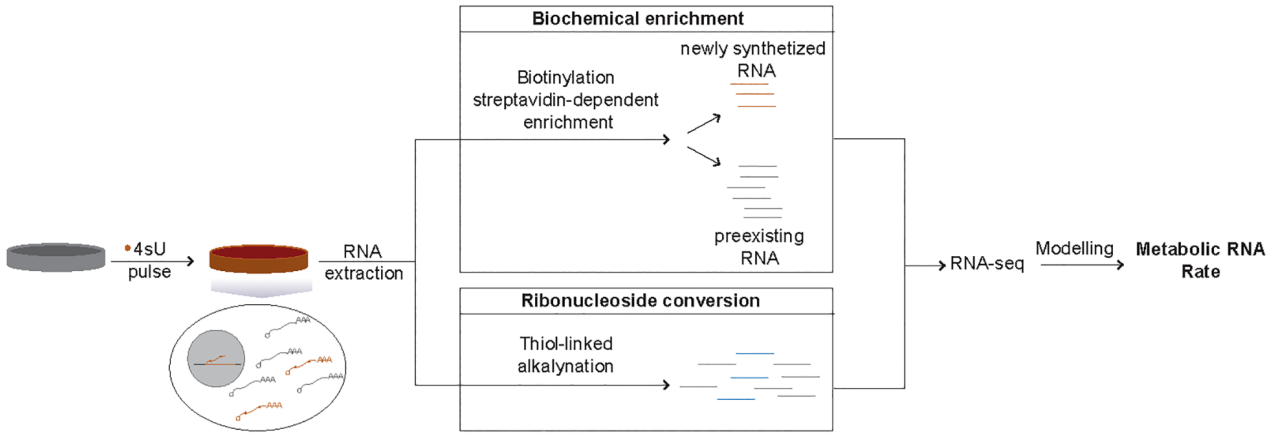

FIGURE 1 | Schematic representation of 4sU metabolic labeling-based inference of RNA metabolic rates. Isolation of newly transcribed RNAs from the total RNA of cells treated with 4sU (orange) can be achieved in two ways. Biochemical enrichment relies on the biotinylation of 4sU incorporated newly transcribed RNAs, followed by streptavidin-dependent separation of the newly synthetized (4sU-incorporated, orange lines) transcripts from the preexisting RNA fraction (gray lines). Ribonucleoside conversion, on the other hand, is based on chemical induced conversion of 4sU into a base that is likely to be read as cytosine (blue lines) and can be distinguished from the preexisting RNA (gray lines) based on the presence of T-C changes. RNA-sequencing-based estimates of the relative levels of newly synthetized transcripts coupled with appropriate computational modeling approaches allows the inference of metabolic RNA rates.

(C) Dot-blot quality control.
  (1) Zeta-Probe Membrane (Biorad, 1620190).
  (2) Biotin-labeled DNA oligo.
  (3) DEPC- H_2O.
  (4) 2 ml Phase Lock tubes (Qiagen, 129056).
  (5) EZ-link Iodoacetyl-PEG2-biotin (Thermo Fisher, 21334) dissolved to 1 mg/ml in DMF (Thermo Fisher, 20673).
  (6) Na_2HPO_4 (Applichem, A1046,1000).
  (7) 20% Sodium Dodecyl Sulfonate (SDS) (Applichem, A0675,0250).
  (8) Phenol:Chloroform:isoamyl alcohol (Sigma, P2069-100ML).
  (9) PBS.
  (10) Streptavidin-horseradish peroxidase (Strep-HRP, Thermo Fisher, 21130).
  (11) Blocking Buffer: (0.5M Na_2HPO_4, 7% SDS, pH 7.2 in DEPC-H_2O).
  (12) Wash solution 1: 40 ml PBS + 10% (v/v) SDS.
  (13) Wash solution 2: 40 ml PBS + 1% (v/v) SDS.
  (14) Wash solution 3: 40 ml PBS + 0.1% (v/v) SDS.
  (15) Advansta WesternBright ECL (advansta, K-12045-D50).

(D) RNA biotinylation for isolation of newly transcribed RNA.
  (1) 1.5 ml Eppendorf tubes (RNAse and DNAse free).
  (2) 10 X Biotinylation Buffer (100 mM Tris-HCl pH 7.4, 10 mM EDTA in DEPC-H_2O).
  (3) 1 mg/ml EZ-link HPDP-Biotin (Thermo Fisher, 21341) in dimethylformamide (DMF) (Sigma, D4551).
  (4) Phenol:Chloroform:isoamyl alcohol (Sigma, P2069-100ML).
  (5) 2 ml phase lock tubes (Qiagen, 129056).
  (6) 75% (v/v) Ethanol in DEPC-H_2O.
  (7) DEPC-H_2O.
  (8) 2.0 ml Eppendorf tubes (DNAse and RNAse free).

(E) Bead preparation.
  (1) Dynabeads MyOne T1 Streptavidin Beads (Thermo, 65601).
  (2) 2X Bind and Wash (B&W) Buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA, 2M NaCl in DPEC-H_2O).
  (3) Solution A (0.1M NaOH, 0.05M NaCl in DEPC-H_2O).
  (4) Solution B (0.1M NaCl in DEPC-H_2O).

(F) Separation of newly transcribed RNA.
  (1) 1X B&W Buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1M NaCl in DEPC-H_2O).
  (2) RNeasy Mini kit (Qiagen, 74104).
  (3) RNase-Free DNase Set (Qiagen, 79254).
  (4) Isopropanol.
  (5) 5M NaCl in DEPC- H_2O.
  (6) Isopropanol.
  (7) 75% Ethanol in DEPC-H_2O.
  (8) RNase-free DEPC-H_2O.
  (9) 70% Ethanol in DEPC-H_2O.
  (10) 100 mM Dithiothreitol (DTT) (Thermo Fisher, R0861) in DEPC-H_2O.

(G) RT-qPCR analysis.
  (1) SuperScript IV First-Strand Synthesis System (Thermo Fisher, 18091200).
  (2) FastStart Essential DNA Green Master (Roche, 06402712001).
  (3) Gene specific primers (sequences can be found in **Supplementary Table 1**).
  (4) DEPC-H_2O

Equipment.

(A) Cell culture Hood (Thermo Fisher, Herasafe KS12).
(B) Chemical Hood (Waldner, Bench-mounted fume hood).

(C) Cell culture incubator at 37°C with 5.0% CO_2 (Thermo Fisher, Direct Heat CO_2 Incubator).
(D) Centrifuge with temperature control (Beckman Coulter, Avanti J-26 XP).
(E) Standard tabletop centrifuge with temperature control (Eppendorf, Centrifuge 5415 R).
(F) Rotating Vertical Mixer (Stuart, Rotator SB3).
(G) DynaMag-2 Magnet Magnetic Rack (Thermo Fisher, 12321 D).
(H) Quantitative PCR Machine (Roche, Light Cycler 96).
(I) See-saw rocker (Stuart, See-saw rocker SSL4).
(J) Chemiluminescence detection apparatus (Vilber Lourmat, FUSION Solo 6S Imaging System).
(K) Thermocycler (Biometra Thermocycler ThermoBlock).
(L) Fluorescent Activated Cell Sorter (Synergy MX flow Cytometer Fluorescent activated Cell Sorter).
(M) Nanodrop (ND-1000 Spectrophotometer).

Methods.

(A) Before You start:
  (1) Centrifuge all 2 ml phase lock tubes and 15 ml phase lock tubes at 12,000 × *g* for 30 s and 1,500 × *g* for 60 s respectively.
  (2) Cool ultracentrifuges and tabletop centrifuges to 4°C.

(B) 4-Thouridine labeling and total RNA extraction.
  (1) The day before labeling, seed mESCs in two gelatin-coated 10 cm plates (12 ml of growth medium). Cells in one plate will be labeled with 4sU whereas cells in the other plate will be untreated and will serve as negative control.
    $NOTE_2$: mESCs should be 70-80% confluent at the time of labeling.
  (2) Transfer 7 ml of medium from one of the overnight mESC culture (4sU-treated mESC) to a 15 ml falcon tube, add 4sU to a final concentration of 200 μM and mix thoroughly by pipetting up and down.
    $NOTE_3$: Negative control plate (containing untreated cells) should be kept in a humidified cell culture incubator at 37°C with 5% CO_2 until RNA is harvested (step 5). From that point onwards RNA from 4sU treated and untreated cells should be handled in parallel.
  (3) Remove the remaining 5 ml of growth medium in the plate and add 7 ml of 4sU supplemented growth medium.
    $NOTE_4$: When planning to use different final 4sU concentrations one should consider the following: (1) High 4sU concentrations can induce nucleolar stress responses and impact proliferation rates (Burger et al., 2013; Radle et al., 2013)*; (2) Low 4sU concentrations may result in low recovery of newly transcribed RNA.*
  (4) Incubate cells in a humidified cell culture incubator at 37°C with 5% CO_2, for the desired pulse duration.
    $NOTE_5$: The "Representative results", described below were obtained from a 15 min 4sU pulse.
  (5) Aspirate media and wash plates with 4 ml PBS.
  (6) Inside a chemical hood, add 5 ml of Trizol reagent to plates. Ensure Trizol covers the whole surface of the plate.
  (7) Incubate at room temperature for 5 min.
  (8) Transfer mESC cell lysate in Trizol from plates to clean 15 ml falcon tubes.
    $NOTE_6$: Lysate- containing Trizol can be stored at 4°C for up to 12 h.
  (9) Add 1 ml of chloroform to lysate-containing Trizol and mix vigorously by pipetting.
  (10) Transfer mixture to the prespun 15 ml phase lock tube.
  (11) Incubate, at room temperature, until clear separation between organic and inorganic phase is obtained (minimum 3 min).
  (12) Centrifuge for 5 min at 4°C at 1500 × *g*.
  (13) Carefully transfer the upper phase (contains RNA) to clean 15 ml falcon tube.
  (14) Add equal volume of Isopropanol (∼3.0–3.5 ml) and mix vigorously by pipetting.
  (15) Incubate mixture at room temperature for 10 min.
  (16) Centrifuge for 15 min at 4°C at 11000 × *g*.
    $NOTE_7$: RNA pellet should be visible at the end of this centrifugation step.
  (17) Remove supernatant and wash RNA pellet with 3.5 ml of 75% Ethanol in DEPC-H_2O.
  (18) Centrifuge for 5 min at 4°C at 7500 × *g*.
  (19) Remove supernatant carefully to avoid dislodging RNA pellet.
  (20) Resuspend RNA pellet in 100 μl of RNAse free DEPC-H_2O and transfer to fresh 1.5 ml tube.
  (21) Remove genomic DNA. To this end, we use on-column DNAse I treatment (RNeasy Mini kit, Qiagen) according to manufacturer's instructions.
    $NOTE_8$: On-column DNase digestion using the RNeasy Mini kit may result in significant loss of small RNA species. If enriching for small RNA species we suggest using an alternative approach [for example TURBO^{TM} DNase (Thermo Fisher, AM2238)].
  (22) Adjust total RNA volume to 100 μl and quantify RNA concentration.
    $NOTE_9$: Proceed directly to RNA Biotinylation or store RNA at −80°C.

(C) Optional dot-blot for assessment of 4sU incorporation.
  (1) Assemble in a 1.5 ml RNAse/DNAse free tube, 300 μl biotinylation reaction by adding in the following order:
    (i) 30 μg of total RNA in 210 μl DEPC-H_2O.
    (ii) 30 μl of 10X Biotinylation Buffer.
    (iii) 60 μl of EZ-link iodoacetyl-Biotin dissolved to 1 mg/ml in DMF.
  (2) Mix vigorously, by pipetting, until solution is homogenous.

$NOTE_{10}$: Ensure to mix immediately after addition of EZ-link iodoacetyl-Biotin to avoid precipitation.

(3) Incubate the mixture on Rotating Vertical Mixer for 2 h at room temperature.

$NOTE_{11}$: During incubation, precool a tabletop centrifuge to 4°C, centrifuge 2 × 2 ml phase lock tubes at 12000 × g for 30 s for each sample being tested and prepare blocking solution and wash solutions 1–3.

(4) Add 300 µl of phenol:chloroform:isoamyl alcohol to biotinylation reaction and mix vigorously by pipetting.

(5) Add biotinylation reaction and phenol:chloroform:isoamyl mixture to prespun phase lock tubes and allow phases to separate at room temperature (minimum 3 min).

(6) Centrifuge for 5 min at 4°C at 12000 × *g*.

(7) Transfer the organic phase (∼280 µl) into a clean 1.5 ml tube.

(8) Add equal volume of phenol:chloroform:isoamyl alcohol, mix by pipetting, transfer to phase lock tube and repeat steps 6–7 once.

(9) Transfer organic phase (generally 275 µl) to clean 1.5 ml tube, add equal volume of isopropanol and 1/10 volume of 5M NaCl. Mix well by pipetting.

(10) Centrifuge for 45 min at 4°C at 16000 × *g*. RNA pellet should be visible following this centrifugation step.

(11) Carefully remove supernatant and resuspend RNA pellet in 300 µl 75% Ethanol in DEPC-H_2O.

(12) Spin for 10 min at 4°C at 13000 × *g*.

(13) Carefully remove supernatant and resuspend pellet in 20 µl of DEPC-treated H_2O.

(14) Quantify RNA concentration, and maintain on ice until use.

(15) Incubate Zeta-membane in 5-10 ml DEPC-H_2O on a see-saw rocker for 10 min.

$NOTE_{12}$: Membrane should be covered by DEPC-H_2O.

(16) Take Zeta-membrane and remove excess liquid by dabbing both sides gently with clean paper towels.

(17) Allow membrane to air dry for 5–10 min.

(18) Prepare, for each RNA sample (including 4sU-untreated control), 10 µl of 1000 ng/ul dilution in DEPC-H_2O.

(19) Prepare four 1:2 serial dilutions (500, 250, 125, 62.5 ng/µl RNA) in 3 µl for each of the RNA samples, in DEPC-H_2O.

(20) Place Zeta-membrane on top of clean glass surface and apply 2 µl of each dilution of RNA to the zeta membrane. Additionally, add 2 µl of 100 ng/µl Biotinylated Oligo as positive control for Strep-HRP activity.

$NOTE_{13}$: To ensure proper spacing between blotted samples, we suggest pipetting the RNA onto the zeta membrane through the holes of an empty pipette tip box.

(21) Air dry membrane for 7 min.

(22) Incubate the membrane for 30 min in 30 ml of freshly prepared blocking buffer (0.5M NaH_2PO_4, 7% SDS, pH 7.2, in DEPC-H_2O) on a see-saw rocker.

(23) Remove blocking solution and incubate the membrane with 10 ml of freshly prepared 1:1000 streptavidin-horseradish peroxidase solution for 15 min (5 ml PBS + 5 ml 20%SDS + 10 µl of streptavidin horseradish peroxidase).

$NOTE_{14}$: Streptavidin horseradish-peroxidase should be thawed on ice and added just before use.

(24) Wash membrane twice with Wash solution 1 for 10 min.

(25) Wash membrane twice with Wash solution 2 for 10 min.

(26) Wash membrane twice with Wash solution 3 for 10 min.

(27) Proceed with chemiluminescent detection using WesternBright ECL solutions according to manufacturer's instructions.

(D) RNA biotinylation.

(1) Thaw RNA (from section B step 22), on ice.

(2) Assemble in a 2.0 ml RNAse/DNAse free tube, 1.0 ml biotinylation reaction by adding in the following order:

(i) 100 ug of RNA in 700 µl of DEPC-H_2O.

(ii) 100 µl of 10X Biotinylation Buffer.

(iii) 200 µl of EZ-link Biotin HPDP dissolved to 1 mg/ml in DMF.

$NOTE_{15}$: Ensure to mix immediately after addition of EZ-link HPDP-Biotin to avoid precipitation.

$NOTE_{16}$: To biotinylate different amounts of 4sU labeled RNA scale reagents proportionally. We do not recommend labeling less than 80 ug of RNA as in our hands and for short pulse durations this leads to < 150 ng newly synthetized RNA.

(3) Mix vigorously, by pipetting, until solution is homogenous.

(4) Incubate the mixture on Rotating Vertical Mixer for 2 h at room temperature.

(5) Add equal volume (in this case 1.0 ml) of phenol:chloroform:isoamyl alcohol to the biotinylation reaction and mix vigorously by pipetting.

(6) Add biotinylation reaction and phenol:chloroform:isoamyl mixture to prespun 2.0 ml phase lock tubes and allow phases to separate at room temperature (minimum 3 min).

$NOTE_{17}$: We recommend using 2.0 ml phase lock tubes as in our experience using 15 ml phase lock tubes leads to considerable loss of material. As the maximum sample volume of 2 ml phase lock tubes is 750 µl, the mix obtained at the end of step 5 (2.0 ml total Volume) should be divided into 3 separate 2.0 ml phase lock tubes and processed separately until precipitation (step 9).

(7) Centrifuge for 5 min at 4°C at 12000 × *g*.

(8) Repeat steps 5–7 to ensure complete removal of unreacted biotin that could otherwise interfere with binding of biotinylated RNA to streptavidin.
(9) Transfer upper phase containing RNA into clean 2.0 ml tube and mix with 1/10 volume of 5M NaCl and an equal volume (∼850 µl) of Isopropanol.
(10) Centrifuge for 45 min at 4°C at 16000 × *g*. RNA pellet should be visible following this centrifugation step.

$NOTE_{18}$: Bead preparation (section E) can be performed during this centrifugation step, assuming that on average 5–10% of RNA is lost during the biotinylation.

(11) Remove supernatant and resuspend in equal volume (850ul) of 75% Ethanol.
(12) Centrifuge for 10 min at 4°C at 16000 × *g*.
(13) Remove supernatant, taking care not to dislodge the RNA pellet.
(14) Resuspend RNA in 100 µl of DEPC-H_2O and transfer to a clean 1.5 ml tube.
(15) Quantify RNA concentration and keep RNA on ice until separation of newly transcribed RNA from preexisting RNA step.

(E) Bead preparation.

$NOTE_{19}$: Beads preparation is based on manufacturer's instructions. For more details please refer to the manual provided with MyOne Streptavidin T1 Dynabeads by Thermo Fisher.

(1) Vortex MyOne Streptavidin T1 Dynabeads for 30 s to ensure complete beads resuspension.
(2) Pipette 2 µl of MyOne Streptavidin T1 Dynabeads for each µg of precipitated biotinylated RNA into a 1.5 ml tube.
(3) Add equal volume (minimum 1.0 ml) of 1X B&W Buffer to beads.
(4) Incubate on Rotating Vertical Mixer for 1 min at room temperature.
(5) Place tube containing resuspended beads on Dynamag Magnetic Rack and separate beads for 1 min.
(6) Carefully remove supernatant and resuspend beads in equal volume of 1X B&W Buffer.
(7) Repeat steps 3-6 for a total of four washes in 1X B&W Buffer.
(8) Resuspend beads in 1.0 ml of Solution A and incubate on a Rotating Vertical Mixer for 2 min.
(9) Place on Dynamag Magnetic rack and separate beads for 1 min.
(10) Remove supernatant carefully.
(11) Repeat steps 8–10 once.
(12) Resuspend beads in 1.0 ml of Solution B and incubate on Rotating Vertical Mixer for 2 min.
(13) Place on Dynamag Magnetic Rack and separate beads for 1 min.
(14) Remove supernatant carefully.
(15) Repeat steps 12–14 once to remove NaOH traces.
(16) Resuspend beads in same volume of 2X B&W Buffer as the volume of beads initially taken from the vial.

$NOTE_{20}$: Beads concentration for optimal coupling with RNA can be optimized for specific applications and pulse durations.

(F) Separation of newly transcribed RNA from preexisting RNA.

(1) Resuspend biotinylated RNA precipitated in section D steps 14–15 to a final concentration of 500 ng/µl in DEPC-H_2O.
(2) Mix previously washed Dynabeads with equal volume of precipitated biotinylated RNA in DEPC-H_2O by pipetting.
(3) Place on Rotating Vertical Mixer and mix for 15 min at room temperature.

$NOTE_{21}$: Beads and RNA solution is viscous! If solution viscosity inhibits even mixing, it is critical to add same volume of 1X B&W to all samples being processed to uniformly reduce bead concentration and ensure even mixing.

(4) Place biotinylated RNA coated beads on Dynamag Magnetic Rack for 3 min to separate beads from solution.
(5) Remove and discard supernatant.
(6) Resuspend beads in 500 µl of 1X B&W Buffer and mix on Rotating Vertical Mixer for 1 min.
(7) Place tube on Dynamag Magnetic rack for 1 min.
(8) Repeat steps 6–7 for a total of three washes with 1X B&W Buffer. Discard supernatant.
(9) Resuspend beads in 100 µl freshly prepared 100 mM DTT and incubate at room temperature for 1 min.
(10) Add 350 µl of Qiagen RNeasy Mini Kit Buffer RLT to DTT bead suspension and mix thoroughly by pipetting.

$NOTE_{22}$:If enriching for small RNAs we suggest Trizol for RNA elution from beads and Qiagen miRNeasy Mini Kit (Qiagen, 217004) according to manufacturer's instructions for RNA purification.

(11) Incubate at room temperature for 5 min.
(12) Place mixture containing beads on Dynamag Magnetic Rack and allow to separate for 2 min.
(13) Transfer supernatant to a clean 1.5 ml tube.
(14) Add equal Volume of 70% EtOH in DEPC-H_2O (450 ul) to tube and mix well by pipetting.
(15) Proceed with RNA purification using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions.
(16) Elute with 25 µl of DEPC-H_2O and quantify concentration.

(G) Optional reverse transcription quantitative PCR analysis of newly transcribed and total RNA ratio.

(1) Reverse transcribe the same volume of total RNA and newly transcribed RNA from each sample using random hexamers.

$NOTE_{23}$: In the "Representative results" section we report on data obtained from reverse transcription of

2 μl of RNA using the SuperScript IV cDNA synthesis kit according to the manufacturer's instructions.

(2) Measure the expression of transcripts that vary across a range of reported stabilities (*Ccne2, Myc, Actin-β, Rplp0, GapdH*) in the newly transcribed and total RNA fractions by RT-qPCR.

NOTE$_{24}$: In the "Representative results" section we report the results for qPCR reactions performed in Roche Lightcycler 96® *according to manufacturer's instructions. qPCR reaction was assembled by adding 2 μl of cDNA diluted 1:4, 5 μl of FastStart Essential DNA Green Master, gene specific primers to final concentration of 0.5 μM and 2.5 μl of DEPC-H_2O.*

(3) To calculate the 4sU enrichment of transcripts in the newly transcribed RNA fraction relative to the total RNA fraction:

(i) Determine normalization factors K_{total} and $K_{newly-transcribed}$ that account for the relative fraction of total volume used for reverse transcription:

- $K_{total} = V_{totalRNA}/V_{qPCRtotalRNA}$;
- $K_{newly-transcribed} = V_{newlytranscribedRNA}/V_{qPCRnewlytranscribedRNA}$;

where

- V_{total} = Volume of total RNA;
- $V_{newly-transcribed}$ = Volume of newly transcribed RNA;
- $V_{qPCRtotalRNA}$ = Volume of total RNA reverse transcribed;
- $V_{qPCRnewlytranscribedRNA}$ = Volume of newly transcribed RNA used for reverse transcription.

(ii) Convert Cq/Ct to normalized expression as following: $Exp_{total} = 2^{-Cqtotal} * K_{total}$ and $Exp_{newlytranscribed} = 2^{-Cq\ newlytranscribed} * K_{newlytrascribed}$

(iii) For each transcript, calculate the ratio of normalized 4sU relative enrichment as following: $4sU_{rel.-enr.} = Exp_{newlytranscribed}/Exp_{total}$.

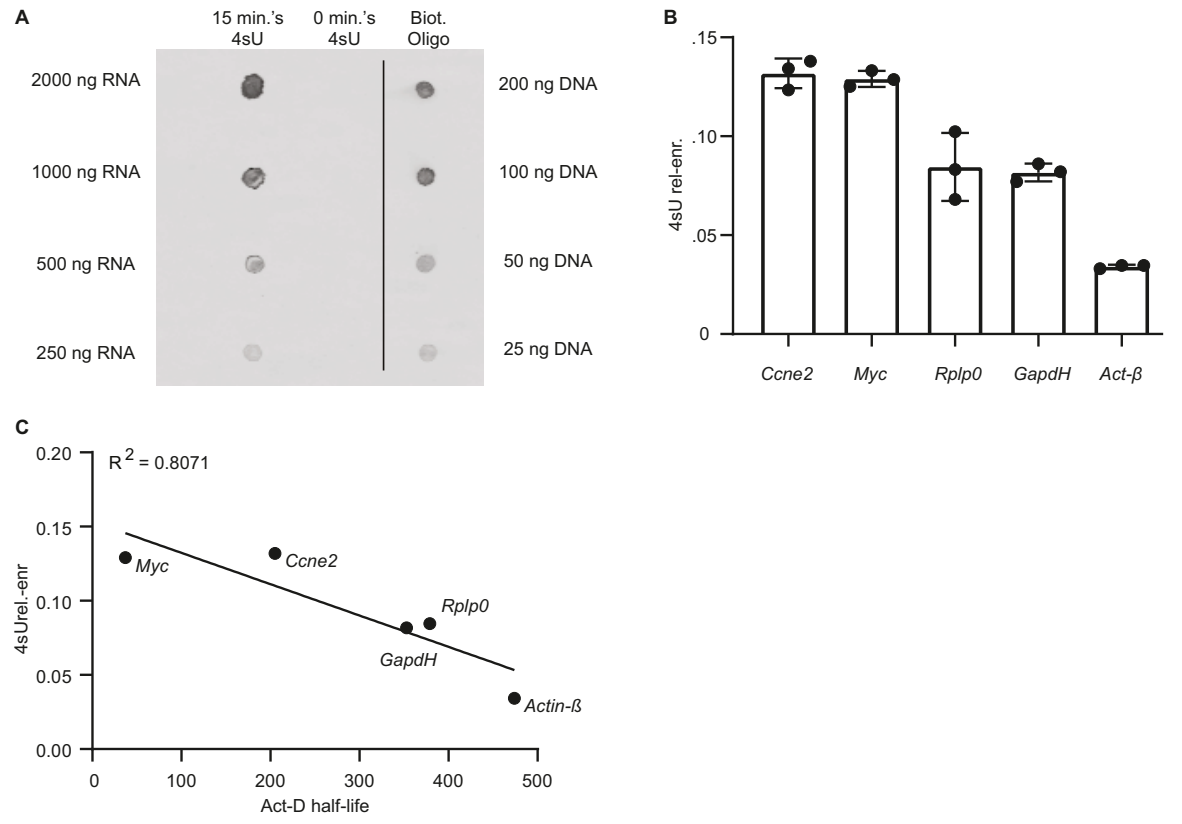

FIGURE 2 | Labeling mESCs with 200 μM of 4sU for 15 min is sufficient for significant incorporation of the modified ribonucleoside in newly transcribed RNA. **(A)** Dot-Blot of RNA extracted from cells labeled with 4sU (15 min 4sU), untreated control (0 min. 4sU) and a biotinylated oligo (Biot. Oligo). 2 μl of RNA (15 min 4sU, 0 min 4sU) and biotinylated oligo (Biot. Oligo) were blotted. The respective concentrations for the blotted samples are indicated on the left and right side of the blot for the RNA samples and Biot. Oligo respectively. **(B)** 4sU relative enrichment ($4sU_{rel-enr}$, Y-axis) of two relatively unstable transcripts (*Ccne2* and *Myc*) and three relatively stable transcripts (*Rplp0*, *GapdH,* and *Act-β*). **(C)** $4sU_{rel.-enr}$ (Y-axis) estimated for five genes (gene names indicated next to data point), represented as a function of half-lifes in mESC estimated using transcriptional inhibition (Sharova et al., 2009) (Act-D half-life, X-axis). The Pearson R^2 is indicated on the top left-hand side of the plot.

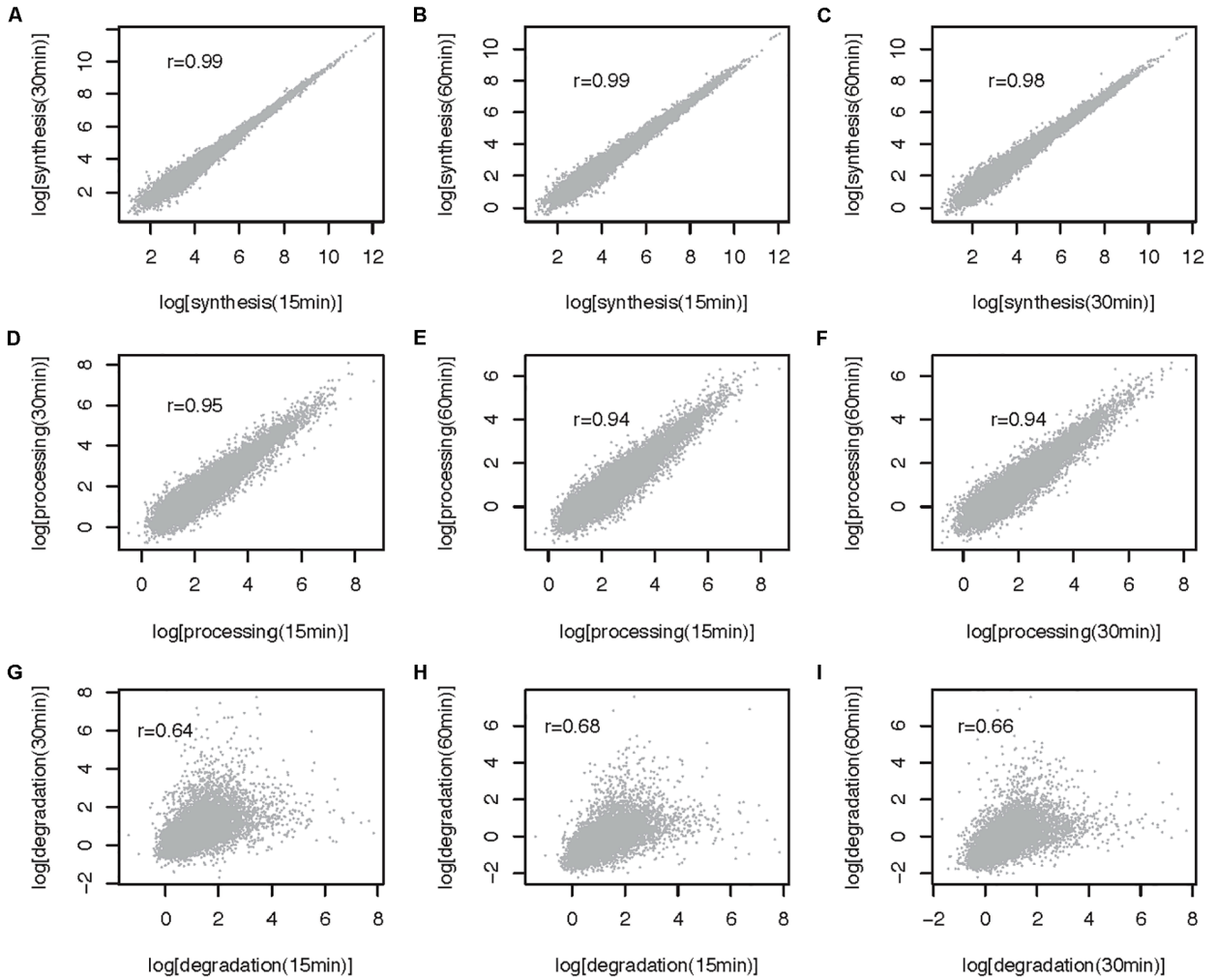

FIGURE 3 | Comparison of RNA metabolic rates obtained after 15, 30, and 60 min of 4sU labeling for multiexonic mESC expressed transcripts (32 641 transcripts). **(A–C)** All against all comparison of synthesis rates (log, min^{-1}). **(D–F)** All against all comparison of processing rates (log, min^{-1}). **(G–I)** All against all comparison of degradation rates (log, min^{-1}). Each point represents one transcript. Pearson correlation (r) for each comparison is noted on the top left-hand side of the relevant panel.

$NOTE_{25}$: Gene specific $4sU_{rel.-enr}$ are inversely correlated with transcript half-life.

RESULTS

We performed a Dot-Blot, as described in section C, to qualitatively assess 4sU incorporation in mESCs following labeling with 200 μM 4sU for 15 min. As expected, no clear signal was detected in the untreated mESC control. In contrast, RNA extracted from cells labeled with 4sU showed significant enrichment in biotinylated residues, as probed using streptavidin horseradish-peroxidase (**Figure 2A**). Following streptavidin-based separation of 4sU labeled RNA, we obtain ∼1.5% relative to input biotinylated RNA ($Mass_{newly-transcribed\,RNA}(ng)/Mass_{biotinylated\,RNA}(ng)*100$) in range with what was previously reported (Marzi et al., 2016). We thus conclude that 15 min of incubation with 200 μM 4sU is sufficient to label a sizable RNA fraction in mESCs.

Subsequently we measured the relative 4sU enrichment ($4sU_{rel.-enr}$) (**Figure 2B**), which correlates with transcript stability (Rabani et al., 2011; Freimer et al., 2018), for a subset of genes with different reported stabilities. As previously reported (Marzi et al., 2016), following normalization, we find that $4sU_{rel.-enr}$ is inversely correlated (R^2 = 0.80) (**Figure 2C**) with previously published stabilities obtained using transcriptional inhibition in mESC (Sharova et al., 2009).

The method described here can be used to estimate RNA metabolic rates at the transcript and transcriptome wide level. To illustrate the use of the approach genome wide and to gain insights into the impact of 4sU pulse duration on rate estimates, we extracted and sequenced RNA from mESCs pulsed for 15, 30, and 60 min and estimated rates using INSPecT [(de Pretis et al., 2015), see

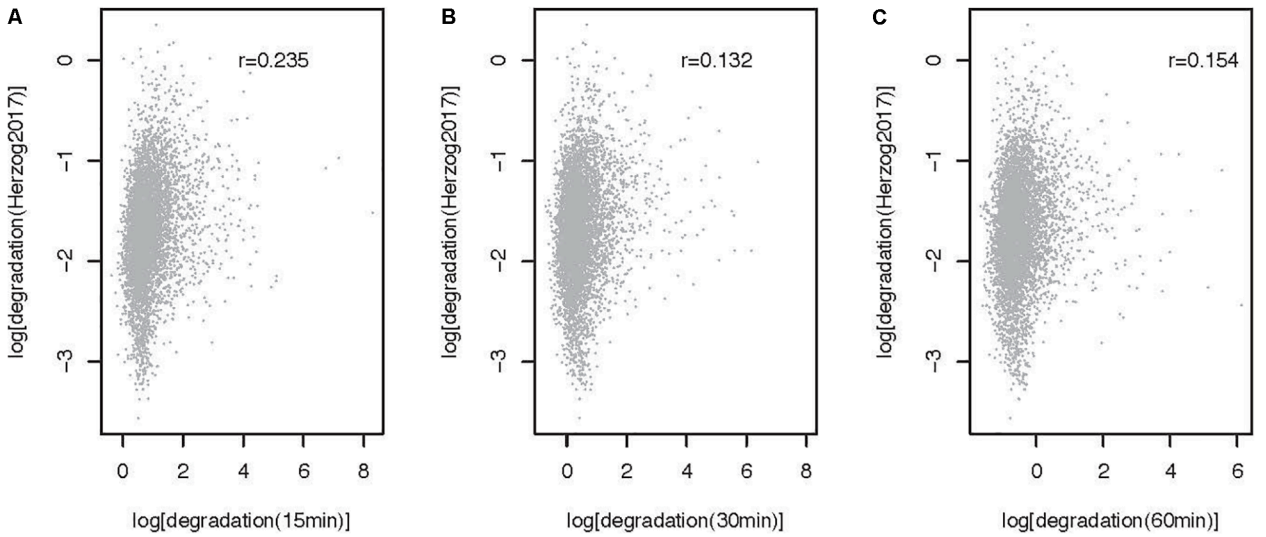

FIGURE 4 | Comparison of degradation rates obtained after 15, 30, and 60 min of 4sU labeling with rates obtained using SLAM-seq (5353 transcripts). Comparison of SLAM-seq based degradation rate (Herzog et al., 2017, log, cpm/h) and rates obtained after **(A)** 15, **(B)** 30, and **(C)** 60 min of labeling with 4sU. Each point represents one transcript. Pearson correlation r for each comparison is noted on the top right-hand side of the relevant panel.

Supplementary Note for a description of the methods]. Synthesis and processing rates are minimally impacted by 4sU pulse length, as highlighted by the high correlation we obtain between estimates obtained from the different experiments (Pearson correlation, $0.99 < r < 0.94$; **Figures 3A–F**). In contrast, degradation rates estimated for cells treated with 4sU for different durations are significantly, yet less well-correlated that are the other two RNA metabolic rates (Pearson Correlation, $0.64 < r < 0.68$, **Figures 3G–I**). This is expected because maximum sensitivity in decay rate estimates, for pulse-only experiments, is achieved using labeling times similar to the transcript half-life (Russo et al., 2017). Given that higher correlation is obtained between rates estimated for the shortest pulse duration (Pearson $r = 0.235$, **Figure 4**) and pulse-chase degradation rates estimated in mESCs using SLAM-seq (Herzog et al., 2017), we conclude that shorter pulse durations provide more accurate genome wide estimates of transcript half-lives in mESCs. The significant, yet relatively low, correlation obtained by this and published data may in part result from the use of different experimental approaches and the relatively simple assumptions, which may not faithfully recapitulate the kinetics of RNA metabolism, used by different algorithms (Duffy et al., 2019).

Furthermore, analysis of the expression of a subset of pluripotency and differentiation markers highlights that longer pulse durations lead to more pronounced differences in these markers' expression (**Supplementary Note Figures 1B,C**). The small, yet significant, decrease we specifically observe in *Nanog* expression after 120 min of pulse with 4sU further underlines the advantages of using short 4sU pulse durations in mESC.

DISCUSSION

Gene expression is controlled by the rates at which genes are transcribed, processed, and degraded. Perturbations to any of these processes can result in changes in gene product abundance. Methods that capture the dynamic processes that control gene expression are therefore paramount to understand gene regulation. Approaches based on metabolic labeling of RNA using modified ribonuleosides, such as 4sU, bypass many of the limitations of common transcription inhibition-based approaches and are now the gold-standard in the field. However, the implementation of 4sU metabolic labeling in different cells has also brought to light some of its pitfalls. For example, differences in doubling times between different cell types, result in variation in 4sU incorporation and impact the methods' sensitivity (Russo et al., 2017; Duffy et al., 2019). As a consequence, labeling duration and 4sU concentration must be optimized for different cell types (Dolken et al., 2008; Duan et al., 2013; Borowski and Szczesny, 2014).

Until recently, isolation of 4sU-incorporating newly synthetized RNA from preexisting RNA has been achieved through biochemical enrichment (**Figure 1**). This enrichment step adds experimental complexity and technical variability between replicates, which in turn decreases the accuracy of the rate estimates (Garibaldi et al., 2017; Duffy et al., 2019). The recent development of chemical-based nucleotide conversion methods, such as SLAM-seq, bypass streptavidin-dependent enrichment and provide promising alternatives to the classical approaches (Herzog et al., 2017). These methods are less labor intensive and are more technically robust. The absence of an enrichment step also ensures maintenance of the cellular ratio of newly transcribed and preexisting RNA, which in turn minimizes

the requirement for normalization and simplifies computational rate inference (Uvarovskii et al., 2019).

Since its development, SLAM-Seq has been used to infer metabolic RNA rates in a variety of cellular contexts (for example, Herzog et al., 2017; Matsushima et al., 2018; Muhar et al., 2018), but still relatively little is known about how enrichment-based and chemical conversion-based methods compare. One exception, is a recent report that suggests that streptavidin-based enrichment may be preferred when using short labeling times, which is generally advised for accurate rate inference of very short or long-lived transcripts (Uvarovskii et al., 2019). Since short 4sU labeling duration results in relatively fewer labeled transcripts, in the absence of an enrichment-step, most sequencing reads will map to unlabeled preexisting RNA and will be biased towards highly expressed transcripts with slow transcription rates (Uvarovskii et al., 2019).

Because isolation of newly transcribed and preexisting RNA fractions in chemical-based nucleotide conversion methods relies on sequencing-based identification of converted sites, this method can seldomly be used to investigate the metabolic RNA rates of individual genes. This reliance on RNA sequencing, also limits the possibility of implementing quality controls prior to transcriptome-wide expression analysis. In contrast, streptavidin-dependent selection approach can be easily quality controlled, and allow testing of rates for individual transcripts as well as transcriptome wide.

The protocol described here provides guidelines for establishment of 4sU metabolic labeling in mESC, and can be adapted to other cell types and experimental designs, underlining the versatility of the technique.

DATA AVAILABILITY STATEMENT

The raw sequencing data is available on the NCBI Gene Expression Omnibus (GEO) under accession number GSE111951.

AUTHOR CONTRIBUTIONS

AM and AB designed the study, wrote the manuscript, read and agreed on the last version of this manuscript. AB performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00097/full#supplementary-material

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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.

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REVIEW
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A New World of Biomarkers and Therapeutics for Female Reproductive System and Breast Cancers: Circular RNAs

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As one of the most recently (re)discovered types of non-coding RNAs (ncRNA), circular RNAs (circRNAs) differentiate from other ncRNAs by a specific biogenesis, high stability, and distinct functions. The biogenesis of circRNAs can be categorized into three mechanisms that permit the back-splicing reaction: exon-skipping, pairing of neighboring introns, and dimerization of RNA-binding proteins. Regarding their stability, circRNAs have no free ends, specific to linear RNA molecules, prompting a longer half-life and resistance to exonuclease-mediated activity by RNase R, bypassing the common RNA turnover process. Regarding their functions, circular transcripts can be categorized into four broad roles: miRNA sponging, protein binding, regulation of transcription, and coding for proteins and peptides. Female reproductive system (including mainly ovarian, corpus, and cervix uteri cancers) and breast cancers are the primary causes of death in women worldwide, accounting for over 1,212,772 deaths in 2018. We consider that a better understanding of the molecular pathophysiology through the study of coding and non-coding RNA regulators could improve the diagnosis and therapeutics of these cancers. Developments in the field of circRNA in regard to breast or gynecological cancers are recent, with most circRNA-related discoveries having been made in the last 2 years. Therefore, in this review we summarize the newly detected roles of circRNAs in female reproductive system (cervical cancer, ovarian cancer, and endometrial cancer) and breast cancers. We argue that circRNAs can become essential elements of the diagnostic and therapeutic tools for female reproductive system cancers in the future.

Keywords: circular RNAs, cancer, cancer therapy, gynecological cancer, breast cancer, female reproductive system

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INTRODUCTION

Contrary to the "central dogma of biology" described by Francis Crick (1970) in which information passes from DNA to RNA and finally to protein, non-coding RNAs (ncRNAs) usually do not participate in protein synthesis (Fabbri et al., 2008, 2019; Bayraktar et al., 2017; Dragomir et al., 2018). Despite not carrying any coding sequences, ncRNAs are well studied across multiple disease disciplines and are sub-classified as microRNAs (miRNAs), transcribed pyknons, small nucleolar RNAs, PIWI-interacting RNAs, long non-coding RNAs, and circular RNAs (circRNAs) (Maxwell and Fournier, 1995; Siomi et al., 2011; Spizzo et al., 2012; Bartel, 2018; Zhang Z. et al., 2018; Dragomir et al., 2019). Among these diverse sub-classes of ncRNAs, circRNA transcripts are the newest addition, recently emerging as a novel class of endogenous RNAs that exist ubiquitously in mammalian cells (Bolha et al., 2017; Dragomir and Calin, 2018a). CircRNAs were primarily detected as viruses in 1970 by using electron microscopy; in 1979 researchers found them to exist in eukaryotic cells (Sanger et al., 1976; Hsu and Coca-Prados, 1979; Chen L. et al., 2015). In 2012, Salzman et al. found abundant circRNA transcripts from different human genes, showing that exons scramble in a non-canonical order and stabilize in a circular conformation (Salzman et al., 2012). Today, liquid biopsies for clinical trials are conducted based on the stable existence of circRNAs in human tissues and fluids: serum and urine (Esteller, 2011).

As one of the most recently discovered ones in the ncRNA world, circRNAs differentiate from other ncRNAs by a specific biogenesis, high stability, and functions. Generally, the biogenesis of circRNAs can be categorized into three distinct mechanisms that permit the back-splicing reaction: exon-skipping, pairing of neighboring introns, and dimerization of RNA binding proteins (the last two being direct back-splicing biogenesis mechanisms) (Li et al., 2018). These varied mechanisms lead to exonic, intronic, and exon-intron circRNAs. Present in a circular form with no free ends specific for linear RNA molecules, circRNAs are more resistant to the enzymatic activity of RNase R, bypassing common RNA turnover process (Suzuki and Tsukahara, 2014). The advantage of this longer life span compared to their messenger RNA (mRNA) counterparts makes circRNAs attractive diagnostic and therapeutic tools in the future. The specific characteristics of circRNA have already been exploited in developing biomarkers for the diagnosis and screening of different pathologies such as atherosclerosis, prion disease, neurological disorders, and human cancers (Braicu et al., 2019).

Recent studies outlined several important roles of circRNAs in different molecular biology pathways: miRNA sponges, regulators of RNA binding protein (RBP), regulators of transcription, and coding for proteins and peptides (Zheng Q.P. et al., 2016; Peng et al., 2017; Zang et al., 2018). Because of their tremendous activities in important genetic pathways, especially oncology, scientists can envision two different therapeutic potentials of circRNAs: inhibiting the circRNAs that are carcinogenic and overexpressed in tumor tissues or restoring circRNAs with tumor suppressor functions that are down-regulated in the tumor (Dragomir and Calin, 2018b). There are many more mechanisms and functions of this new class of transcript yet to be learned, however, with the vast development of computational strategies, the kernels of circRNAs in cancer biology are being discovered.

THE BIOGENESIS OF circRNAs

CircRNAs are usually generated from pre-mRNAs, process-facilitated by RNA Polymerase II (Pol II) (Chen, 2016). What makes circRNAs so special is the covalent closed loop, without poly(A) tails at 3′ end, that usually decides the fate of many RNA transcripts (Jeck and Sharpless, 2014). Interestingly, a high degree of conservation in circRNAs gene expression is found across eukaryotic species (Memczak et al., 2013; Wang et al., 2014). Although most of them are not well expressed, there are multiple circRNAs more abundantly present than their linear mRNA analogs. The formation of circRNAs stems from intronic, exonic, and intergenic regions, or even 5′ and 3′ untranslated segments (Memczak et al., 2013; Zhang et al., 2013; Lei et al., 2018). In general, we categorize circRNAs into three types: exonic, intronic, and exon-intron circRNAs, based on their distinct composition and circularization mechanism (Xu S. et al., 2018). The splicing regulatory mechanisms of circRNA biogenesis are diverse from the linear isoforms. Although there remain unanswered clarifications about circRNA biogenesis, we define the main process as back-splicing (Nigro et al., 1991). CircRNAs display distinct and diverse back-splicing events under catalysis of the canonical spliceosomal mechanism across different cell lines (Ashwal-Fluss et al., 2014; Starke et al., 2015; Wang and Wang, 2015; Zhang et al., 2016). Three models have been proposed to specify each mechanism of circRNA formation: exon-skipping, intron pairing, and RNA-binding protein interactions (Vicens and Westhof, 2014; Barrett et al., 2015; Dong et al., 2017).

The first model that can give rise to back-splicing is exon skipping, in which one or multiple exons of the mature mRNA will be missing. In this model, the lariat-driven circularization proceeds as two non-adjacent exons join together, finally producing a mRNA with skipped exons, a circular RNA transcript and a lariat structure. Additionally, intronic lariats can form intronic circRNAs (ciRNAs) if these circular loops escape from the activity of debranching enzyme (DBR1 debranching RNA lariats 1). The existence of ciRNAs depends on a 7-nt GU-rich motif, located in the proximity of the 5′ splice site and a 11-nt C-rich motif close to the branchpoint (Zhang et al., 2013; Kristensen et al., 2019; **Figure 1A**). The relationship between the circRNA and exon skipping transcription has been demonstrated by Jeck et al.; his group has characterized non-colinear exons of more than 25,000 different RNA species in human fibroblasts via high-throughput sequencing that follows this mechanism (Jeck et al., 2013; Barrett et al., 2015).

The second biogenesis mechanism of circRNAs is intron pairing-driven circularization. In this biogenesis mechanism, two introns flanking the exon/exons of a pre-mRNA have a structure capable of joining each other. The flanking introns approach each other creating a secondary conformation that makes the

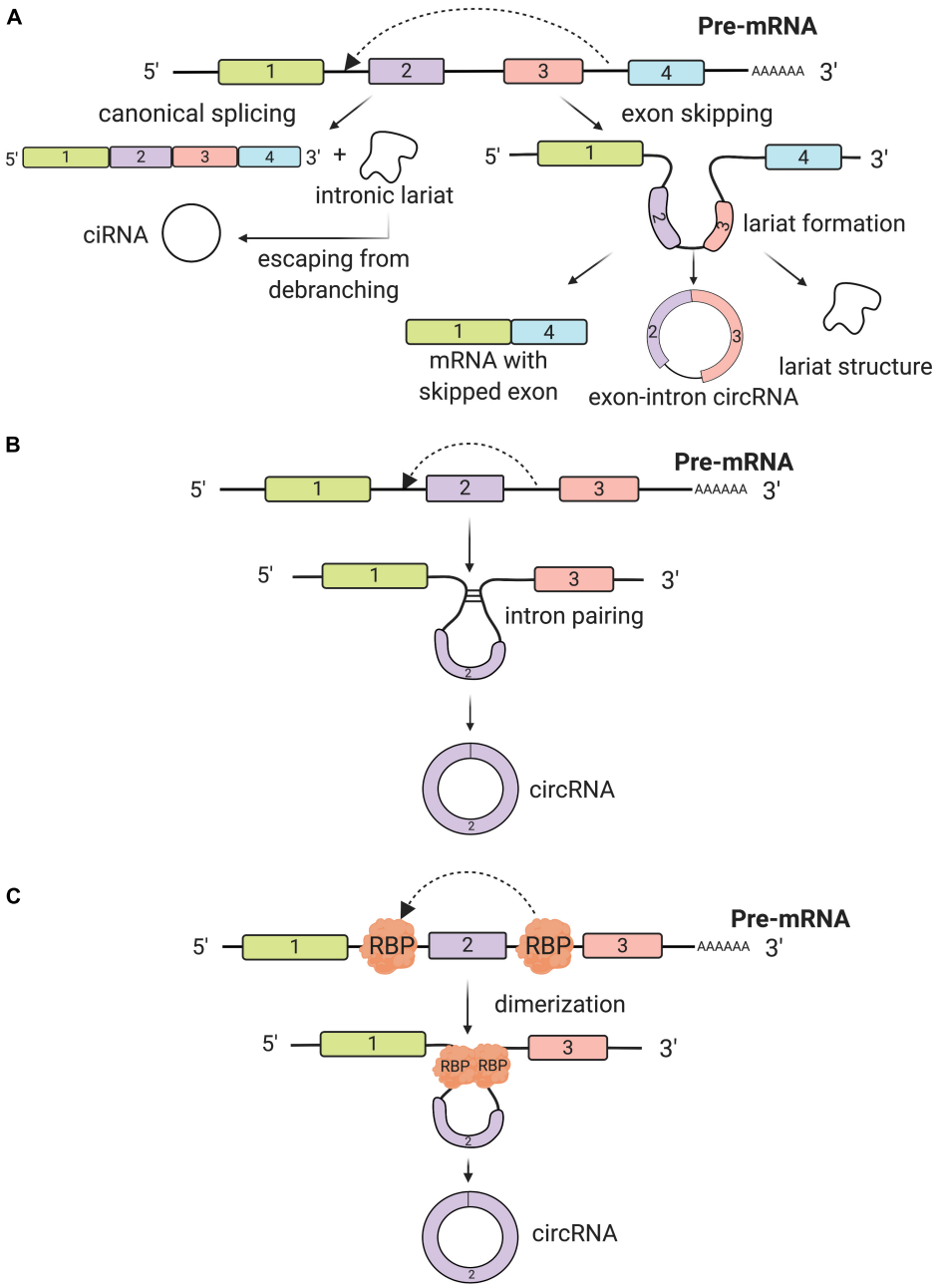

FIGURE 1 | Biogenesis of circRNAs. **(A)** The back-splicing process can take place because of exon skipping mechanism, which leads to lariat formation. This process is a non-canonical splicing pathway and three different products are synthesized: a circRNA, a mRNA with skipped exons and a lariat structure. Additionally, intron lariats, by escaping from debranching, can form intronic circRNAs (ciRNAs). **(B)** Back-splicing can be induced by intron pairing (often Alu repeats). Introns covalently bind together and a circRNA is synthesized. **(C)** RNA binding proteins (RBP) bind usually introns flanking the exon(s) that will form the circRNA. RBP dimerize promoting the back-splicing process.

splice sites possible to carry on back-splicing (**Figure 1B**). Most of the intron-pairing patterns are promoted by ALU repeats. By using the bioinformatics database from UCSC, Ivanov et al. predicted human circRNAs genome-wide, based on the sequences correlated with these ALU consistent repeats (Ivanov et al., 2015). In a follow-up study, Zhang et al. (2014) found that the length of flanking introns does not necessarily control the biogenesis of circRNAs. However, the more extended length of introns, the more chances for them to have more ALU elements, consequently enhancing exon circularization (Zhang et al., 2014). Adenosine deaminase 1 acting on RNA (ADAR1) is involved in the intron-pairing process of circRNA formation. Known to interact with ALU repeats, ADAR can decrease the pairing activity of ALU repeats, which prevents the formation of

circRNAs (Athanasiadis et al., 2004). In another study, ADAR1 was proven to be a double-stranded RNA (dsRNA) binding protein that interrupts miRNA processing (Chen T. et al., 2015). Therefore, there is a possibility that this protein factor regulates circRNA formation through a direct mechanism of dsRNA binding activity.

Thirdly, another circRNA formation mechanism is by RNA-binding proteins (RBPs). This mechanism involves protein factors that are able to bind to pre-mRNAs to connect the flanking introns together. This process is induced by protein dimerization, which creates an RNA loop. One of the most popular RBPs responsible for circRNA biogenesis is muscleblind like splicing regulator 1 (MBNL1) protein (Chen and Yang, 2015). CircRNA MBL/MBNL1 contains conserved MBL binding sites on its own so that it is easily bound to MBL protein (Ashwal-Fluss et al., 2014; Du et al., 2017; Taylor et al., 2018). This binding interaction promotes circMBL biosynthesis, and the MBL levels are crucial in determining the circularization rates of bracketed exons. MBNL1 proteins tie to neighboring introns of their own pre-mRNA and by dimerizing they link the introns together and prompt circularization (**Figure 1C**). There are also other important RBPs controlling circRNA biogenesis, such as nuclear factor 90 and 110 (NF90/NF110) (Li et al., 2017), QKI (Conn et al., 2015), and FUS (Errichelli et al., 2017), all of which promote the back-splicing process.

Over the past decade, extensive research on circRNA biogenesis with various proposed mechanisms was carried out. Although circRNAs were first believed to be just transcriptional noise from the RNA splicing process, more and more discoveries have confirmed that they have a strictly regulated biosynthesis. However, the biogenesis is not yet fully characterized, which opens broader space for researchers to carry out further investigations. More research is needed in the future to decipher the multiple aspects of circRNA biogenesis so that we can better categorize and detect them via computational genomic strategies. The reasons and mechanism behind how different circRNAs are formed are also vital in studying their relationship with other gene targets for different diseases such as cancer.

FUNCTIONS OF circRNAs

A general and in-depth overview of the characteristics and functions of circRNAs is still lacking. However, the high degree of conservation between different species may suggest some important roles in the physiological cellular mechanisms (Wang et al., 2014). CircRNAs' transcriptional expression is cell-specific and differentially detected between healthy and disease samples, which turns them into a potential candidate of illness-related biomarkers (Salzman et al., 2013). Also, circRNAs' functionality has been suggested by their long half-life compared to other RNA counterparts. Endogenous circRNAs lack the 5′ and 3′ ends due to circularization, so they can escape from the exonuclease-mediated degradation, being actively resistant to multiple RNA turnover mechanisms (Enuka et al., 2016; Kaczmarek et al., 2017). Additionally, for some circRNAs, complex *in vivo* studies were performed and proved their functionality. Recently, a CDR1 as knockout mouse model was developed and showed that this circRNA binds miR-7 and miR-671 and deregulates their expression *in vivo*, leading to impaired brain function (Piwecka et al., 2017). Such *in vivo* models are highly necessary to understand the complex functions of circRNAs in female reproductive system and breast cancers.

With all of these arguments, scientists are more objective that circRNAs actually carry out important roles in regulating different molecular pathways by four possible functions: miRNA sponging, protein binding, direct/indirect regulation of transcription, or coding for proteins and peptides.

miRNA Sponging

One of the most studied functions of ncRNAs is miRNA sponging, defined as the anti-sense partial complementarity interaction between a ncRNA (other than a miRNA) and a miRNA. Belonging to the small ncRNAs class, miRNA down-regulates gene expression at mRNA level (Calin and Croce, 2006; Friedman et al., 2009; Fabbri and Calin, 2010; Almeida et al., 2012; Dragomir et al., 2018). Mathematical modeling indicated that the miRNA sponging depends on the intracellular mobility mechanism of miRNAs, which is characterized to be of intermittent active transport type (Vasilescu et al., 2016). The miRNA–circRNA interaction was brought into attention due to its complex cascade of gene expression regulation. One of the first discoveries on circRNA as miRNA sponging was from Hanssen's lab, when they found more than 70 conserved miRNA interaction sites (for the same miRNA) on ciRS-7 (CDR1as) (Hansen et al., 2013). Because of their specific structure, circRNAs can stay away from miRNAs' destabilization and degradation via miRNA-mediated deadenylation (Hansen et al., 2011). CircRNAs can block the binding of miRNA base-pairing to its target mRNA. By sequestering the negative regulatory activities of miRNAs on mRNAs, circRNAs indirectly affect the expression level of these translational brake (**Figure 2A**). Salmena et al. (2011) and Tay et al. (2014) also suggested mRNAs and circRNAs compete with each other for binding the same miRNAs, via miRNA response elements (MREs).

Despite being considered a classical model of circRNA functions, scientists still raise many arguments regarding miRNA sponging as a general function of all circRNAs. Some recent studies lean toward a controversial view, that some circRNAs cannot always act as a miRNA sequester. For example, Militello et al., using computational algorithms, showed that only two circRNAs out of 7112 human circRNAs have more predicted miRNA-binding site than expected by chance (Militello et al., 2017). However, more and more concrete evidence on individual circRNAs sponging miRNAs are validated in several cancer models. There are up to 822 studies available on PubMed based on the search terms "circRNAs," "miRNA sponge," and "cancer" accessed by July 2019. Hence, in this review, we mostly analyze the roles of circRNAs in gynecological and breast cancers via miRNA sponging mechanism.

Protein Binding

In addition to sponging miRNAs, circRNAs also bind to different RBPs and have different potential roles: inhibiting

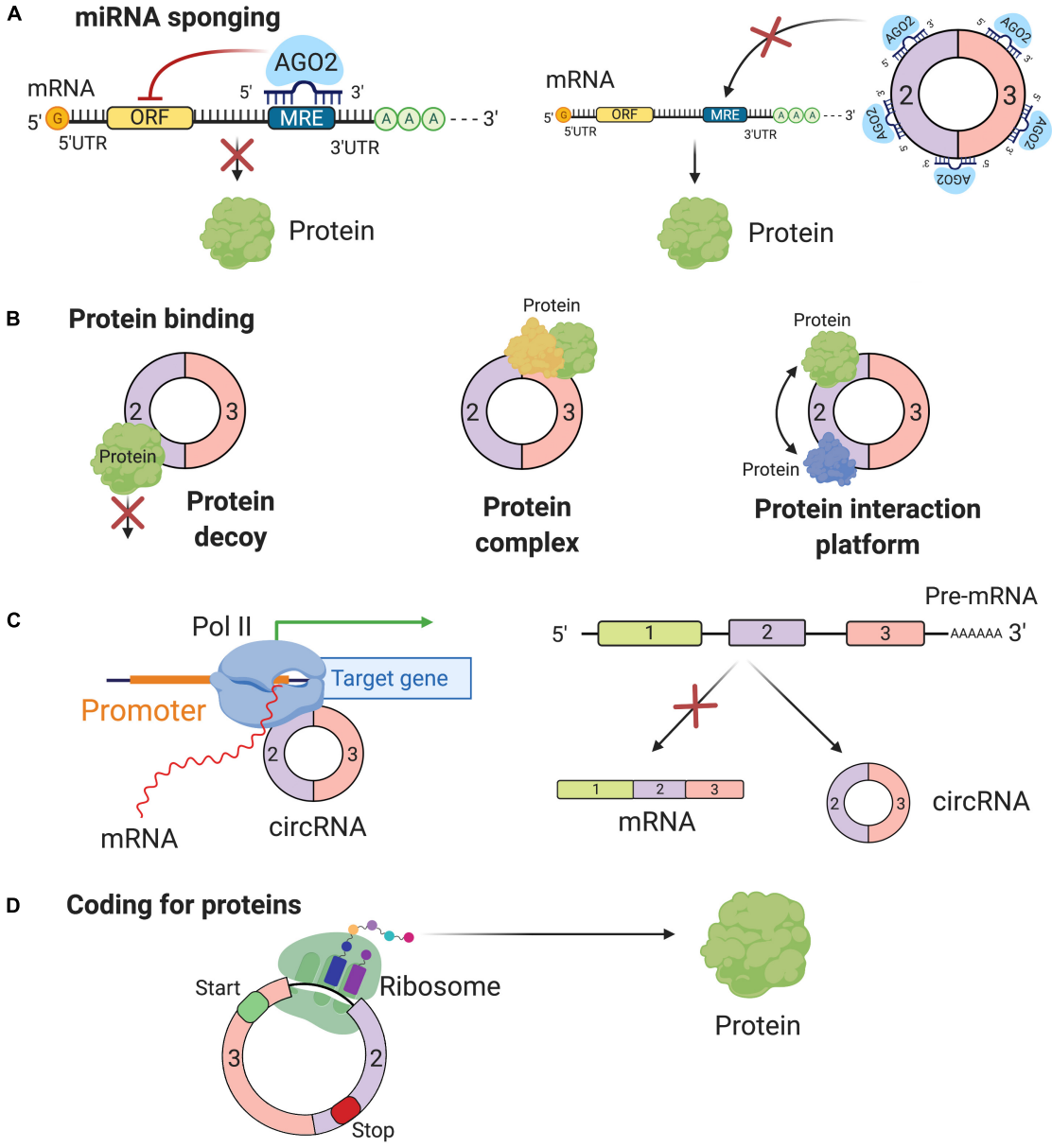

FIGURE 2 | Function of circRNAs. **(A)** The most common function of circRNAs is miRNA sponging. By sponging miRNAs, circRNAs inhibit miRNA capacity to perform their post-transcriptional inhibition. **(B)** By binding proteins circRNAs can block their function (protein decoy), can build protein complexes, which include multiple proteins and have complex regulatory functions or are scaffolds for protein-protein interactions. **(C)** CircRNAs can affect the biogenesis of other genes by directly interacting with the promoter region at the DNA level or simply the preferential biogenesis of circRNAs inhibits the formation of functional mRNAs. **(D)** Recently, it was reported that some circRNAs have coding potential and are translated into proteins.

the function of proteins (protein decoys), facilitating the formation of protein complexes, and permitting the interaction between different proteins (interaction platform) (Zang et al., 2018; **Figure 2B**). Several well-known circRNAs associate with RBPs; the best-known examples are circ-MBL, circ-Foxo3, and hsa_circ_0000020 (Ashwal-Fluss et al., 2014; Du et al., 2016; Dudekula et al., 2016).

Ashwal-Fluss demonstrated that the splicing factor muscleblind (MBL/MBNL1) circRNA (circ-MBL) and its neighboring introns have conserved MBL interaction sites. Specifically, circRNAs can get tuned in their biosynthesis, depending on the level of MBL proteins. There is convincing evidence showing that circRNAs production is co-transcriptional and competes with the canonical pre-mRNA splicing, suggesting its potential role in gene regulation (Ashwal-Fluss et al., 2014).

Circ-Foxo3 is another circRNA that has a capability of interacting with different proteins related to cell progression. Two common target proteins of circ-Foxo3 are cyclin-dependent

kinase inhibitor 1 (or p21) and cyclin-dependent kinase 2 (CDK2). CDK2 coordinates the activities of G1/S and S/G2 changeover in the cell cycle (Peng et al., 2016). In contrast, p21 retards the cell cycle progression by restraining various interactions of cyclin A and cyclin E (Karimian et al., 2016). Circ-Foxo3 forms a complex together with these two proteins, which facilitates the communication between p21 and CDK2, inhibiting the normal function of the latter (Du et al., 2016).

Using computational methods, Dudekula et al. (2016) confirmed the footprints of flanking sequences of hsa_circ_0000020 on binding to some RBPs, including HuR, FMRP, and ElF4A3 at a very high frequency and hypothesized that this circRNA may act as a protein decoy. More than 117,000 circRNAs were found to bind with eukaryotic translation initiation factor 4A3 (ElF4A3). However, most of the uncertainties are based on how this binding between circRNAs and RBPs contribute to RBP-related functions.

Regulation of Transcription

CircRNAs also play a role in transcription regulation, enhancing transcription at the transcriptional level. Focusing on transcriptional level, circRNAs have two regulatory paths: one at the initiation step and one at the elongation step. During the initiation step, the role played by circRNAs involves the formation of the pre-initiation complex. Li et al. (2015) found two exon-intron circRNAs (EIciRNAs) exclusively localized in the nucleus, and the knockdown assay of these circular transcripts results in decreased levels of their parental genes. Subsequent experiments revealed a particular collaboration *in cis* between the EIciRNAs and U1 snRNA, which forms a complex together with Pol II at the DNA level at the site of the promoter. During the elongation phase, the interaction of Elongating Polymerase II with ciRNAs was described. CiRNAs accumulate at their site of transcription and increase parental genes transcription elongation by interacting with RNA polymerase II (Zhang et al., 2013). These features distinguish them from circRNAs, localized in the cytoplasm, functioning mainly as miRNA sponges. Additionally, the biogenesis of circRNAs via exon skipping can be seen as a passive function of the circular transcripts. The production of a circRNA also leads to the synthesis of a mRNA, which misses one or multiple exons and most likely alters its coding capacity (Dragomir and Calin, 2018a; **Figure 2C**).

Coding for Proteins or Peptides

In general, the translation of mRNAs into proteins begins with the recognition in 5′ UTR of the initiation codon and ends with in the 3′ region the stop codon (Hershey et al., 2012). Since circRNAs do not have this initiation codon, it was long thought that they could not be translated. Most probably, circRNAs do not need large polyribosomes, but just a limited number ribosomes that are sufficient to translate the circRNAs into peptides and proteins (Panda et al., 2017; **Figure 2D**). Confirming these statements, several studies reported the presence of small peptides, often less than 100 amino acids encoded by supposedly non-coding regions of the genome. Among these "non-coding" regions, some are recognized as circRNAs and contain short ORFs, similar to lncRNA, which can actually generate small proteins or micropeptides (Panda et al., 2017; Yang Y. et al., 2017; Pan et al., 2018). Furthermore, the origin of these circRNAs, mostly exonic, and their cellular compartmentalization, mostly cytoplasmic, add evidence for their translation into functional peptides (Jeck et al., 2013).

circRNAs AND FEMALE REPRODUCTIVE SYSTEM AND BREAST CANCERS

Female reproductive system (including cervical cancer (CC), ovarian cancer (OC), and endometrial cancer (EC), as well as more uncommon neoplasia, such as vulvar or vaginal cancers, categorized also as gynecological cancers) and breast cancers (BC) are a leading cause of death worldwide. Their incidence increases continuously and is expected to reach 109,000 women for gynecological cancers and 268,600 for BCs in 2019 in the United States. Mortality is also significant among these patients, although it varies between countries. In the United States, <41,760 women will die from BC and 33,100 from gynecological cancer in 2019 according to the American Cancer Society (Bray et al., 2018).

These cancers have a strong genetic predisposition. Hereditary BC and OC are syndromes, with an autosomal-dominant pattern of transmission, which involves an increased predisposition to OC, BC, or both. BRCA1 and BRCA2 are the genes most often found mutated and increase the risk for early age onset BC and/or OC, and often after a first cancer a second cancer is common (Miki et al., 1994; Wooster et al., 1995; Hartmann and Lindor, 2016). Recent data shows that not only other coding genes (Calin et al., 2005), but also non-coding ones, including circRNAs, play a role in the genetic predisposition for breast and reproductive system cancers (**Table 1**).

Breast Cancer

Breast cancers remains the most frequent form of women's cancer and ranks second for cancers related to death in women (Bray et al., 2018). Emerging from the cells in the breast secretory system, made of lobules and galactophoric channels, BCs are mainly ductal or lobular adenocarcinomas. The development of targeted anti-HER2 therapies has made a significant improvement in the prognosis of metastatic BC, and the emergence of immunotherapy raises hopes for a better management of these patients (Libson and Lippman, 2014; Nanda et al., 2016). Despite this, BC still remains an important challenge for physicians and scientists in the battle of finding the most efficient diagnosis and treatments for BC patients. Most deaths caused by BC stem from the relapse and metastasis to other distant organs when conventional treatments such as surgery or curative chemotherapy are no longer an option–only 26% of stage 4 patients reach 5 years of survival (Braden et al., 2014; Peart, 2017).

Therefore, it is urgent to investigate the cascade of molecular events leading to breast malignancies, especially before metastasis development, in order to target the tumors as soon as possible. Understanding the mechanistic basis of genetic and epigenetic

TABLE 1 | Summary of deregulated circRNAs in breast and female reproductive system cancers.

Disease	circRNA	Target	Up/Down	Function	References
Breast cancer	circFBXW7	miR-197-3p and encodes a tumor suppressor protein, FBXW7-185aa	Down	Tumor Suppressor	Ye et al., 2019a
	circ-ABCB10	miR-1271	Up	Oncogene	Liang et al., 2017
	circ_0103552	miR-1236	Up	Oncogene	Yang L. et al., 2019
	hsa_circ_0004771	miR-653 and indirectly ZEB2	Up	Oncogene	Xie R. et al., 2019
	hsa_circ_0072309	miR-492	Down	Tumor Suppressor	Yan et al., 2019
	hsa_circ_001783	miR-200c-3p and indirectly ZEB1, ZEB2, and ETS1	Up	Oncogene	Liu et al., 2019
	circ_0005230	miR-618 and indirectly CBX8	Up	Oncogene	Xu Y. et al., 2018
	hsa_circ_00052112	miR-125a-5p and indirectly BAP1	Up	Oncogene	Zhang H.D. et al., 2018
	hsa_circ_0007534	miR-593 and indirectly MUC1	Up	Oncogene	Song and Xiao, 2018
	hsa_circ_0001982	miR-143	Up	Oncogene	Tang et al., 2017
	circGFRA1	miR-34a and indirectly GRAF1	Up	Oncogene	He et al., 2017
	circ-Foxo3	miR-22, miR-136*, miR-138, miR-149*, miR-433, miR-762, miR-3614–5p and miR-3622b–5p and indirectly Foxo3	Down	Tumor Suppressor	Yang et al., 2016
	circANKS1B	miR-148a-3p and miR-152-3p and indirectly USF1	Up	Oncogene	Zeng et al., 2018
	circTADA2As	miR-203a-3p and indirectly SOCS3	Down	Tumor Suppressor	Xu et al., 2019
	circAGFG1	miR-195-5p and indirectly CCNE1	Up	Oncogene	Yang R. et al., 2019
Cervical cancer	circAMOTL1	miR-485-5p and indirectly AMOTL1	Up	Oncogene	Ou et al., 2019
	circE7	Encoding the viral oncoprotein E7	Up	Oncogene	Zhao J. et al., 2019
	hsa_circ_0018289	miR-497	Up	Oncogene	Gao et al., 2017
	circEIF4G2	miR-218 and indirectly HOXA1	Up	Oncogene	Mao et al., 2019
	hsa_circRNA_101996	miR-8075 and indirectly TPX2	Up	Oncogene	Song et al., 2019
	hsa_circ_0000263	miR-150-5p and indirectly MDM4	Up	Oncogene	Cai et al., 2019
	hsa_circ_0067934	miR-545 and indirectly EIF3C	Up	Oncogene	Hu et al., 2019
	circRNA8924	miR-518-5p and miR-519-5p	Up	Oncogene	Liu J.M. et al., 2018
	hsa_circ_0023404	miR-136 and indirectly TFCP2	Up	Oncogene	Zhang J.H. et al., 2018
	circRNA-000284	miR-506 and indirectly Snail-2	Up	Oncogene	Ma et al., 2018
	circSMARCA5	miR-620	Down	Tumor Suppressor	Tian and Liang, 2018
	circ-ATP8A2	miR-433 and indirectly EGFR	Up	Oncogene	Ding and Zhang, 2019
Ovarian cancer	CDR1as	miR-1270	Down	Tumor Suppressor	Zhao Z. et al., 2019
	circHIPK3	Not confirmed	Down	Tumor Suppressor	Teng et al., 2019
	circRNA1656	N/A	Down	Tumor Suppressor	Gao et al., 2019
	circ-ITCH	miR-145 and indirectly RASA1	Down	Tumor Suppressor	Hu et al., 2018
	hsa_circ_0061140	miR-370 and indirectly FOXM1	Up	Oncogene	Chen Q.Z. et al., 2018
	circEPSTI1	miR-942 and indirectly EPSTI1	Up	Oncogene	Xie J. et al., 2019
Endometrial cancer	hsa-circ-0039659	hsa-miR-542-3p and hsa-let-7c-5p	Up	Oncogene	Ye et al., 2019b
	circ-ZNF91	miR-23b and miR-199	Up	N/A	Chen B.J. et al., 2018

changes in BC can guide us to develop novel diagnostic and therapeutic strategies for treating this fatal disease. CircRNAs were proved to be involved in different hallmarks of BC such as proliferation, apoptosis, and activating invasion and metastasis (Zhou et al., 2019).

One of the best characterized circRNAs in BC is circFBXW7. Carrying two functions, protein coding and miRNA sponging, circFBXW7 was discovered to be down-regulated and negatively correlate with tumor development and lymph node metastases in triple-negative breast cancer (TNBC). CircFBXW7 is located mainly in the cytoplasm, suggesting its potential relation to miRNA activities. Based on computational analysis, Ye et al. (2019a) have analyzed and confirmed miR-197-3p as one target of circFBXW7. As a miRNA sponge, circFBXW7 decreases the expression level of miR-197-3p and inhibits tumor progression. In 473 TNBC samples, a Spearman correlation analysis provided a positive correlation between circFBXW7 and its host gene FBXW7, which is regulated by miR-197-3p. CircFBXW7 also encodes FBXW7-185aa protein, which up-regulates the tumor suppressor FBXW7 and degrades c-Myc oncogene, further inhibiting TNBC proliferation and migration. These data have strengthened the concept of using circFBXW7 as a potential diagnostic and therapeutic tool for TNBC patients in the future.

By using microarray analysis and RT-qPCR, Liang et al. (2017) conducted a screening on different circRNAs expressed in BC tissues and adjacent non-cancerous tissues. Across a total of 2,587 circRNAs analyzed, circ-ABC10 is overexpressed in cancerous tissues, five to ten times higher than in healthy tissues. Knockdown of circ-ABC10 prevents BC cells from proliferation and initiates apoptosis, suggesting its oncogenic characteristic.

Mechanistically, circ-ABC10 was suggested to bind miR-1271 and inhibit its functional activity. The same pattern was observed in circ_0103552, a 920-nucleotide circRNA. Yang L. et al. (2019) detected an up-regulation of its expression in BC cells *in vitro*, and associated its level with higher TNM stages and lymph node invasion in patient samples. The rate of apoptosis increases noticeably in the absence of circ_0103552, while overexpressing this circRNA leads to a boost in cell growth ability in multiple BC cell lines. This study also showed that this circRNA does not act alone, but under a negative association with miR-1236. Furthermore, Xie R. et al. (2019) used high-throughput circRNA sequencing to examine the expression level of hsa_circ_0004771. Not only being up-regulated in BC tissues, this circRNA was proved to target miR-653 and decrease its expression and inhibit its function. They showed that, if not sponged, miR-653 directly binds to 3′-UTR of the mRNA of ZEB2 (Zinc finger E-box binding homeobox 2).

Another circRNA called hsa_circ_0072309 is expressed at abnormally high levels in patients with BC and is linked to poor overall survival (OS) rates (Yan et al., 2019). In order to uncover the mechanism of hsa_circ_0072309, Yan et al. used a luciferase reporter assay to confirm the direct interactions between this circRNA and miR-492. The overexpression and knockdown experiments proved the oncogenic role of hsa_circ_0072309, endorsing the potential of using it as a biomarker for BC diagnosis. Liu et al. (2019) analyzed 923 circRNAs and 100 miRNAs with over 37,000 possible interactions. Among 923 investigated circRNAs, hsa_circ_001783 was established to have the highest rank score among all BC-associated circRNAs. Using bioinformatic data analysis and multiple biochemical tools, they also discovered that hsa_circ_001783 is necessary for BC progression and metastatic spread, mechanistically sponging miR-200c-3p. Circ_0005230 is another candidate that has a significant increase in expression when comparing both BC tissues and cell lines with normal tissue and cells, respectively (Xu Y. et al., 2018). Some clinical characteristics were taken into consideration, showing that patients with raised expression of circ_0005230 have lower 5-year OS rates. Also functioning as a miRNA sponge, this circRNA reduces miR-618 expression, indirectly elevating the expression of CBX8.

In some circumstances, circRNAs can even induce the spreading of tumors to adjacent organs such as hsa_circ_0052112 (Zhang H.D. et al., 2018). Functioning as an oncogenic circRNA, hsa_circ_0052112 enhances tumor cells to invade and migrate by sponging miR-125a-5p that acts as a tumor suppressor, inhibiting BAP1 oncogene. Similar to hsa_circ_0052112, hsa_circ_0007534 shows an inverse expression with miR-593 (Song and Xiao, 2018). The tumor suppressive role of this circRNA was confirmed both through overexpression and knockdown experiments, which proved that hsa_circ_0007534 can function as an oncogenic regulator in BC.

Knowing that the high expression of circ-Foxo3, Foxo3, and Foxo3 pseudogene decreases cell viability, Yang et al. planned to study their roles in breast carcinoma development. They found that the mRNA of Foxo3, the circRNA of Foxo3 and Foxo3 pseudogene are regulated by eight miRNAs: miR-149*, miR-136*, miR-138, miR-22, miR-433, miR-3614-5p, miR-762, and miR-3622b-5p. Furthermore, they discovered that the ectopic expression of these three transcripts could decrease cancer cell proliferation and cancer cell survival and tumor growth, confirming their tumor suppressive role (Yang et al., 2016).

CircTADA2As were also confirmed to have a tumor suppressive role in triple negative breast cancer (TNBC). In a large cohort of BC patients, both circTADA2A-E6 and circTADA2A-E5/E6 were found to be significantly decreased, and their low levels were linked to short survival rates. Focusing on circTADA2A-E6, Xu et al. (2019) demonstrated that this circRNA suppresses cellular growth, invasion, migration, and colony formation. Furthermore, circTADA2A-E6 has been shown to complementarily bind to miR-203a-3p and inhibit its repressive function on SOCS3, inducing a less aggressive cancer.

On the contrary, Tang et al. (2017) identified hsa_circ_0001982 to be up-regulated in BC cell lines and tumors, using microarrays analysis. *In vitro* gene modulation studies showed that hsa_circ_0001982 inhibits BC cell growth, invasion, and induce cell death by sponging miR-143. Similarly, He et al. proved the oncogenic role of a circRNA called circGFRA1 in TNBC. While up-regulation of circGFRA1 is correlated with poor prognosis, its knockdown inhibits proliferation and promotes apoptosis in TNBC. To assess the mechanism behind the functionality of this circRNA, He et al. (2017) used luciferase reporter assay and concluded that circGFRA1 and GFRA1 directly bind to miR-34. Taken together, these findings suggest that circGFRA1 regulates GFRA1 expression through sequestering miR-34 and may function as a sponge, confirming its regulatory function in TNBC.

Also, in TNBC, Zeng et al. identified circANKS1B pro-metastasis effect. They first demonstrated that this circRNA is overexpressed in TNBC tumors and cell lines. Furthermore, they demonstrated that it induces epithelial-to-mesenchymal transition (EMT) promoting BC metastasis in a murine cancer model. Mechanistically, circANKS1B sponges two miRNAs: miR-152-3p and miR-148a-3p, indirectly up-regulating USF1. USF1, being a transcription factor, induces higher levels of TGF-β1, activating the pro-metastatic signaling pathway TGF-β1/Smad. In summary, these results confirm the pro-tumorigenic function of circANSK1B in BC (Zeng et al., 2018).

Another circRNA up-regulated in TNBC, circAGFG1, was shown to promote cell proliferation, mobility and invasion *in vitro*, oncogenesis, and distant metastasis *in vivo*. According to these results, the level of this circRNA correlates with pathological grade, clinical stage and poor prognosis of TNBC patient. Functional studies showed that circAGFG1 may block the function of miR-195-5p relieving its repressive effect on cyclin E1 (CCNE1) mRNA, confirming its oncogenic role (Yang R. et al., 2019).

Cervical Cancer

Often associated with HPV16 and 18 infection, CC arise from the transitional zone between the cylindrical and the squamous epithelium; most of them are squamous cell carcinomas or adenocarcinomas (Bosch et al., 1995). CC can remain loco-regional for a long time with a cervical extension from close to close, while invasive cancers are defined with basal

membrane crossing (Waggoner, 2003). As genetic and epigenetic variations can predispose to this malignant gynecological tumor (Cordeiro et al., 2018), understanding the underlying molecular mechanisms remains a necessity.

CircAMOTL1 is one of the best-characterized circRNAs in CC. CircAMOTL1 is up-regulated in CC tissues compared to healthy adjacent tissues, and its expression is especially high in metastatic tumor tissues. At a phenotypical level, circAMOTL1 induces tumor development both *in vitro* and *in vivo*. Three databases, PITA, miRmap, and microT, suggested 61 potential binding miRNAs of circAMOTL1. MiR-485-5p, a binding candidate, was found significantly down-regulated in tumor tissues, therefore suggesting that circAMOTL1 enhances oncogenic activities in CC via the circAMOTL1/miR-485-5p axis. Further experiments provided evidence that the up-regulation of circAMOTL1 induces the overexpression of its host gene, AMOTL1. With all of the supporting data, circAMOTL1 is believed to play an oncogenic role in CC (Ou et al., 2019).

Also, very well-characterized is the role of circE7 in CC. A newly published paper has described the role of this viral circRNA with coding potential. HPV16 circE7 back-splicing junction was detected and characterized using Northern blotting and inverse RT-PCR of HPV16-infected cell lines. The circRNA was shown to be essential for coding E7 oncoprotein, which induces tissue proliferation and invasion in CC *in vitro* and *in vivo*. Because HPV plays regulatory roles in transcriptional and post-transcriptional activities in response to the differentiation state of epithelial cells, this circRNA formation can affect how HPV coordinates infection and immune evasion (Zhao J. et al., 2019). From this study, it is possible to further investigate the diagnostic and therapeutic implications of circE7 in CC.

Gao et al. (2017) elucidated the molecular basis of hsa_circ_0018289 on CC tumor formation. Among 45 up-regulated circRNAs detected by microarray, hsa_circ_00018289 was the one most significantly overexpressed in 35 CC tumors compared to the adjacent normal tissues. The loss-of-function experiments revealed its function in cancer cell proliferation and invasion. Via luciferase reporter assay, Gao et al. (2017) validated that hsa_circ_0018289 targets miR-497 and suppresses its expression. A circRNA isoform of eukaryotic translation initiation factor 4γ2 (circEIF4G2) was found to be up-regulated in CC tissues and was linked to unfavorable prognosis (Mao et al., 2019). *In vivo* and *in vitro* evidence highlighted the plausibility of circElF4G2 cancerous characteristics such as cell proliferation, colony formation, and metastasis. Mechanistically, this circRNA proved to inhibit miR-218, further influencing the downstream target of the miRNA, the transcription factor homeobox A1 (HOXA1). The increasing expression level of circEIF4G2 indirectly induces the expression of HOXA1 both at transcriptional and at translational levels. Although the axis of circEIF4G2/miR-218/HOXA1 has not been yet well elucidated, early findings have shown that HOMO genes family are associated with carcinogenesis and low survival rates in CC patients (Bitu et al., 2012; Eoh et al., 2017).

Xenopus kinesin-like protein 2 (TPX2) is another example of the many indirect targets of circRNAs via miRNAs. Jiang et al. (2014) previously reported an abnormal behavior of this microtubule-associated protein in CC progression and invasion via immunohistochemistry and RT-qPCR. Because miR-8075 can inhibit TPX2, the sponging effects of hsa_circRNA_101996 on miR-8075 indirectly generates more TPX2. This molecular mechanism was further suggested by Song et al. (2019) describing how the increased level of hsa_circRNA_101996 is associated with different stages of CC and induced proliferation, migration, and invasion.

After examining the characteristics and circRNA expression, Cai et al. (2019) confirmed the inducible patterns of hsa_circ_0000263 on tumor cell growth. This circRNA can affect post-transcriptional gene regulation, especially restraining the activity of miR-150-5p, indirectly regulating murine double minute 4 (MDM4) gene expression. By inhibiting miR-150-5p, this oncogenic circRNA eventually decreases the expression of p53 tumor suppressor, because MDM4 is a critical inhibitor of p53 (Wade et al., 2013). Hsa_circ_0067934 is another circRNA that displays tumorigenic properties in CC. In CC tissues, the expression of this circRNA is significantly up-regulated and is linked to lymphatic metastases (Hu et al., 2019). Knockdown experiments of hsa_circ_0067934 validated its capacity to induce tumor proliferation, colony formation, and EMT features. By providing luciferase assay data, Hu et al. (2019) showed that this circRNA mainly targets miR-545 and down-regulates its expression. This miRNA subsequently regulates the eukaryotic initiation factor 3C (EIF3C), which has been previously reported to suppress cell growth and induce cancer cells apoptosis (Hao et al., 2015; Zhao et al., 2017). Sometimes the dysregulated expression of a circRNA can affect more than one miRNA at the same time. The sponging effects were observed both for miR-518-5p and miR-519-5p by circRNA_8924 simultaneously, which induces the aggressive characteristics of CC tumors such as metastasis (Liu J.M. et al., 2018). Similarly, hsa_circ_0023404 is up-regulated in CC and restrains the activity of miR-136 (Zhang J.H. et al., 2018). Zhang J.H. et al. (2018) further knocked down this circRNA showing that it significantly suppresses proliferation, arrests cell-cycle progression, and inhibits cell migration and invasion in CC. *In vivo* research was used to understand the function of circRNA-000284, the authors reported that when decreasing the non-physiologically high levels of this circRNA, it prevents cells from proliferating and invading to adjacent organs. Moreover, if cirRNA-000284 expression is suppressed in CC cells, cell cycle arrest is promoted in G0/G1 phase and cancer growth is slowed down (Ma et al., 2018).

CircSMARCA5 was found to be down-regulated in CC while its high-levels slow-down CC cell growth, migration, invasion, and prompt cell cycle arrest *in vivo*. Furthermore, circSMARCA5 binds to miR-620 and significantly down regulates its expression. Tian et al. have demonstrated that this circSMARCA5/miR-620 regulatory axis leads to a suppression of invasion and proliferation confirming its involvement in CC (Tian and Liang, 2018).

Conversely, circ-ATP8A2 is up-regulated in CC tumors and cell lines, suggesting an oncogenic role. According to this, the down-regulation of circ-ATP8A2 inhibits cell growth, migration, and invasion and increases apoptosis, while

overexpression circ-ATP8A2 results in a reverse phenotype. Mechanistically, circ-ATP8A2 blocks the function of miR-433, indirectly derepressing epidermal growth factor receptor (EGFR) mRNA (Ding and Zhang, 2019).

Ovarian Cancer

Ovarian cancer is one of the most lethal gynecological cancers with very low survival rates because of its deep localization, leading to late and non-specific symptoms (Holschneider and Berek, 2000). Numerous diagnoses are made at the peritoneal carcinomatosis stage where the volume of the primary tumor and its intraperitoneal extension are considerable, requiring surgical treatment and chemotherapy. Only women with a nil or minimal post-surgical tumor residue have a chance of prolonged survival (Rosen et al., 2009; Malvezzi et al., 2016). Hence, modern OC management has shifted toward developing potential biomarkers for early diagnosis, risk assessment, prediction of treatment success, and treatment toxicity (Yang W.L. et al., 2017; Zhu et al., 2019). Because OC is highly controlled by different genetic pathways with multiple molecular characteristics, it is the utmost importance to understand dysregulation in cancer cells using genome wide screening methods. Having a long half-life in body fluids and specificity in cancer, circRNAs are being actively explored as biomarkers for OC diagnosis (Memczak et al., 2015; Zhang et al., 2017).

One of the best-characterized circRNA in OC is CDR1as, which has a potential function in cisplatin chemoresistance. In this study, CDR1as was lowly expressed in cisplatin-resistant OC patient tissues. The function of CDR1as in the acquisition of cisplatin chemoresistance was more strongly validated thanks to *in vitro* and *in vivo* experiments. Bioinformatics prediction analysis by two different databases suggested miR-1270 as a molecular target of CDR1as. Consistently, this miRNA was highly expressed in cisplatin-sensitive cells, obviously opposite to the trend observed with CDR1as. Furthermore, miR-1270 targets a tumor suppressor gene, SCAI—by binding to the putative binding sites on its 3′ UTR, it decreases SCAI expression level in cisplatin-sensitive cells. Data were more promising when Zhao's group also detected a low level of CDR1as in serum exosomes, which suggested using this circRNA as a stable tool for detecting cisplatin-resistant OC tumors (Zhao Z. et al., 2019).

In an early OC-associated circRNA publication that was released in 2016, scientists detected numerous circRNA isoforms in primary OC specimens and matched peritoneal carcinomatosis and metastasized lymph nodes (Ahmed et al., 2016). Some genes involved in important signaling pathways such as NF-kB, PI3k/AKT, and TGF-β were found to be expressed differentially between the linear and the matched circRNAs, using paired-end RNA-Seq libraries from primary ovarian tumors, matched peritoneum, and lymph node metastases. CircRNA and mRNA levels exhibit an opposite trend; for example, the mRNAs of NF-kB, PI3k/AKT, and TGF-β are usually up-regulated in metastatic tissues, while the corresponding circRNAs are down-regulated. This differential expression pattern opens a promising direction in using these circRNA forms as biomarkers for highly heterogenous cancer transcriptomes.

Recently, Teng et al. published a comprehensive analysis of 7,333 circRNAs related to OC regulatory activities, in which the expressions of 2,431 were noticeably increased, and those of 3,120 were significantly decreased (Teng et al., 2019). Among all validated circRNAs, circHIPK3 showed the most down-regulated signals based on the sequencing data and displayed various tumor suppressive functions. Specifically, depleting circHIPK3 in OC cell lines promotes cell growth and migration to adjacent tissues, as well as negatively regulates the programed cell death mechanism. In high-grade serous OC (SOC), the most common pathological subtype of OC, there are up to 710 differentially expressed circRNAs. CircRNA1656 is the most differentially expressed among all and highly associates with OC patients tumor stage (Gao et al., 2019). Hu et al. (2018) demonstrated that circ-ITCH sponges miR-145 increase the expression of RASA1 and therefore inhibit tumor progression both *in vitro* and *in vivo*. Functionally, the down-regulation of miR-145 gives rise to RASA1 protein expression, inducing tumor proliferation, invasion, and migration. As a miR-370 sponge, hsa_circ_0061140 promotes cell proliferation and metastasis in OC cell lines SKOV3 and A2780, subsequently decreasing FOXM1 expression (Chen Q.Z. et al., 2018). Via RNA fluorescence *in situ* hybridization (FISH) and luciferase reporter assays, Chen et al. were able to detect the up-regulation of hsa_circ_0061140 and how tumor growth is promoted through the expression of this circRNA.

Finally, circEPSTI1 was found remarkably up-regulated in OC. A series of experiments were performed by Xie J. et al. (2019) showing that circEPSTI1 regulates EPSTI1 levels and OC development by inhibiting miR-942. They showed that circEPSTI1 inhibition suppresses cancer cell growth and invasion capacity, and induces programed cell death in OC, confirming its oncogenic role (Xie J. et al., 2019).

Endometrial Cancer

The number of patients with EC has increased recently, while the age of diagnosis is earlier than before (Moore and Brewer, 2017). A recent report of EC showed that in 2017, in the United States, there were 61,380 newly diagnosed cases and over 10,920 deaths[1]. EC is categorized into two subtypes: type 1 lesions, often low-grade and hormonal sensitive, are one of the most frequent and have an optimal prognosis, while the second type is rare but more aggressive and at risk of recurrence, even for early stage tumors (Amant et al., 2005). Due to the severe mortality of EC in recent years, scientists are trying to develop specific predictive biomarkers for endometrial malignancies. The diagnosis and treatment of ECs has recently been greatly improved thanks to advances in the knowledge of regulatory pathways involved in tumor initiation and progression. Precise molecular characterization of the disease led to the developing of targeted therapies and diagnostic tools for EC patients, such as PP2A, a tumor suppressive heterotrimeric protein phosphatase type 2A (Remmerie and Janssens, 2019). Preventing endometrial tumorigenesis and tumor invasion, PP2A has been shown to be altered in more than 40% of EC.

Compared to BC, OC, and CC, the function of circRNAs in EC is less characterized. The most characteristic study on

[1]https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html

circRNAs in EC was performed by Ye et al. (2019b). Using circRNA sequencing, the authors conducted a screening on the expression of 75,928 different circRNAs in grade 3 EC tumors versus adjacent healthy endometrial tissues. Among all, 25,735 circRNAs were overexpressed and 36,432 were down-regulated. They also ranked the top five circRNAs that have the highest expression and lowest expression in EC tissues. Hsa_circ_0039569, hsa_circ_0001523, hsa_circ_0001610, hsa_circ_0001400, and hsa_circ_0007905 were substantially overexpressed, while hsa_circ_0000437, hsa_circ_0001776, hsa_circ_0009043, hsa_circ_0000471, and hsa_circ_0014606 were the most down-regulated in their analysis. Further bioinformatic analysis demonstrated that circRNAs and miRNAs build a complex regulatory network; deregulated circRNAs potentially sponge 451 miRNAs. Based on this regulatory network, the hsa-circ-0039659/hsa-miR-542-3p/hsa-let-7c-5p pathway has been suggested as the most important one in predicting grade 3 EC (Ye et al., 2019b).

Another circRNA has been recently revealed to play a role in the tumorigenesis of EC: circ-ZNF91 (Chen B.J. et al., 2018). Circ-ZNF91 functions as a miRNA sponge, inhibiting the expression of miR-23b and miR-199. Moreover, Chen B.J. et al. (2018) performed a global expression profile of circRNAs to compare cancer and healthy endometrial tissues. Based on their findings, the ratio of circRNAs to linear RNA isoforms was lower in EC than in healthy endometrial tissues, 23.9 and 30.1%, respectively. There were up to 120 circRNAs differentially expressed in EC tissues, out of which HSPG2 and RP11-255H23.4, two ciRNAs, were found to be expressed only in healthy tissues. Surprisingly, their mRNA isoforms increased significantly in EC tissues. Although this study did not establish further details on how each individual circRNA expression leads to tumor initiation and progression, it provided a foundation for future investigations on EC-associated circRNA functions and mechanisms.

circRNAs AS POTENTIAL CIRCULATING BIOMARKERS AND THERAPEUTIC TARGETS

CircRNAs are potential non-invasive biomarkers. In BC, Yin et al. (2018) investigated plasma circRNAs' expression with the aim of discovering valuable diagnostic biomarkers. They identified hsa_circ_0001785 as a stable biomarker for the diagnosis and progression of BC. Data showed that this circRNA has an AUC of 0.784, and if combined with two other established biomarkers, carcinoembryonic antigen (CEA) and carcinoma antigen 15-3 (CA 15-3), the AUC increases to 0.839 (Yin et al., 2018).

In CC, Li et al. (2019) developed a new microarray capable of profiling circRNAs. Interestingly, the newly developed tool seemed to be superior to RNA-seq and the authors also tested it when profiling the circRNAs from plasma of patients with CC. It was possible to detect around 18,000 circRNAs in the plasma of CC patients and the expression of 2,787 circRNAs was deregulated after surgery for tumor removal. The diagnostic and prognostic power of circRNAs was not further tested, but it is plausible to speculate that they can be used as non-invasive biomarkers for CC (Li et al., 2019).

Knowing that serum circSETDB1 is a tumor-promoting circRNA and is up-regulated in SOC, Wang W. et al. (2019) chose to investigate the potential role of this circRNA as a biomarker. First, they assessed the capacity of this circRNA to separate SOC patients from healthy controls. A ROC curve analysis showed that serum circSETDB1 expression can be used to discriminate SOC patients from healthy controls, with an AUC of 0.8031 and a sensitivity of 78.33% and specificity of 73.33%. The same circulating circRNA was used to separate primary chemoresistant SOC patients from primary chemosensitive ones. Data showed that circSETDB1 can be used to diagnose chemosensitivity with an AUC of 0.8107 and a specificity of 76.74% and sensitivity of 77.78%. Finally, Wang W. et al. (2019) investigated if serum circSETDB1 levels can be used as a predictive tool for progression free survival (PFS). Patients with low levels of circSETDB1 had a mean PFS of 18.9 months and patients with high levels had a mean PFS of 13.2 months (Log Rank = 6.815, P = 0.006). Taken together, these data prove that circSETDB1 is a promising non-invasive diagnostic and prognostic biomarker for SOC (Wang W. et al., 2019). Serum circMAN1A2 was found up-regulated in several cancers including OC, which was investigated as a potential biomarker by Fan et al. Even if data appear less promising in OC than in other malignancies, circMAN1A2 still has an AUC of 0.694, a sensitivity of 0.583, and a specificity of 0.806. According to the authors, further studies are necessary to confirm or infirm the potential role of this circRNA as a biomarker for OC (Fan et al., 2019).

In EC, Xu H. et al. (2018) conducted a pilot study and discovered that serum circulating extracellular vesicles from EC patients contain 209 up- and 66 down-regulated circRNAs compared to matched healthy volunteers. The authors further validated the up-regulation of has-circ_01090406 and has_circ_0002577, but did not test the diagnostic power of these circular transcripts.

The CircRNAs described above represent only a fraction of the promising diagnostic and prognostic potential of these molecules in BC and female reproductive system cancers. However, it is important to note that further studies will be required before these circRNAs can be used in further clinical settings as diagnostic or prognostic biomarkers.

Similar to coding genes or well-studied non-coding genes (i.e., miRNAs), circRNAs can be classified as tumor suppressors and oncogenes. Therefore, we can envision two different therapeutic approaches: inhibiting the circRNAs, which are carcinogenic and overexpressed in tumor tissues, or restoring circRNAs with tumor suppressor functions, which are down-regulated in tumors.

Tumor suppressor circRNAs were reported to act as potent endogenous sponges that bind oncogenic miRNAs (oncomiRs) and inhibit their function (Dragomir and Calin, 2018a). Artificially synthesized tumor suppressor circRNAs are promising anti-miRNA therapy. Because it is known that not only one type of miRNA is overexpressed in specific cancer type, these artificial constructs can be designed to bind and inhibit multiple oncomiRs simultaneously. Additionally, artificial circRNAs can have multiple binding sites for the same miRNA,

similar to the well-known sponge of miR-7, that harbors over 70 binding regions for this miRNA (Hansen et al., 2013; Memczak et al., 2013). Hence, circRNAs seem to be the ideal inhibitors for oncomiRs. Recently, synthetic circRNA based-therapy for gastric and esophageal cancers was developed and proved to be efficient (Liu X. et al., 2018; Wang Z. et al., 2019), and so similar strategies may also be used for BC and female reproductive system cancers. Moreover, it was also reported that endogenous circRNA have an anti-viral function at the cellular level (Tagawa et al., 2018). Artificial circRNAs could be designed to inhibit the replication of oncoviruses and to design preventive therapies for cancers caused by viral infections (i.e., CC).

There are some clear advantages of circRNA restoration therapies compared to other RNA therapies: circRNAs have a longer half-life compared to mRNAs, so the dose can be reduced and the administration of the treatment can be infrequent. There are also possible disadvantages. For example, it is not yet clear if artificial circRNAs can activate the immune system, like miRNAs, and induce systemic inflammatory response syndrome (SIRS), one of the most frightening adverse events of RNA therapy. Because of the similar structure to some viral particles [the first described circRNAs were viroids (Sanger et al., 1976)], this approach could be very dangerous.

Oncogenic circRNAs are overexpressed and usually inactivate tumor suppressor miRNAs. Inhibiting oncogenic circRNAs has not yet been explored, but we can imagine several strategies. First, it is possible to block the biogenesis of circRNAs using small molecules similar to the therapies developed for miRNAs. Second, we can envision complementary, small RNA molecules that bind the sites of the circRNA responsible for sponging and inhibiting tumor suppressor miRNAs. It is important to mention that this single strand RNA molecule needs to have a higher affinity for the circRNAs which target miRNAs. This therapy is similar to miRNA mask therapy. Finally, it is possible to directly induce the degradation of circRNAs using RNA inference. One possible solution is short interfering RNAs (siRNAs), which can be further chemically modified to increase efficiency (i.e., locked nuclei acids) (Shah et al., 2016; Petrescu et al., 2019). It is important to mention that, in order to induce the knockdown of circRNAs, siRNAs need to target the back-splice junction of the circular transcript (Kristensen et al., 2019).

STUDYING circRNAs VIA DATABASE

To make research more accessible, it is urgent to create comprehensive databases for circRNAs and their related diseases and/or targets to be able to generate more in-depth analyses. In the past decade, using next-generation sequencing and bioinformatics, various circRNA studies were collected and integrated into circRNA databases. Two well-known circRNA datasets, circBase, and circFunBase, provide general information on each circRNA expression (Glazar et al., 2014; Meng et al., 2019). These data collections consist of all circRNA-related research from 2013 until the present as well as over 150,000 circRNA genomes sequences from diverse species. More specifically, some databases introduce visualized circRNA–miRNA interaction networks to create a detailed platform of circRNA–disease relationships such as Circ2Traits (Ghosal et al., 2013). CircNet is one of the most widely used databases, gathering the genomic annotation and sequence of circRNA isoforms, tissue-specific expression levels, and miRNA or gene-related interactome maps (Liu et al., 2016). An example of a more exhaustive database, CSCD (cancer-specific circRNA database), is derived from a total of 228 RNA or polyA(-) RNA-seq samples from malignant and normal *in vitro* models. This powerful dataset contains 272,152 cancer-specific circRNAs in different types of tumors (Xia et al., 2018). MiOncoCirc is another dataset of circRNAs that represents all circRNAs regulating primary tumors, metastasis, and even rare cancers (Vo et al., 2019). There are other circRNA databases that have been used in the past decade to detect and validate targets

TABLE 2 | CircRNAs databases.

Name	Website	Description	References
CircInteractome	http://circinteractome.nia.nih.gov/	Predicts and maps the binding sites for RBPs and miRNAs on reported circRNAs.	Dudekula et al., 2016
CircBase	http://www.circbase.org/	Public circRNA datasets and custom python scripts to discover circRNA.	Glazar et al., 2014
CircFunBase	http://bis.zju.edu.cn/CircFunBase/	Utilizes 7,000 manually curated functional circRNA entries.	Meng et al., 2019
Circ2trait	http://gyanxet-beta.com/circdb/index.php	CircRNAs and their related diseases regulation.	Ghosal et al., 2013
CircNet	http://circnet.mbc.nctu.edu.tw/	Utilizes transcriptome sequencing datasets from circRNA expression in 464 RNA-seq samples.	Liu et al., 2016
CSCD	http://gb.whu.edu.cn/CSCD/#	Cancer-specific circRNA database.	Xia et al., 2018
MiOncoCirc	https://mioncocirc.github.io/	Cancer circRNA database constructed from clinical cancer samples.	Vo et al., 2019
CIRCpedia v2	http://www.picb.ac.cn/rnomics/circpedia/	CircRNA annotations retrieved from 180 RNA-seq datasets in six different species.	Dong et al., 2018
TCSD	http://gb.whu.edu.cn/TSCD	Tissue-specific circRNA database.	Aghaee-Bakhtiari, 2018
CircRNADb	http://reprod.njmu.edu.cn/circrnadb	circRNA database annotated (in particular in humans.)	Chen et al., 2016
Circ2Disease	http://bioinfo.snnu.edu.cn/CircR2Disease/	739 manually curated circRNA entries associated with 100 different diseases.	Fan et al., 2018

in numerous studies related to this new member of the ncRNA family such as CircRNADb, CircInteractome, CIRCpedia v2, and TCSD (Chen et al., 2016; Dudekula et al., 2016; Zheng L.L. et al., 2016; Aghaee-Bakhtiari, 2018; Dong et al., 2018). A more user-friendly platform is Circ2Disease, which can be used to search how many circRNAs are deregulated in various diseases including malignant tumors. This database provides thorough information about the expression patterns of circRNAs, the experimental technique of detection, and literature reference on PubMed (Fan et al., 2018).

We have summarized 11 circular RNA databases in **Table 2**. However, most of the current databases have not been updated since 2017–2018, while, in the field of breast and gynecological cancers, most circRNA-related discoveries have been made in late 2018 and 2019. Presently, over 700 scientific papers have been published investigating the association of circRNAs in regulating malignant pathways, but we still lack a thorough and complete perspective on how different circRNAs function and how they are regulated. We believe it is of great significance to update the databases more frequently and develop their systems to be more generic, organized, and viable. It will make it easier for scientists to get access and link the rules of circRNA biology and reveal their functions in studying cancer.

CONCLUSION

CircRNAs are belived to be involved in the tumorigenesis of different types of malignancies, including female reproductive system and breast cancers, and similar to other ncRNAs (Jin et al., 2013; Zhou et al., 2014), they have prognostic, diagnostic, and therapeutic potential. The high conservation between species, the specific expression, and the variety of their roles suggest that circRNAs are specifically involved in physiological cellular mechanisms (Ebbesen et al., 2017). As demonstrated in this review, the dysregulation in gene expression of circRNAs is believed to be one of the major mechanisms leading to the development and progression of gynecological cancers. In the blossoming era of exploiting genetic determinants in cancer biology, the significance of circRNAs is beginning to be recognized and their functionalities are beginning to be elucidated. There is copious evidence indicating that circRNAs will play a tremendous role in the future care of cancer patients via diagnostic and therapeutic approaches. If the footprints of circRNAs are yet to be confirmed, circular transcripts might be a major, ground-breaking target for therapies: either to block overexpressed, pro-tumorigenic circRNAs or to restore down-regulated tumor suppressive circRNAs. With the assistance of a growing number of circRNA databases available, scientists will have access to established studies and can further develop their research directions.

AUTHOR CONTRIBUTIONS

AT, GMC, MD, and GAC: conception and design. CI, MD, and GAC: provision of study materials. AT, GMC, LB, MC, and MD: collection and assembly of data. AT, GMC, LB, PR, SJ, EG, MD, and GAC: manuscript writing. MC and MD: figure design. All authors gave the final approval of the manuscript.

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Non-coding RNAs as Putative Biomarkers of Cancer-Associated Cachexia

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Cachexia is a complex metabolic syndrome that determines a severe body weight loss characterized by a marked reduction in muscle mass. About 80% of patients with advanced cancer develop cachexia due to both the tumor itself and cancer treatment (radiotherapy and/or chemotherapy), which is associated to a worse prognosis. Despite its clinical relevance, this syndrome is still under-diagnosed and it lacks effective treatments. Radio-chemotherapy treatment is essential in patients with advanced head and neck cancers (HNSCC). Although this treatment has improved patients' life expectancy, it has also dramatically increased their need for assistance and support. The management of adverse symptoms, including cachexia, is of great importance in order to avoid delays in therapy, reduction of dosages and hospitalizations. MicroRNAs (miRNAs) are small non-coding RNA molecules, which have emerged as powerful biomarkers in stratifying human cancers. Due to their high stability in body fluids, miRNAs might be excellent non-invasive biomarkers for the early detection and follow-up of cancer patients. Here, we will summarize the current knowledge and debate the strong need to identify circulating biomarkers for the early diagnosis of cachexia. We will propose circulating non-coding RNAs as biomarkers for detecting early cachexia and implementing specific treatment. We will also discuss the potential use of circulating miRNAs as biomarkers of cachexia in HNSCC patients' blood samples collected before and after radio-chemotherapy treatment. Our intent is to pave the way to the identification of specific circulating miRNAs associated to cachexia occurrence and to the design of specific interventions aimed at improving the quality of life of cancer patients.

Keywords: miRNA, cachexia, liquid biopsy, HNSCC (head and neck squamous cell carcinoma), myomiR

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CANCER-ASSOCIATED CACHEXIA IN ADVANCED CANCER PATIENTS

Cachexia is a complex multifactorial syndrome characterized by the loss of mass and functionality of skeletal muscle and by the loss of adipose tissue, with consequent progressive loss of body weight (DeWys, 1982). Unlike malnutrition, cachexia cannot be resolved simply with conventional nutritional support (Fearon et al., 2011).

This syndrome affects the majority of patients with advanced cancer treated with radio-chemotherapy, including in particular patients with advanced head and neck cancers (HNSCC) (Gorenc et al., 2015; Kwon et al., 2017). It is also associated with a poor prognosis, an altered quality of life and a reduced tolerance and response to anticancer therapies (DeWys, 1982; Fearon et al., 2011).

Weight loss is a common feature in patients with HNSCC and can occur before, during and after radio-chemotherapy treatment.

The correct diagnosis of cachexia and the maintenance of muscle mass represent an important goal in the care of cancer patients.

The development of cachexia varies according to the nature, the stage and the site of the tumor, the type of treatment, but it is also based on individual characteristics (genetic predisposition, initial BMI and body composition, physical activity, food intake, comorbidity and gut microbiota) (Bindels and Delzenne, 2013; Johns et al., 2013). Therefore, due to the heterogeneity of its manifestation and to the lack of a precise definition, the diagnosis of cachexia is often not performed promptly.

The need for an early diagnosis determines the need to identify biomarkers able to reflect the process of muscular atrophy that characterizes cachexia. The ideal biomarker should be easy to quantify without the need for an invasive muscle biopsy.

CURRENT KNOWLEDGE ABOUT POTENTIAL BIOMARKERS FOR CANCER-ASSOCIATED CACHEXIA

Cancer-associated cachexia is a very complex and still poorly characterized syndrome whose molecular pathways remain to be still elucidated.

Muscle atrophy, that characterizes cachexia, is the result of an imbalance between muscle protein synthesis and degradation that determine a decrease in myofibrillar and sarcoplasmic proteins leading to muscle fibers shrinkage.

What is taken for granted is the key role of systemic inflammation in the occurrence of cachexia, determined both by the presence of the tumor itself and by the host-derived factors (Argiles et al., 2019).

For this reason, to date, the majority of the studies aimed at the discovery of powerful biomarkers for cachexia focused on pro-inflammatory cytokines released by tumor, immune and stromal cells.

In particular the cytokine tumor necrosis factor α (TNFα), previously called cachectin, has been demonstrated to be a major player in cancer related cachexia as it is able to induce muscle wasting through NFkB pathway (Han et al., 1999).

Different studies in animal models and cancer patients demonstrated that high levels of interleukin 6 (IL-6) correlate with muscle wasting, inhibition of protein synthesis, promotion of protein degradation and autophagy in myotubes (White et al., 2011; Pettersen et al., 2017).

Supporting the key role of inflammation in cancer-related cachexia occurrence, recently Penafuerte et al. (2016) reported an increase in neutrophil-derived proteases (NDPs), angiotensin II (Ang II), transforming growth factor beta 1 (TGFβ1) and C-reactive protein (CRP) plasma levels in cachectic and pre-cachectic cancer patients.

Collectively, the release of these pro-inflammatory cytokines has been demonstrated to promote ubiquitin-proteasome and autophagy lysosome pathways in skeletal muscle cells thereby determining muscle wasting.

Despite these evidences, there is still an incomplete understanding of the underlying biology of cancer-associated cachexia, and there is still the need to find powerful biomarkers useful for the diagnosis and management of such complex metabolic syndrome.

MICRORNAs

MicroRNAs (miRNAs) are highly conserved single strand RNA molecule of about 17–22 nucleotides in length whose role in the regulation of wide range of biological processes has been widely characterized (Gebert and MacRae, 2019).

miRNAs' biogenesis starts with the transcription by RNA polymerase II that generates a primary transcript with a hairpin double helix structure of about 300 nucleotides called pri-miR (Treiber et al., 2019). The pri-miR is processed by the endonuclease Drosha and the cofactor DGCR8 into a smaller precursor called pre-miR in the nucleus. The pre-miR is exported to the cytosol by the exportin 5 enzyme (Treiber et al., 2019). In the cytosol, the endonuclease Dicer cleaves the pre-miR to generate the miRNA duplex, of which only one strand will be the mature miRNA (Treiber et al., 2019). The mature miR is then included in the RNA-induced silencing complex (RISC), through which it is brought to the target mRNAs (Treiber et al., 2019) (**Figure 1**).

The peculiar mechanism of action of miRNAs consists in the ability to bind to the 3′ UTR of their target mRNAs and to promote their degradation or the inhibition of mRNA translation. This leads to a decrease of the expression of specific proteins.

Due to the multiplicity of mRNAs target for each miRNA, fine modulation in their expression levels can determine relevant changes in the cell. Indeed miRNAs' deregulation may be the cause of several pathological conditions, such as cancer (Tufekci et al., 2014).

MICRORNAs AS POTENTIAL CANCER BIOMARKERS IN TISSUES AND IN BODY FLUIDS

Growing evidence has demonstrated the broad implication of miRNAs in cancer occurrence and allowed the classification of miRNAs in two main categories according to their target mRNAs: the so called "oncomiRNAs" and the "tumor suppressor miRNAs" (Fearon et al., 2011; Blandino et al., 2014; Frixa et al., 2015; Verduci et al., 2019). Under physiological conditions there is a balance between these two classes of miRNAs. At

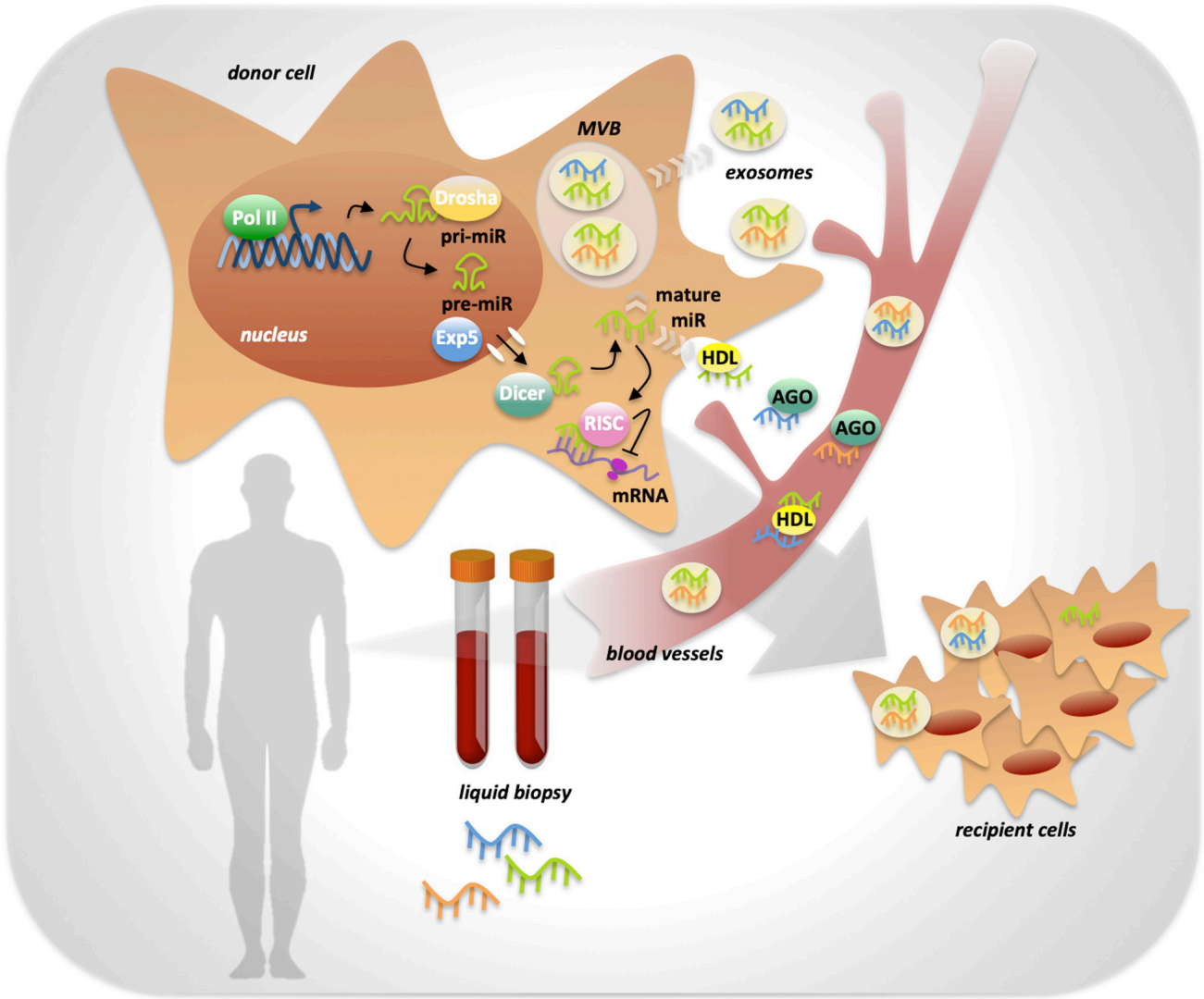

FIGURE 1 | miRNAs processing, activity and release in body fluids. MiRNA genes are transcribed by RNA polymerase II into a primary transcript with a hairpin double helix structure of about 300 nucleotides called pri-miR. The endonuclease Drosha is responsible of pri-miR processing into a smaller precursor called pre-miR, that is exported from the nucleus to the cytoplasm by Exportin 5 enzyme (Exp5). Into the cytoplasm the endonuclease Dicer completes miRNA biogenesis by processing the pre-miR in the mature miRNA. miRNA activity, consisting in the inhibition of target mRNAs translation, is determined by its inclusion in the RISC complex. Mature miRNAs can be released by the cell into the extracellular space in two ways: through the inclusion in protein complexes or in exosomes that preserve them from RNases activity. Once in body fluids, miRNAs can reach distant sites in the body and act as mediators of cell-to-cell communication. miRNAs' peculiar high stability makes them ideal powerful biomarkers easily detectable trough liquid biopsy.

the onset of cancer there are alterations that can unbalance the ratio between oncomiRNAs and tumor suppressor miRNAs fostering tumorigenesis. Deregulation of miRNAs expression can be established by alterations that affect their biogenesis, such as: (a) epigenetic modification on their regulatory regions; (b) changes in the activity of specific transcription factors; (c) alterations in processing enzymes involved in miRNAs' maturation steps (mainly Drosha and Dicer) (Rakheja et al., 2014; Lin and Gregory, 2015; Frixa et al., 2018; Ramassone et al., 2018).

The initial studies, concerning the role of miRNAs in cancer occurrence, focused on patients-derived tumoral tissues and allowed the identification of specific signature able to stratify human cancers (Lu et al., 2005; Calin and Croce, 2006; Di Leva and Croce, 2013). In particular, miRNAs discriminate tumoral from non-tumoral tissues, or metastatic from non-metastatic tumors, or different histotypes of the same tumor type, or, in treated patients, responder from non-responder individuals (Donzelli et al., 2015; Lindholm et al., 2019; Sokilde et al., 2019).

One of the most characterized oncogenic miRNAs is miR-21, whose expression has been found to be up-regulated in several solid and hematological malignancies (Feng and Tsao, 2016). The widespread pro-tumorigenic function of miR-21 depends on its ability to bind to the 3′UTR of several tumor suppressor mRNAs

independently from the tissue context. Its oncogenic activities include promotion of cell proliferation, migration, invasion, metastasis and resistance to chemotherapeutic treatments (Zhu et al., 2008; Pfeffer et al., 2015). Moreover, miR-21 has been demonstrated to be an important biomarker of poor prognosis in several cancer types (Yan et al., 2008; Zhou et al., 2014; Arantes et al., 2017; Labib et al., 2017). For this reasons miR-21 represents one of the actionable miRNAs for novel therapeutic strategies and it is considered a robust biomarker to be implemented in clinical practice (Bonneau et al., 2019; Hanna et al., 2019).

Conversely, miR-145-5p is one of the most studied tumor-suppressor miRNAs as it results to be down-regulated in several types of cancer (Cui et al., 2014; Donzelli et al., 2015). Most of the findings revealed that miR-145-5p regulates the expression of several oncogenes in particular genes involved in cell invasion, migration and metastatization (Ye et al., 2019). Notably, the attempt to disclose a therapy for the replacement of miR-145-5p in tumor cells is actively pursued. These and other studies highlighted the promising role of miRNAs as powerful biomarkers for cancer diagnosis and prognosis and as novel actionable targets for more effective cancer therapies.

Since 2008, with the first evidence of the presence of miRNAs in plasma and in others biological fluids, and thus in the extracellular environment, the potential role of miRNAs as mediators of cell-to-cell communication has been deeply investigated (Mitchell et al., 2008; Kosaka et al., 2010; Li et al., 2019).

Indeed, it has been demonstrated that miRNAs exert paracrine functions and are essential for tissue communication.

In regard of cancer, the release of miRNAs by tumoral cells in the extracellular environment has been assessed for several types of cancer (Izzotti et al., 2016).

The release of miRNAs from tumoral cells can occur in two different ways: miRNAs can be included in microvesicles that are released from the cells through blebbing of the plasma membrane, otherwise cells can actively release miRNAs in microparticle-free form and they can bind to high-density lipoproteins or to RNA-binding proteins such as Ago2 (Kosaka et al., 2013) (**Figure 1**).

A peculiar feature of circulating miRNAs is their remarkable stability, due to their small size and to the inclusion in protein complexes or in microvesicles that preserve them from RNase activity. Indeed, miRNAs are present in almost all biological fluids such as plasma, serum, saliva, urine, cerebrospinal liquid, milk, amniotic fluid and tear (Cortez et al., 2011; Izzotti et al., 2016).

MiRNAs' high stability, the non-invasive way to collect (i.e., blood collection), to detect and to quantify (i.e., Real-Time PCR) them, are all features that overlap to those of an ideal biomarker. Indeed, the majority of the studies, aimed to the discovery of novel powerful biomarkers for cancer screening, diagnosis, prognosis and monitoring of the effectiveness of therapies in cancer patients, are focusing on genome wide approaches for the evaluation of miRNAs in body fluids (Hamam et al., 2017; Cui et al., 2019).

Intriguingly, miR-21 represents one of the circulating miRNAs whose concentration is increased in the serum of patients with the most varied types of cancer, suggesting the potential use of miR-21 as non-invasive diagnostic markers (Wu et al., 2015; Pulito et al., 2017; Cui et al., 2019).

ARE SKELETAL MUSCLE MICRORNAS POTENTIAL BIOMARKERS FOR CANCER-ASSOCIATED CACHEXIA?

Several genome wide studies revealed that miRNAs are differentially expressed in human tissues. In particular, a small group of miRNAs, referred to as *myomiRs*, that are enriched or exclusively expressed in the striated muscle has been identified. This muscle-specific group of miRNAs includes miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, and miR-499 (Kirby and McCarthy, 2013). The tissue-specificity is due to the presence of muscle-specific transcription factor binding sites on the regulatory regions of these miRNAs and, for some of them, to the genomic localization within the myosin heavy chain (MyHC) genes. The role of myomiRs in the regulation of muscle homeostasis, development and functionality has been extensively characterized (Horak et al., 2016; Sjögren et al., 2017). Moreover, deregulation of their expression resulted to be associated with muscle atrophy, one of the main hallmark of cancer-associated cachexia (Suzuki and Springer, 2018).

This suggests the possibility for myomiR to be novel mediators and powerful biomarkers for cancer-associated cachexia. In the last years, different studies on muscle tissue from mice models of cachexia or from cachectic cancer patients revealed a correlation between myomiRs deregulation and cachexia occurrence and maintenance (Soares et al., 2014; Lee et al., 2017; Fernandez et al., 2019).

The muscle-specific miR-206, together with miR-21, was one of the first miRNAs identified to be positively associated to muscle wasting by performing miRNA profiling of muscles derived from different atrophic mice models (Soares et al., 2014).

Recently, Lee et al. (2017), by performing miRNA sequencing from tibialis anterior muscles of cachectic lung carcinoma mice and of healthy mice, identified a signature of 9 miRNAs differentially expressed: miR-147, miR-205-3p, miR-229a3p, miR-233-3p, miR-431-5p, miR-511-3p, miR-665-3p, miR-1933-3p, and miR-3473d. Gene ontology analysis of these miRNAs indicated an involvement in cellular development, cell cycle, cell morphology, cell death and survival, and inflammatory responses, supporting their role in muscle wasting (Lee et al., 2017).

More recently, Fernandez and collaborators, performed an integrated genome wide analysis by combining miRNA/mRNA sequencing from the same set of skeletal muscles derived from mice models of cancer-associated cachexia (Fernandez et al., 2019). In particular, they identified 18 miRNAs differentially expressed in skeletal muscle of cachectic mice compared to the controls. Among the 18 miRNAs, 13 resulted to be up-regulated and 5 to be down-regulated. The integrative analysis allowed the authors to generate a miRNA-mRNA network composed of 171 interactions between 18 miRNAs and 131 target mRNAs. This analysis revealed enrichment for genes involved in extracellular

matrix organization, highlighting their contribution to cancer-associated cachexia (Fernandez et al., 2019).

Genome wide studies performed in muscles derived from cachectic cancer patients enlarged the knowledge about miRNAs' contribution to cancer-associated cachexia.

Interestingly, Narasimhan et al. (2017) analyzed miRNAs expression of human skeletal muscle in cachectic and non-cachectic pancreatic and colorectal cancer patients and identified 8 novel deregulated miRNAs: miR-3184-3p, miR-423-5p, let-7d-3p, miR-1296-5p, miR-345-5p, miR-532-5p, miR-423-3p, and miR-199a-3p. These miRNAs resulted to be up-regulated in skeletal muscle of cachectic patients, and pathway analysis of their potential mRNA targets identified pathways related to myogenesis and inflammation (Narasimhan et al., 2017).

More recently, Van de Worp et al. (2019), by performing miRNA profiling in skeletal muscle of cachectic lung cancer patients compared to matched healthy controls, identified a signature of 28 miRNAs differentially expressed. Interestingly, 4 miRNAs our of 5 which resulted to be up-regulated in cachetic patients, belong to the same cluster (miR-450a-5p, miR-450b-5p, miR-424-5p, and miR-424-3) (Van de Worp et al., 2019).

Adipose tissue loss also occurs in cancer-associated cachexia. This feature has been scarcely investigated. Kulyte et al. (2014) identified a signature of 5 miRNA differentially expressed in abdominal subcutaneous adipose tissue from gastrointestinal cancer patients with or without cachexia. In particular, miR-483-5p, miR-23a, miR-744, and miR-99b were down-regulated, whereas miR-378 was significantly up-regulated in cachectic patients. In details, the authors demonstrated that miR-378 up-regulation in adipose tissue is required to promote catecholamine-stimulated lipolysis.

TABLE 1 | miRNAs demonstrated to be related to cancer-associated cachexia.

Tissue miRNAs	Tissue	References
miR-206, miR-21	Mice muscles	Soares et al., 2014
miR-147, miR-205-3p, miR-229a3p, miR-233 3p, miR-431-5p, miR-511-3p, miR-665-3p, miR-1933-3p, miR-3473d	Mice muscles	Lee et al., 2017
miR-10b-5p, 1249-3p, miR-144-3p, miR-144-5p, miR-146a-5p, miR-146b-5p, miR-181c-3p, miR-183-5p, miR-1843a-3p, miR-223-3p, miR-29b-3p, miR-338-5p, miR-350-3p, miR-3535, miR-379-3p, miR-382-5p, miR-451a, miR-671-3p	Mice muscles	Fernandez et al., 2019
miR-3184-3p, miR-423-5p, let-7d-3p, miR-1296-5p, miR-345-5p, miR-532-5p, miR-423-3p, miR-199a-3p	Human skeletal muscle	Narasimhan et al., 2017
miR-483-5p, miR-23a, miR-744 and miR-99b, miR-378	Human skeletal muscle	Kulyte et al., 2014
miR-450a-5p, miR-424-5p, miR-450b-5p, miR-424-3p, miR-335-3p, miR-103-3p, miR-483-5p, mir-409-3p, miR-15b-5p, miR-370-3p, miR-20a-3p, miR-451a, miR-517c-3p, miR-144-5p, miR-766-3p, miR-1255b, miR-517a-3p, miR-512-3p, miR-522-3p, miR-520g-3p, miR-483-3p, miR-519a-3p, miR-26a-2-3p, miR-485-3p, miR-379-5p, miR-518b, miR-520h, miR-656-3p	Human skeletal muscle	Van de Worp et al., 2019

Circulating miRNAs	Type of tumor	References
miR-1	Advanced hepatocellular carcinoma	Koberle et al., 2013
miR-486	Breast cancer	Chen et al., 2014
miR-21	Colorectal cancer	Okugawa et al., 2018
miR-203	Colorectal cancer	Okugawa et al., 2019
miR-130a	Head and neck cancer patients	Powrozek et al., 2018

POTENTIAL USE OF CIRCULATING MICRORNAS AS BIOMARKERS FOR CANCER-ASSOCIATED CACHEXIA

Despite genome wide studies performed either in muscle or adipose tissues highlight the involvement of miRNAs in cancer-associated cachexia, the validation in large cancer patient cohorts is lacking and the molecular mechanisms underlying their specific involvement need to be deeply elucidated (**Table 1**).

Moreover, reasoning about muscle or adipose tissue miRNAs as potential biomarkers for cancer-associated cachexia is poorly feasible, because biopsies are too invasive for routine analysis and cannot be applied for the monitoring of cachexia during cancer patients treatment. Therefore, there is the urgent need to identify alternative strategies and design clinical studies to validate miRNAs as potential biomarkers of cancer associated cachexia. Recent studied have started to look at body fluid circulating miRNAs as promising and powerful biomarkers for the diagnosis and the monitoring of cancer-associated cachexia (**Table 1**).

In particular, several studies have reported that low concentrations of myomiRs in blood correlate with poor prognosis in non-small cell lung cancer, melanoma, astrocytoma, or osteosarcoma patients (Hu et al., 2010; Li et al., 2014; Zhang C. et al., 2014; Tian et al., 2015). This because skeletal muscle, under physiological conditions, continuously releases exosomes contained myomiRs in the blood (Guescini et al., 2015), but in advanced cancers, when a substantial reduction of muscle mass occurs, there is a consequent decrease in circulating myomiRs. Indeed, Koberle et al. (2013) demonstrated that low levels of circulating miR-1

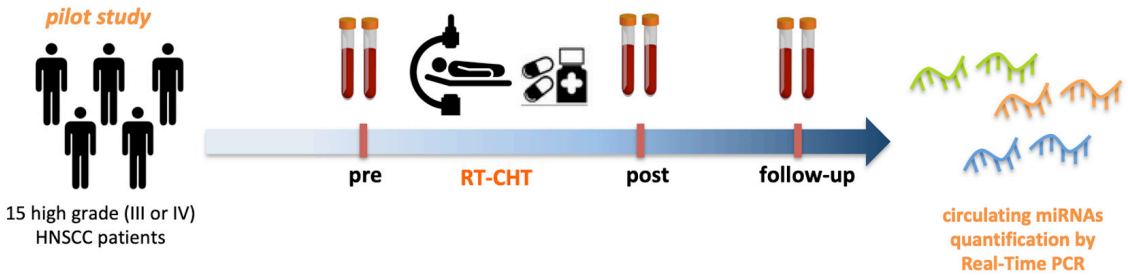

FIGURE 2 | Circulating miRNAs as powerful biomarkers for radio-chemotherapy treatment related cachexia in HNSCC patients. Pilot study design. Serum samples from 15 high grade (III or IV) HNSCC patients before (pre) and after (post- and follow-up) radio-chemotherapy treatment have been collected and subjected to miRNAs expression analysis by Real-Time PCR to asses the feasibility in the quantification of circulating miRNAs.

associated with cachexia in advanced hepatocellular carcinoma patients. Moreover, Chen et al. (2014) reported the circulating muscle enriched miR-486 to be lower in breast cancer patients compared with healthy subjects. More recently, Van Westering et al. (2019), demonstrated that the amount of released myomiR in the serum is dependent on dystrophin protein levels and on its distribution at the sarcolemma.

Not only myomiRs, but also miRNAs released by the tumor itself hold the promise to be considered in the next future robust circulating biomarkers for cancer-associated cachexia. Originally, Fabbri et al. (2012) demonstrated that miR-21 and miR-29a released by lung cancer cells bind to receptors of the Toll-like receptor (TLR) family (murine TLR7 and human TLR8) in immune cells; thereby promoting a TLR-mediated pro-metastatic inflammatory response. As inflammation is one of the main causes of muscle wasting in cancer-associated cachexia, circulating miR-21 and miR-29a seem to be good candidates for cachexia diagnosis and monitoring. The potential use of circulating miR-21 as biomarker for cancer-associated cachexia is supported by other two studies (He et al., 2014; Okugawa et al., 2018). In particular, He et al. (2014) reported that miR-21 contained in lung cancer- and pancreatic cancer-derived microvesicles was able to induce TLR7-mediated cell death in murine myoblasts. More recently, Okugawa et al., demonstrated that circulating miR-21 levels increased in cachectic colorectal cancer patients (Okugawa et al., 2018). Recently they also reported that circulating miR-203 is able to predict myopenia in metastatic colorectal cancer patients (Okugawa et al., 2019).

Looking at the circulating miRNAs that can predict cachexia in radiotherapy-treated head and neck cancer patients, Powrozek et al. (2018), recently identified miR-130a as a good candidate. Indeed, by evaluating the circulating levels of miR-130a in 70 head and neck cancer patients, they observed that patients with low levels of miR-130a were at higher risk to be classified as cachectic, compared to those with high levels of miR-130a. In agreement with a previous study that reported the cytokine TNFα to be a direct target of miR-130a in cervical cancer cell lines (Zhang J. et al., 2014), the authors observed a correlation between low levels of miR-130a and high plasma levels of TNFα in head and neck cancer patients, confirming the key role of inflammation in the onset of cancer-associated cachexia.

The hypothesis that circulating miRNAs could represent powerful biomarkers for the diagnosis and monitoring of cancer-associated cachexia appears to have a strong rationale. In particular, due to the relevant contribution of cancer treatments to cachexia, we aim to evaluate the role of circulating miRNAs in a cohort of advanced head and neck squamous cell carcinoma (HNSCC) patients treated with radio-chemotherapy. To this purpose we firstly performed a retrospective analysis on sera samples of 15 patients enrolled in a prospective study funded by Italian Association for Cancer Research (AIRC, project No.17028) with pathologically confirmed squamous cell carcinoma of the oropharynx, Karnofsky Performance Status >80, stage III or IV without distant metastases, treated with radiotherapy + chemotherapy. Cigarette smoking, alcohol consumption and p16/HPV status, basal weight and variation during treatment, were recorded.

Patient sera samples (n = 15) were collected before the treatment (pre) and 3 months after the treatment (post). Sera collected at 6 months after the treatment (follow-up) were available for 8 out of 15 patients (**Figure 2**).

As a proof of concept we evaluated, by Real-Time PCR, the expression levels of miR-21, due to the previously mentioned evidence about its potential use as biomarker for cancer-associated cachexia. This analysis allowed us to test the quality of the samples and to confirm the presence of miRNAs in the serum of patients affected by head and neck tumors. In particular we confirmed that miR-21 is highly expressed in sera samples of HNSCC patients and it is modulated by radio-chemotherapy treatment.

Prospectively we aim to evaluate the expression of circulating miRNAs (in particular miR-21 and myomiRs) in a prospective cohort of patients with histologically confirmed squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx and larynx, stage III o IV, treated with definitive or adjuvant radio-chemotherapy. The parameters related to cachexia will be evaluated (i.e., weight and its variation over time, variation Body Mass Index, determination of the degree of systemic inflammation by measuring proinflammatory cytokines). Furthermore, the assessment of nutritional status, fatigue, quality of life through questionnaires, will be recorded at baseline during and at follow-up, in order to identify predictive biomarkers of the onset and progression of cachexia.

CONCLUSION

Cancer-associated cachexia is a serious limitation for cancer patients that severely worsen their quality of life, especially for those treated with radio-chemotherapy. Up to date there are no effective treatments for this complex metabolic syndrome. This is mainly due to the lack of a deep characterization of the molecular mechanisms underlying cachexia.

The unmet clinical need that makes very difficult to diagnose timely and properly cachexia is the lack of specific biomarkers. Inflammatory cytokines, such as TNFα and IL-6 might be envisaged as biomarkers for diagnosis and monitoring of cachexia, but further confirmatory studies using larger casuistries than those already analyzed are needed.

MiRNAs are emerging as promising biomarkers not only in cancer but also in other diseases due to their involvement in key biological processes and to their higher stability.

Recently, the evidence of the involvement of miRNAs in cancer-associated cachexia is emerging. Despite the findings are still preliminary they might pave the way for the consideration of miRNAs as useful tools in the diagnosis and monitoring of cachexia.

AUTHOR CONTRIBUTIONS

SD, SS, and GB contributed to the writing and revision of the manuscript. AF, LM, and GS contributed to the writing of clinical issue of cancer-associated cachexia and provided HNSCC patients serum. SD and FG processed the serum samples and performed qRT-PCR analysis. AS performed the bioinformatics analysis. All authors read and approved the final version of the manuscript.

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REVIEW
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Non-coding RNAs in Nervous System Development and Disease

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The rapid advance of RNA sequencing technologies contributed to a deep understanding of transcriptome composition and has allowed the discovery of a large number of non-coding RNAs (ncRNAs). The ability of these RNA molecules to be engaged in intricate and dynamic interactions with proteins and nucleic acids led to a great expansion of gene expression regulation mechanisms. By this matter, ncRNAs contribute to the increase in regulatory complexity that becomes highly specific between tissues and cell types. Among the ncRNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) are especially abundant in nervous system and have been shown to be implicated in its development, plasticity and aging as well as in neurological disorders. This review provides an overview of how these two diverse classes of ncRNAs control cellular processes during nervous system development, physiology, and disease conditions with particular emphasis on neurodegenerative disorders. The use of ncRNAs as biomarkers, tools, or targets for therapeutic intervention in neurodegeneration are also discussed.

Keywords: ncRNAs, circRNAs, neurodegenerative diseases, biomarkers, therapeutics, neuronal development, synaptic activity

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INTRODUCTION

The development and function of the nervous system relies on complex and well-orchestrated gene expression regulation occurring at multiple levels, from transcription to RNA processing, translation, and decay. New discoveries in transcriptomics, facilitated by technical advances in next generation sequencing and computational biology, have revealed the existence of a plethora of transcripts lacking coding potential but exerting an intense regulatory activity in a wide range of biological processes including neuronal development, differentiation, and function. These transcripts belong to the heterogeneous family of non-coding RNAs (ncRNAs) composed by several classes of genes, producing smaller molecules such as microRNAs (miRNAs), and longer transcripts that can be processed to form long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). Studies in the field of lncRNA and circRNA molecules have accelerated considerably during the last few years and major interest has grown in the use of these molecules as diagnostic and therapeutic targets in neurodegenerative diseases (Derrien et al., 2012; Salta and De Strooper, 2017).

LncRNAs are defined as a heterogeneous class of molecules longer than 200 nucleotides (nts) with no protein coding capacity. Their biogenesis is similar to that of mRNAs with RNA Polymerase II (RNAPII) engaging in their transcription. Nascent non-coding transcripts are often subjected to capping, canonical and alternative splicing as well as polyadenylation (Quinn and Chang, 2016). LncRNAs show tissue-specific expression, are generally present at low levels compared to the coding counterparts and show relatively low evolutionary conservation (Cabili et al., 2011; Derrien et al., 2012). Despite few examples, they generally lack an open reading frame (ORF) and influence gene expression at different levels through a variety of mechanisms of action, including recruitment of chromatin modifiers and transcription factors, regulation of three-dimensional chromatin folding, control of mRNA processing, translation and decay (Rinn and Chang, 2012; Fatica and Bozzoni, 2014).

In addition to these linear ncRNAs with distinct 5′ and 3′ ends, a group of circRNAs with covalently closed ends has recently gained attention. Initially discovered as plant viroids and hepatitis delta virus (Sanger et al., 1976; Kos et al., 1986) only in recent years the high-throughput RNA sequencing coupled with circRNA-specific bioinformatic algorithms revealed that 1000s of circRNA molecules are produced from a large fraction of genes in metazoans (human, mouse, zebrafish, worm, fruit fly) (Salzman et al., 2012; Jeck et al., 2013; Jeck and Sharpless, 2014; Salzman, 2014; Wang et al., 2014). Their biogenesis relies on a peculiar splicing reaction called back-splicing that joins a downstream 5′ splice site to an upstream 3′ splice site. Not much is known about their function, however, the few examples that have been characterized have revealed that circRNAs can potentially regulate gene expression both at transcriptional and post-transcriptional level (Kristensen et al., 2019).

Notably both lncRNAs and circRNAs are preferentially expressed in the nervous system and resulted to be dynamically regulated during neuronal development as well as in response to neuronal activity (Kim et al., 2010; Mercer et al., 2010; Derrien et al., 2012; Lipovich et al., 2012; Aprea et al., 2013; Rybak-Wolf et al., 2015; You et al., 2015; Biscarini et al., 2018). They also show highly restricted expression in various anatomic brain regions and cell structures (Mercer et al., 2008; Rybak-Wolf et al., 2015; You et al., 2015). These dynamics and region-specific expression patterns strongly suggest that lncRNAs and circRNAs may play key roles in nervous system development and function. Moreover, recent studies have also shown that, similar to protein coding genes, dysregulation of ncRNA molecules can affect proper nervous system development and function thus contributing to the onset and progression of neurological diseases (Shao and Chen, 2016; Wang et al., 2018a).

This review provides a comprehensive description of lncRNA and circRNA biogenesis and function highlighting their involvement in nervous system development and physiology. It also underlines the implication of ncRNA deregulation in diverse neurodegenerative disorders and ultimately how ncRNAs might serve as suitable diagnostic biomarkers and therapeutic targets.

LONG NON-CODING RNAs: IDENTIFICATION AND GENOMIC CHARACTERIZATION

Analysis of transcriptomes through a high-resolution RNA sequencing (RNA-seq) is one of the most robust methodologies for the *de novo* identification of lncRNAs (Mortazavi et al., 2008).

Large scale studies from multiple sources of data such as DNAse hypersensitivity and chromatin state maps released from the ENCODE (Encyclopedia of DNA Elements) consortium, revealed that 93% of the human genome is actively transcribed and 39% consists of transcriptional units composed by promoter and poly(A) signals: strikingly only a little more than 1% is protein coding. Analysis on multiple human cell and tissue types confirmed that lncRNAs largely outnumber the coding elements, are highly expressed in the nervous system and although their gene body is poorly conserved, the promoter regions and their structural motifs show higher evolutionary constraints (Mercer et al., 2008; Guttman et al., 2009; Derrien et al., 2012, 2014; Hon et al., 2017). Ambitious projects for the functional annotation of the mammalian genome (FANTOM) confirmed that lncRNAs are pervasively transcribed, producing a comprehensive understanding on the genomic organization of more than 50,000 lncRNA loci. Capped Analysis of Gene Expression (CAGE) linked with computational analyses shows that lncRNAs have a heterogeneous genomic organization and can be found: (i) as independent transcriptional units (intergenic RNAs or lincRNAs); (ii) transcribed divergent from coding genes thus sharing the same promoter (divergent lncRNAs); (iii) transcribed from intronic regions (intronic lncRNAs) or enhancer regions (eRNAs); (iv) transcribed as antisense RNAs with respect to coding genes (natural antisense transcripts, NATs) (**Figure 1**). Notably, around 70% of the mammalian coding genes show evidence of antisense transcription, producing ncRNAs that partially or completely overlap with their sense coding strand, their promoter or their regulatory regions (Zhang et al., 2006; Wanowska et al., 2018). Upon evidence of such widespread presence, the importance of NATs and their regulatory relationship with their sense counterparts were deeply dissected for many disease-associated genes and were shown to be particularly relevant in neurodegeneration and for the repeat expansion phenomenon, as it will be described in the following paragraphs.

CIRCRNAS: IDENTIFICATION AND BIOGENESIS

The identification of circRNAs in mammals happened in a serendipitous manner when RNA-seq of libraries prepared from ribosome-depleted RNA were computed in order to map non-canonical RNAs derived from genome rearrangements. The work by Salzman et al. (2012) led to the identification of a class of transcripts derived from coding loci and made by exons joint in a reverse order with respect to the one encoded in the genome. These transcripts were demonstrated to be circular in

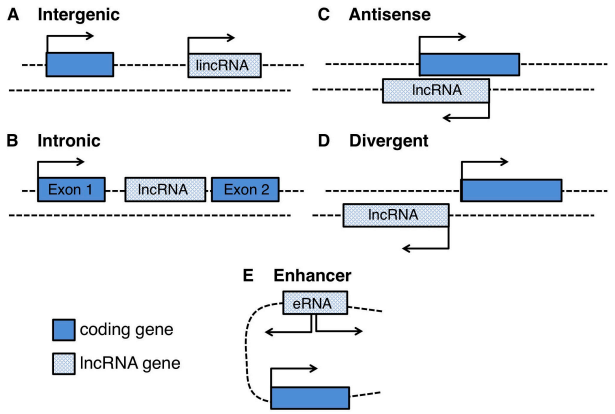

FIGURE 1 | Schematic representation of the genomic loci of different long non-coding RNAs: **(A)** intergenic RNAs (lincRNAs), located between two protein coding-genes, **(B)** intronic lncRNAs, located inside introns of protein coding-genes, **(C)** natural antisense lncRNAs (NATs), transcribed in an antisense orientation with respect to a protein coding-gene **(D)** divergent lncRNAs originated from bidirectional transcription of protein-coding genes, and **(E)** enhancer RNAs (eRNAs) transcribed from bidirectional transcription of enhancer regions.

shape and to derive form a non-canonical splicing event named back-splicing (Salzman et al., 2012; Jeck et al., 2013); indeed, as a consequence of this event, circRNAs result to contain a downstream splice donor joint to an upstream splice acceptor (the back-splice junction, BSJ) (**Figure 2A**). Nowadays, the standard procedure adopted in order to identify circRNAs is the high depth RNA-seq of Ribosomal RNA depleted samples (Glazar et al., 2014). Eventually, the addition of an exonuclease treatment (for instance RNAse R) or poly(A) plus selection limits the presence of linear RNAs thus improving the sequence coverage of circRNAs (Jeck et al., 2013; Panda et al., 2017). Moreover, several pipelines have been developed so far in order to compute the RNA-seq datasets for identifying circRNAs, and is worth mentioning: find_circ (Memczak et al., 2013), CIRCexplorer (Zhang et al., 2014), circBase (Glazar et al., 2014), circRNA_finder (Westholm et al., 2014), and CIRI2 (Hansen, 2018). Since these algorithms differ significantly in the pool of circRNA species they predict, it is recommended to use at least two independent algorithms to ensure proper annotation of the BSJs (Szabo and Salzman, 2016).

Two elements have been described, so far, to facilitate back-splicing: intronic *cis*-elements and/or protein factors acting *in trans* (**Figures 2B,C**).

Analyses of the intronic sequences of genes producing circRNAs revealed that introns flanking circularizing exons are longer than the average and often contain complementary inverted repeats (Jeck and Sharpless, 2014; Liang and Wilusz, 2014; Westholm et al., 2014; Zhang et al., 2014). In humans the repetitive elements are frequently represented by ALU sequences (Jeck et al., 2013; Ivanov et al., 2015) and recent studies showed that the circularization of such exons is affected by the activity of the exonucleases DHX9 and of the editing enzyme ADAR (Rybak-Wolf et al., 2015; Aktaş et al., 2017) both interfering with intron pairing (**Figure 2D**). Notably, a global decrease in ADAR mediating editing of ALU sequences has been observed during the differentiation of human embryonic stem cells toward the neuronal fate (Osenberg et al., 2010); this might explain the overall upregulation of circRNA production occurring during neuronal differentiation observed in both flies and mammals (Rybak-Wolf et al., 2015).

Nevertheless, the presence of intronic repeats *per se* is not sufficient to explain the dynamic and tissue specific expression of circRNAs, that instead relies on the activity of RNA binding proteins (RBPs). In most of the cases, RBPs bind introns in a sequence specific manner and, through dimerization, promote the back-splicing reaction (**Figure 2C**). Muscle blind (MBL) in Drosophila, Quaking (QKI) and Fused in Sarcoma (FUS) in mammals as well as multiple hnRNPs (heterogeneous nuclear ribonucleoproteins) and SR (serine-arginine) proteins are directly involved in facilitating circRNA biogenesis (Ashwal-Fluss et al., 2014; Conn et al., 2015; Kramer et al., 2015; Errichelli et al., 2017; Fei et al., 2017).

It has been also demonstrated that the back-splicing reaction can be further regulated by exon skipping events: the lariat containing the skipped exon can be re-spliced thereby producing a mature circRNA (**Figure 2E**) (Barrett et al., 2015). This mechanism allows the generation of both linear and circular RNAs from a single precursor transcript.

Finally, intron lariats that escape debranching can give rise to a different class of circRNAs, named ciRNAs (circular intronic circRNAs) (**Figure 2A**). Even though, the molecular mechanisms of ciRNA biogenesis is still unknown it has been shown that a consensus RNA motif near the 5′ splice site confers intron lariats the resistance to the debranching activity (Zhang et al., 2013).

LONG NON-CODING RNA FUNCTIONS

At the beginning of the post-genomic era scientists realized that the genome is pervasively transcribed (Lander et al., 2001). By that time, the pioneering studies on H19 and Xist were the only few examples showing the functional role of lncRNAs on imprinting (Bartolomei et al., 1991; Brown et al., 1991). However, the remainder of the full plethora of ncRNAs, their transcriptional significance and functional role, remained controversial for a long time. It was only after RNA-seq techniques were fully available that we could appreciate innovative studies that explored and characterized lncRNA molecular functions in different cellular and molecular contexts. For instance, the studies on MEG3, MALAT1, HOTAIR, and linc-MD1 have revealed the critical and versatile role of lncRNAs in shaping the complex mammalian regulatory networks, through different mechanisms of action (Rinn et al., 2007; Tripathi et al., 2010; Cesana et al., 2011).

Structural features in the lncRNA sequence play a key role in the assembly and regulation of multi-molecular complexes, by controlling the affinity for DNA, RNA, and proteins (Wang and Chang, 2011; Fatica and Bozzoni, 2014). It has been shown that, due to the lack of a functional ORF, the poor conservation of lncRNA molecules only reflects the lower sequence constraint

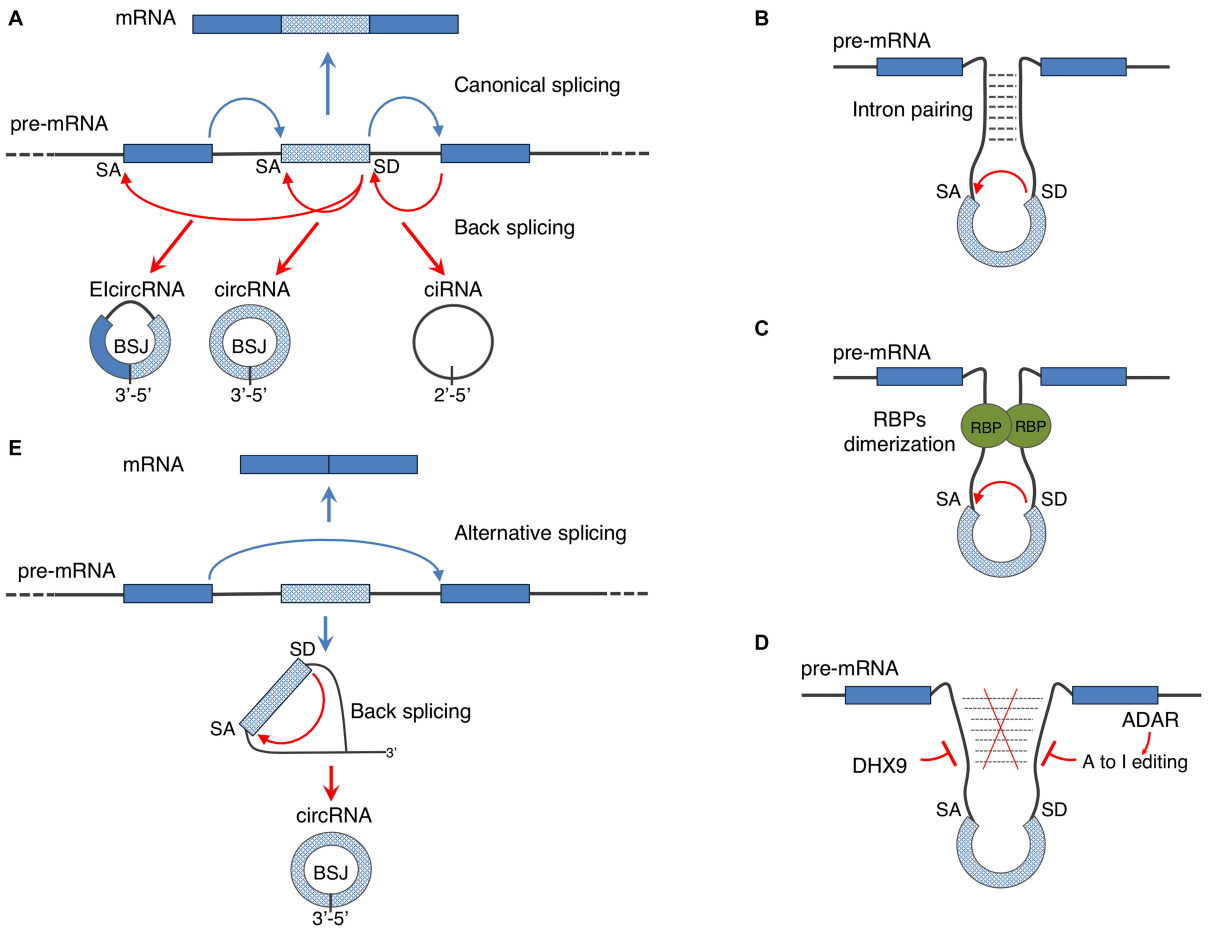

FIGURE 2 | Regulation of circular RNA biogenesis: **(A)** canonical splicing generates mature mRNA while back-splicing can give rise to different kinds of circular transcripts: circRNAs can derive from exonic regions (circRNA), from introns (ciRNA) or from both exons and introns (EIcircRNA). **(B,C)** Back-splicing mechanism can be driven by intron pairing or RNA binding proteins (RBPs) dimerization. **(D)** Inhibitory activity of ADAR and DHX9 enzymes on back-splicing driven by intron pairing. **(E)** As a consequence of alternative splicing events, lariat containing the skipped exon can be re-spliced producing a mature circRNA. Through this mechanism both mature mRNA and a circRNA can be produced from a single pre-mRNA.

and that, instead, conservation of their secondary structures is important for maintaining the functionality of these molecules (Smith et al., 2013; Derrien et al., 2014). On the other hand, lncRNAs show high sequence conservation in their promoter region where binding sites for important regulatory transcription factors allow their tissue-specific expression patterns (Guttman et al., 2009). Indeed, in human and murine embryonic stem cells 60% of lncRNAs identified are divergently transcribed with respect to coding genes and share the same promoter, leading to a coordinated expression of coding and non-coding transcripts during development and differentiation (Sigova et al., 2013).

LncRNAs can be localized in the nucleus, in the cytoplasm and even in or with the mitochondria, and their localization may anticipate their mode of action (Rackham et al., 2011; Cabili et al., 2015; Leucci et al., 2016).

Inside the nucleus the scaffolding property of lncRNAs allows to guide protein factors or complexes to specific genomic loci, thus regulating their transcription and maturation in a positive or negative manner (Engreitz et al., 2016; Morlando and Fatica, 2018) (**Figures 3A–C**). Among these lncRNAs are Xist (recruits PRC2 for H3K27me3 and RYBP-PRC1 for H2A ubiquitylation; Zhao et al., 2010; Tavares et al., 2012), HOTTIP (recruits WDR5/MLL complex for histone H3 lysine 4 trimethylation; Wang et al., 2011) and eRNAs (recruit transcription factors and RNAPII; Kim et al., 2010; Lai et al., 2013). Cytoplasmic lncRNAs regulate gene expression at post-transcriptional level recruiting the appropriate protein machineries affecting the stability (lncRNA TINCR; Kretz et al., 2013), decay (1/2-sbsRNAs lncRNA; Gong and Maquat, 2011), translational activation (lnc-31; Dimartino et al., 2018) and repression (linc-p21; Yoon et al., 2012) of mRNAs (**Figure 3D**). Notably, many evidence reports that NATs have an impact on the sense coding strand in the cytoplasm by using their sequence complementary in order to mask miRNA binding sites (BACE1-AS; Faghihi et al., 2008) or to influence translation (Uchl1-AS; Carrieri et al., 2012). LncRNAs can also act as decoy molecules, which may inactivate transcription factors in the nucleus or sequester miRNAs and RBPs in the cytoplasm thus preventing them to bind their natural

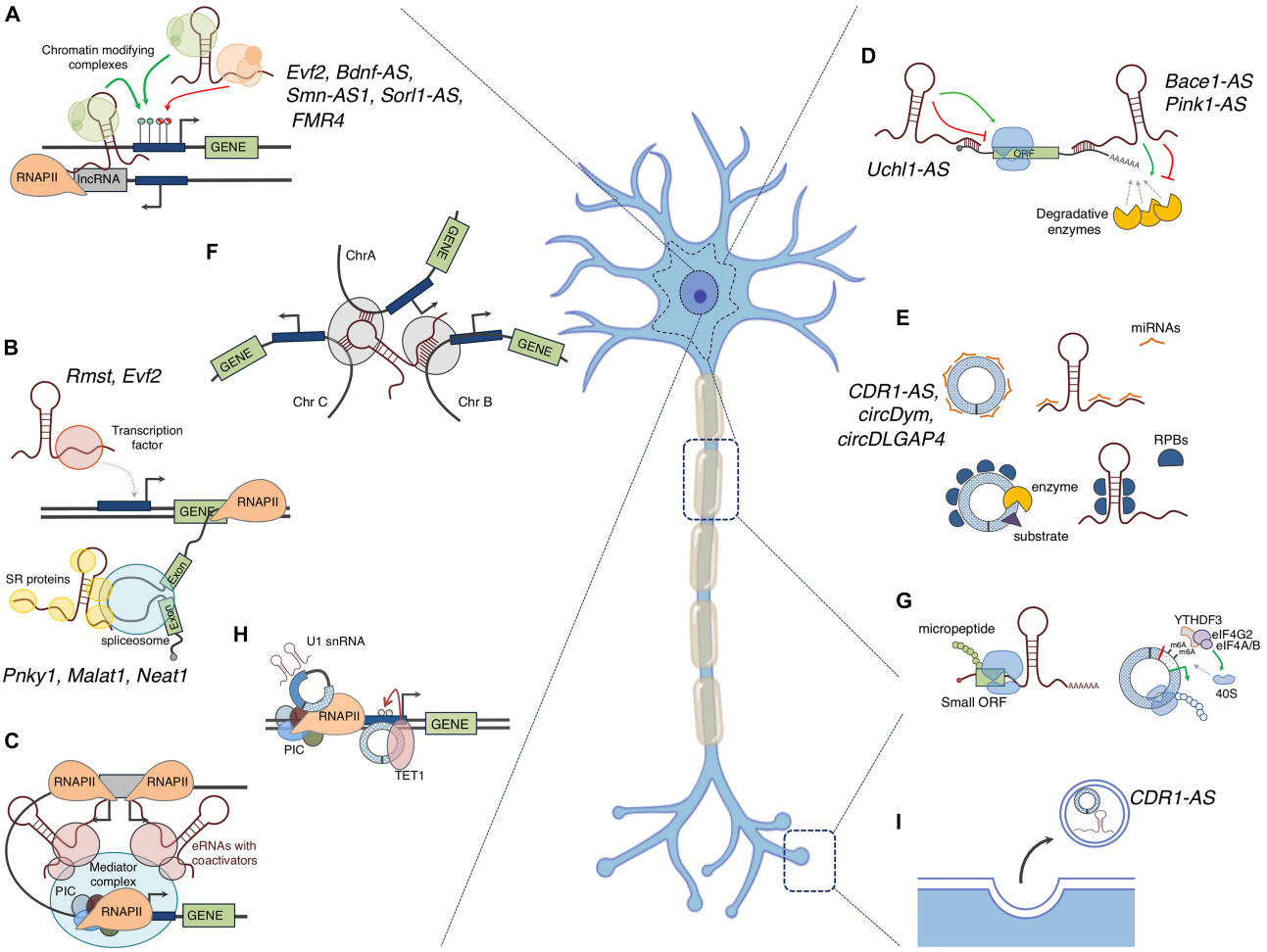

FIGURE 3 | Mode of action of non-coding RNAs: The figure depicts the putative functions ncRNAs in the nucleus and in the cytoplasm of a neuron. **(A)** LncRNAs can act as scaffolds for recruiting chromatin-modifying complexes to gene promoters, thus silencing or inducing gene expression; *cis*- and *trans*- active mechanisms are shown; **(B)** LncRNAs can influence transcription of specific genes by acting as molecular decoys for transcription factors or can induce preferential inclusion or exclusion of exons, thus affecting the mRNA splicing patterns. **(C)** eRNAs can recruit transcriptional activators to distant promoters to activate gene expression; **(D)** LncRNAs can bind to mRNAs thus increasing their stability or inducing their decay. Induction or inhibition of translation is another outcome of the binding of lncRNAs to mRNAs. **(E)** CircRNAs and lncRNAs can interact with microRNAs (miRNAs) and RNA binding proteins (RBPs) titrating them away from their physiological targets or delivering them to the cell periphery (such as synapses). As scaffolds, they can also favor the interaction between enzymes and substrates. **(F)** LncRNAs can participate in genome architecture by coordinating the expression of genes located on different chromosomes. **(G)** LncRNAs can contain a small ORF that can be translated into functional micropeptides. In addition, an ORF can be generated upon circularization of AUG-containing exons, in this case circRNAs are translated in a Cap-independent manner. This translation can occur in the neuronal body or at the periphery, thus contributing to the protein content in this subcellular compartment. **(H)** CircRNAs can regulate transcription of their host genes by interacting with the transcriptional machinery or by recruiting epigenetic factors (TET1); **(I)** LncRNAs and circRNAs can be encapsulated and secreted into extracellular vesicles, for instance in response to neuronal activity, and might act as signal molecules for cell–cell communication. The names of lncRNAs and circRNAs with known functions in the nervous systems are indicated.

targets (Jpx lncrna, Sun et al., 2013; linc-ROR, Wang et al., 2013; linc-MD1, Cesana et al., 2011; lncMyoD, Gong et al., 2015) (**Figures 3B,E**).

Moreover, mRNA processing can be modulated by lncRNAs (Romero-Barrios et al., 2018). NEAT1 and MALAT1 are two examples of lncRNAs able to regulate the splicing of specific pre-mRNAs by modulating the phosphorylation state of splicing factors (Tripathi et al., 2010; Cooper et al., 2014) (**Figure 3B**).

For nuclear lncRNAs, *cis*- and *trans*- regulatory mechanisms have been described. *Cis*-acting lncRNAs affect the expression of proximal loci; examples are the NATs that interfere with the expression of the antisense coding gene by repressing or promoting its expression. The *cis*-regulatory mechanism is mediated via NAT transcription *per se* or through RNA-RNA interactions with the overlapping transcript. In this latter case, splice sites can be masked leading to alternative splicing events or transcript availability can be reduced through nuclear retention (Wanowska et al., 2018). A very common mechanism of *cis*-activity of NATs is the recruitment of epigenetic machineries, like the PRC2 thus influencing the chromatin state of their

antisense coding gene (Yu et al., 2008; Modarresi et al., 2012) (**Figure 3A**).

Differently, *trans*-active lncRNAs affect the expression of target genes that are located in different chromosomes (Chu et al., 2011; Vance et al., 2014) (**Figure 3A**).

In the last few years several evidence has emerged tightly linking transcription and the three-dimensional organization of the genome, so adding an additional layer of complexity in gene expression regulation (Mele and Rinn, 2016). However, the cause-consequence relationship between transcription and genome re-organization was not univocally determined (Osborne et al., 2004; Zink et al., 2004). Many lncRNAs might participate in genome architecture through the simple act of transcription, independently of the mature RNA product or of their generally low abundance and lack of sequence conservation (Hangauer et al., 2013; Ballarino et al., 2018). Moreover, some mature lncRNA transcripts, such as XIST, FIRRE, and NEAT1, are known to play a role in organizing nuclear architecture (Mao et al., 2011; Engreitz et al., 2013; Hacisuleyman et al., 2014) (**Figure 3F**).

Ultimately, many apparent non-coding transcripts were revelaled to be associated to the ribosome and to produce small peptides (Hube and Francastel, 2018) (**Figure 3G**). The non-coding definition of lncRNAs relies on the arbitrary threshold of an ORF shorter than 100 aminoacids (Carninci et al., 2005); however, ribosome profiling revealed that translation is more pervasive than previously thought (Ingolia, 2014). Examples of lncRNA-derived micropeptides are myoregulin (encoded by the LINC00948 RNA in humans and by the 2310015B20Rik RNA in mice) and DWORF (encoded by LOC100507537 gene in humans and by NONMMUG026737 gene in mice) which regulate muscle performance by affecting the activity of the key calcium pump SERCA in a negative and positive manner respectively (Anderson et al., 2015; Nelson et al., 2016). Notably, DWORF may be useful therapeutic molecule in improving the cardiac muscle function of mammals with heart disease (Nelson et al., 2016). Ribosome associated lncRNAs have also displayed a dual function both as peptide coding and as ncRNA and are now termed bifunctional (Li et al., 2017). The lncRNA Oskar, involved in oocyte development is a prototypical example in this expanding research area (Jenny et al., 2006).

CIRCULAR RNA FUNCTIONS

The majority of circRNAs identified shows cytoplasmic localization even though there are some examples of nuclear localized molecules (Salzman et al., 2012; Jeck et al., 2013; Li et al., 2015a; Errichelli et al., 2017; Chen et al., 2018a). In these two compartments circRNAs control gene expression at different levels through mechanisms that have not been fully characterized.

Indeed, the function of few circRNAs has been unveiled so far. Several studies reported circRNAs exerting miRNA "sponge" activity, thereby altering the expression of the miRNA targets (Hansen et al., 2013; Memczak et al., 2013; Zheng et al., 2016; Bach et al., 2019) (**Figure 3E**). The most notable cases are the mouse Sry and the human CDR1-AS (also known as ciRS-7) which respectively, possess 16 binding sites for miR-138 and more than 70 evolutionary conserved binding sites for miR-7 (Hansen et al., 2013; Memczak et al., 2013). In particular CDR1-AS is expressed much more than any other housekeeping gene in mouse and human brain and this, together with the high number of miR-7 binding sites, makes the competing activity of CDR1-AS stoichiometrically relevant in neuronal tissue (Hansen et al., 2013). More recently, Piwecka et al. (2017) showed that CDR1-AS may modulate the activity of miR-7 by acting on its stability. Consistently with these data, the depletion of CDR1-AS causes the modulation of miR-7 targets mRNAs both *in vivo* and *in vitro* (Hansen et al., 2013; Memczak et al., 2013).

In addition to miRNAs, circRNAs can interact with RBPs. They may sequester them from other targets or regulate their stability/activity (Ashwal-Fluss et al., 2014; Du et al., 2017; Liu et al., 2019; Rossi et al., 2019); circRNAs may also act as molecular scaffolds to allow enzymes and substrates to become adjacent (Du et al., 2017; Zeng et al., 2017) (**Figure 3E**).

Examples of circRNAs containing an ORF, thus serving as templates for translation, have also been described (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2018; Zhang et al., 2018a; Liang et al., 2019). Given their circular structure, the translation of these circRNAs relies on a Cap-independent mechanism and on the presence of the N6-methyl-adenosine modification. This latter promotes the binding of the reader protein YTHDF3 and the IRES-specialized translation initiation factor eIF4G2 (Legnini et al., 2017; Yang et al., 2017) (**Figure 3G**).

In the nucleus, circRNAs can participate in gene expression regulation at transcriptional level. For instance, circPAIP2 and FECR1 circRNA have been described to regulate the transcription of their parental genes through two diverse mechanisms: interacting with transcription machinery, whilst the latter by recruiting the TET1 DNA demethylase (Li et al., 2015a; Chen et al., 2018a) (**Figure 3H**).

ROLE OF NON-CODING RNAs IN THE PHYSIOLOGY AND DEVELOPMENT OF THE NERVOUS SYSTEM

LncRNAs show a crucial role in many stages of neuronal differentiation and specification by participating in gene expression control at the epigenetic, transcriptional, and post-transcriptional levels. LncRNA molecules may regulate the exit from the pluripotency state, influence cell fate choice during neural development and contribute to the synaptic activity in mature cells (Zalfa et al., 2003; Zhong et al., 2009; Guttman et al., 2011; Ng et al., 2012; Vance et al., 2014). The lncRNA mechanism of action is strictly linked to their secondary structure and their scaffolding activity, which translates into the ability to regulate gene expression by binding and recruiting secondary factors onto regulative domains. Here we report some examples that are noteworthy for the study of nervous system differentiation and in the maintenance of its physiological functionality (**Table 1**). RMST is a lncRNA controlled by the master regulator REST

during neural differentiation (Ng et al., 2013). Depletion of RMST prevents cells from exiting the embryonic stem cell state and inhibits the initiation of neural differentiation. Indeed, RMST acts as a scaffold RNA by guiding the transcription factor Sox2 to the promoter of key neurogenesis-promoting genes, such as DLX1, ASCL1, HEY2, and SPS (Ng et al., 2013).

Evf2 lncRNA also regulates cell-fate choice and is a clear example of how RNA secondary structure may contribute through a variety of modes of action. This lncRNA is a NAT to the DLX6 gene and recruits the DLX transcription factor and the methyl-CpG-binding protein (MECP2) to the promoters of interneuron lineage genes (DLX5, DLX6, and GAD1) through both *cis*- and *trans*- acting scaffolding mechanisms, ultimately controlling the excitatory-inhibitory neurons balance *in vivo* (Bond et al., 2009). In addition, the Evf2 transcript is implicated in controlling the methylation state of DLX5/6e enhancer through a chromosomal looping mechanism, thus regulating the expression of DLX5/6 locus (Berghoff et al., 2013). However, how the Evf2 methylation control is combined with the recruitment of DLX and MECP2 is still unknown.

Pnky is a lncRNA that is few kilobases divergent from the Pou3f2 gene. This lncRNA, whose expression peaks in dividing neural stem cells (NSCs) and decreases during neuronal differentiation, is essential for self-renewal of the NSC population. Pnky interacts with the splicing regulator PTBP1 and controls the expression and alternative splicing of a core set of targets involved in neurogenesis (Ramos et al., 2015). Through this mechanism Pnky regulates the balance between self-renewal and differentiation of NSCs.

An interesting variation of this mode of action has been described for the lncRNA Cyrano that is highly expressed in the nervous system and recently shown to be implicated in a whole non-coding regulatory circuitry (Kleaveland et al., 2018). Kleaveland et al. (2018) identified a post-transcriptional regulatory network in which Cyrano binds miR-7, leading to its degradation through a target RNA-directed miRNA degradation mechanism (TDMD). Mir-7 degradation in nervous tissue blocks the repression of its RNA targets among which the circRNA CDR1-AS. Notably, Cyrano knock-down in zebrafish causes a neurodevelopmental phenotype (Ulitsky et al., 2011).

LncRNAs specifically expressed in *in vitro* derived murine motor neurons have also been identified (Biscarini et al., 2018). Two interesting cases are Lhx1os and lncMN-1 that are divergent from Lhx1 and Pcdh10 protein-coding genes respectively. Lhx1 encodes for a morphogenetic factor of the LIM family involved in lateral motor neuron differentiation, head development and motor neuron axon guidance, while Pcdh10 codes for a protocadherin involved in motor neuronal cell adhesion (Tsuchida et al., 1994; Hunter and Rhodes, 2005; Machado et al., 2014). Notably, Lhx1os and lncMN-1 show strong co-regulation with two divergent coding transcripts in both mouse and human motor neurons.

LncRNAs were shown to participate in mature neuron plasticity and physiology. Indeed, despite being post-mitotic cells, neurons need to maintain a high level of plasticity in order to be able to respond to stimuli and to re-arrange their synaptic network for accompanying processes such as learning, memory, cognition and recovery from injury or insult (Costa-Mattioli et al., 2009; West and Greenberg, 2011). The lncRNA BC1/BC200 regulates synaptogenesis. This transcript is expressed in the developing and adult nervous system where is transported to dendrites (Muslimov et al., 1997). In these cellular structures BC1/BC200 interacts with FMRP and the translational machinery in order to control the formation of the 48S complex leading to a repression of local translation at synapses (Wang et al., 2002; Zalfa et al., 2003). Moreover, the expression of BC1/200 is dynamically upregulated at specific synapses in response to neuronal activity, thus shaping the synaptic protein content (Muslimov et al., 1998).

MALAT1, initially characterized for its role in cancer metastasis, was then identified to have a role in synapse formation (Ji et al., 2003). This lncRNA is highly expressed in neurons and it is localized in nuclear speckles; MALAT1 controls the processing of synaptogenesis-related genes through the recruitment of SR-protein family members to transcription sites (Bernard et al., 2010).

Finally, some interesting mechanisms involving NATs have been shown to contribute to the regulation of neuronal plasticity. BDNF-AS, whose expression is dependent on neural activity also contributes to the decision between synaptic maintenance or elimination in response to the levels of stimulation by controlling the expression of its sense overlapping gene BDNF (Lipovich et al., 2012; Modarresi et al., 2012).

Similarly to lncRNAs, circRNAs have been recognized to play important roles in development and function of neuronal tissue (van Rossum et al., 2016) (**Table 1**). Recent studies have demonstrated that circRNAs are specifically enriched in brain tissue and in particular, they revealed that compared to other tissues, the mammalian brain contains the highest percentage of genes hosting circRNAs and that these genes hold the ability to produce the greatest number of distinct circRNAs (Rybak-Wolf et al., 2015).

Notably, neuronal circRNAs resulted to be regulated during embryonic development and conserved between rodents, pigs and humans (Westholm et al., 2014; Rybak-Wolf et al., 2015; Venø et al., 2015; Chen et al., 2018b). In a model system of neuronal differentiation the overall expression of circRNAs resulted to be significantly upregulated and gene ontologies of their host genes showed their enrichment in neuronal specific pathways such as neuron development, differentiation, and synaptic transmission (You et al., 2015). Other studies further showed that circRNAs result differentially expressed in various anatomic brain regions and at specific embryonic or postnatal stages (Rybak-Wolf et al., 2015; Venø et al., 2015). Moreover, based on differential expression between adult and aged brains, these studies also showed that the expression of specific circRNAs is aging-related (Westholm et al., 2014; Gruner et al., 2016; Xu et al., 2018). Some examples are the murine circRims2 and circDym which are expressed more than 50% in adult cerebellum and circPldxnd1 which instead is predominantly expressed in prefrontal cortex (>60%) with respect to the other brain regions (Rybak-Wolf et al., 2015). In addition, Venø et al. (2015) found that during the porcine embryonic brain development the expression of clusters of specific circRNAs coincides with distinct

TABLE 1 | List of ncRNAs with a known or potential function (asterisk) in neuronal physiology and disease.

Name	Classification	Proximal/overlapping coding gene	Molecular function	Biological function	Disease	References
RMST	lincRNA	N/A	Decoy for transcription factor	ESC self-renewal and inhibition of neural differentiation	N/A	Ng et al., 2013
Evf2	NAT	Dlx6	Scaffold RNA for transcription factors and chromatin remodeling	Neuronal differentiation and development	N/A	Bond et al., 2009
Pnky	Divergent	Pou3f2	Decoy for splicing factors	Neural Stem Cell self-renewal	N/A	Ramos et al., 2015
Cyrano	lincRNA	N/A	target RNA-directed miRNA degradation	ESC self-renewal	N/A	Kleaveland et al., 2018
Lhx1os	Divergent	Lhx1	Unknown	Neuronal Differentiation	ALS	Biscarini et al., 2018
LncMN-1	Divergent	Pcdh10	Unknown	Neuronal Differentiation	ALS	Biscarini et al., 2018
BC200	Intronic	Epcam-DT	Scaffold RNA for translation factors	Regulates translation at synapsis	AD	Muslimov et al., 1998; Wang et al., 2002; Zalfa et al., 2003
MALAT1	lincRNA	N/A	Decoy for splicing factors, paraspeckle	Neuronal Stress Response	ALS, HD, FTD, AD	Ji et al., 2003; Bernard et al., 2010
BDNF-AS	NAT	Bdnf	Scaffold RNA	Synaptogenesis	HD	Lipovich et al., 2012; Modarresi et al., 2012
SMN-AS1	NAT	Smn	Scaffold RNA	Neuronal Differentiation	SMA	d'Ydewalle et al., 2017
SORL1-AS	NAT	Sorl1	Scaffold RNA	Unknown	AD	Ciarlo et al., 2013
BACE1-AS	NAT	Bace1	RNA-RNA interaction for miRNA masking	Beta amyloid formation	AD	Faghihi et al., 2008; Faghihi et al., 2010
UCHL1-AS	NAT	Uchl1	RNA-RNA interaction through SINEUP	Neuronal Stress Response	AD, PD	Carrieri et al., 2015
NEAT1	lincRNA	N/A	Scaffold RNA for splicing factors, Paraspeckle	Neuronal Stress Response	ALS, HD, FTD, AD	Nishimoto et al., 2013; An et al., 2018
C9ORF72-AS	NAT	C9ORF72	RNA foci	Unknown	ALS	DeJesus-Hernandez et al., 2011; Cooper-Knock et al., 2015b
PINK1-AS	NAT	Pink1	RNA-RNA interaction in *cis* on Pink1	Mithocondrial function	PD	Scheele et al., 2007
FMR4	NAT	Fmr1	Scaffold RNA in trans for chromatin remodeling	Neural precursor proliferation	FXS, FXTAS	Khalil et al., 2008; Peschansky et al., 2016
HTT-AS	NAT	Htt	Decoy for transcription factor in *cis* on HTT	Unknown	HD	Chung et al., 2011
ATXN8-OS	NAT	Sca8	RNA foci	Unknown	SCA	Moseley et al., 2006
SCAANT1	NAT	Sca7	Decoy for transcription factor in *cis* on Sca7	Unknown	SCA	Sopher et al., 2011
CDR1-AS	circRNA	CDR1	miRNA sponge	Synaptic transmission	AD, PD	Hansen et al., 2013; Memczak et al., 2013; Piwecka et al., 2017
circRims2	circRNA	Rims2	Unknown	Unknown	N/A	Rybak-Wolf et al., 2015
circDym	circRNA	Dym	miRNA sponge	Microglial activation	Depressive-like behavior	Rybak-Wolf et al., 2015; Zhang et al., 2018b
circPldxnd1	circRNA	Pldxnd1	Unknown	Unknown	N/A	Rybak-Wolf et al., 2015
circStau2	circRNA	Stau2	Unknown	Unknown	N/A	Rybak-Wolf et al., 2015
circHomer1_a	circRNA	Homer1	Unknown	Homeostatic synaptic plasticity*	N/A	You et al., 2015
mmu_circRNA_017963	circRNA	Tbc1d30	miRNA sponge*	Apoptotic process, transport and RNA splicing, synaptic functions*	AD	Huang et al., 2018
circDLGAP4	circRNA	Dlgap4	miRNA sponge	Cell viability, apoptosis, mitochondrial damage, and autophagy	PD	Feng et al., 2019

N/A is for "Not Applicable."

developmental transitions and that the maximum expression and complexity of circRNAs is reached at a time corresponding to the period of major neurogenesis (form day 48 to day 60). Finally, it has been also demonstrated that circRNA expression is modulated during *in vitro* differentiation of murine motor neurons with specific circRNAs exclusively expressed in this cell type (Errichelli et al., 2017).

All these data strongly support the idea that circRNAs play important biological functions during development and specification of the nervous system.

Besides the expression modulation, neuronal circRNAs also show an intriguing subcellular localization since they have been found to be enriched in synaptoneurosomes more than their linear counterparts (Rybak-Wolf et al., 2015; Venø et al., 2015; You et al., 2015). An example is circStau2 that is mainly located at synapses while the linear Stau2 is primarily cytoplasmic (Rybak-Wolf et al., 2015). Furthermore, the most abundant neuronal circRNAs derive from genes encoding for proteins associated with synaptic functions (Venø et al., 2015; You et al., 2015). In this regard, You et al. (2015) demonstrated that circHomer1_a, which originates from the Homer1 gene encoding for a key protein in post-synaptic density regulation, reaches its highest expression and synaptic localization during synaptic plasticity in cultured hippocampal neurons (Meyer et al., 2014; You et al., 2015). In the same study, You et al. (2015) also analyzed the expression of circRNAs at various stages of brain development in mice (from E18 to P30) and showed that in hippocampal neurons an abrupt postnatal shift in circRNA expression (and not of the linear host transcripts) occurs when synaptogenesis begins. Lastly, mice carrying a deletion of the CDR1-AS genomic region exhibited defects in excitatory synaptic transmission and impaired sensorimotor gating; an increased spontaneous vesicle release was also observed in the neurons of these mice, suggesting that CRD1-AS might have a role in regulating synaptic transmission (Piwecka et al., 2017).

Even though, the molecular mechanism allowing the circRNAs delivery at the neuronal periphery is still unknown, all the findings suggest a role for these RNAs in the formation and activity of specific neuronal structures: circRNAs might be selectively transported to synapses to regulate their activity functioning as sponges/cargo for miRNAs and RBPs (**Figure 3E**). In this way they might indirectly regulate the expression of the miRNA/RBP-targeted mRNAs at synapses. Alternatively, synaptic circRNAs might function as signal molecules since they can also be packaged into vesicles and released into the synaptic cleft to influence neighboring cells (Li et al., 2015b; Lu and Xu, 2016) (**Figure 3I**).

LONG NON-CODING RNAs IN NEURODEGENERATIVE DISEASES

Alteration of lncRNA expression has been extensively described for many neuronal diseases together with their wide implication in the formation of aberrant molecular pathways (Salta and De Strooper, 2017). In addition, among the non-coding genes, lncRNAs are highly expressed in the nervous system and have been often observed to be located in proximity to neuronal genes and loci associated with neurodegenerative diseases (Qureshi et al., 2010; Wei et al., 2018). Starting from their genomic localization (intergenic, antisense, intronic, etc.) researchers focused on understanding the role that these molecules may play in *cis* or *trans* to control gene expression. As described above, several mechanisms of action have been observed and here we report some evidence where lncRNA activity is altered in neurodegenerative diseases (**Table 1**). Numerous examples are of NATs involved in neuronal processes and associated with neurodegeneration. NATs can regulate gene expression by recruiting chromatin modifiers (i.e., SMN-AS1) or by impacting the splicing of the sense coding-strand (i.e., SORL1-AS). Other lncRNAs work independently from their neighboring genes and may act as scaffolds in specific stress related paraspeckles (i.e., NEAT1), rather than regulate mRNA stability by forming RNA-RNA interactions (i.e., BACE1-AS, UCHL1-AS, PINK1-AS).

In spinal muscular atrophy (SMA) mutations in the SMN1 gene, encoding for the survival motor neuron protein (SMN), is the cause of the disease. In addition to SMN1 gene, SMN protein can be also produced by a splicing variant of SMN2, a gene present in variable copies for every individual. When mutations of SMN1 occur, depending on SMN2 copy number, SMA develops showing diverse clinical severities (Gavrilov et al., 1998). d'Ydewalle et al. (2017) identified a SMN antisense transcript (SMN-AS1), whose expression levels increased in neuronal differentiation, inversely correlating with the SMN protein. They found that SMN-AS1 recruits the chromatin modifier PRC2 to the SMN2 promoter repressing its gene expression. SMN-AS1 knock-down dissociates PRC2 from the promoter, thus increasing the overall SMN protein levels in neurons. This indicates that the levels of SMN-AS1 play an important role in the balance of residual SMN protein, thus impacting on the clinical outcome of the SMA disease (d'Ydewalle et al., 2017).

SORL1 antisense RNA (SORL1-AS) is produced from the Sortilin Related Receptor 1 (SORL1), a gene involved in amyloid-β formation in neuronal cells (Massone et al., 2011). Observations in post-mortem cerebral cortices of Alzheimer disease (AD) compared to healthy individuals showed that SORL1 levels increase and inversely correlate with SORL1-AS (Ciarlo et al., 2013). Ciarlo et al. (2013) found that SORL1-AS expression drives a splicing shift of SORL1 from the synthesis of the canonical long protein variant A to an alternatively spliced protein isoform. This process, resulting in a decreased synthesis of SORL1 variant A, is associated with impaired processing of amyloid precursor protein (APP) leading to increased amyloid β formation. The level and the activity of SORL1-AS in the etiology of the disease becomes crucial and may represent an interesting target for future therapeutic strategies (Ciarlo et al., 2013).

NEAT1 is a mammalian lncRNA that is ubiquitously expressed and has a scaffold role in the formation of subnuclear bodies termed paraspeckles. It presents two major isoforms, a 3.7 kb NEAT1_1 and 23 kb NEAT1_2 (Hutchinson et al., 2007). Nishimoto et al. (2013) observed NEAT1_2 to be upregulated during the early stages of amyotrophic lateral sclerosis (ALS) pathogenesis and found it to be present in paraspeckles of ALS patients, providing, for the first

time, a direct association between paraspeckle formation and neurodegenerative disease (Nishimoto et al., 2013). Paraspeckles are stress-responsive nuclear bodies, which increase in size and number and accompany several physiological as well as pathological stressful conditions (An et al., 2018). Mechanistically, increased paraspeckle formation is observed in ALS and is accompanied by nuclear depletion of TDP-43, a protein frequently dysregulated in ALS (Mackenzie et al., 2010). Indeed, TDP-43 binds NEAT1_2, and when mutated or downregulated affects NEAT1_2 accumulation and paraspeckle assembly (Nishimoto et al., 2013). In Huntington disease (HD), frontotemporal dementia (FTD), and AD an increase of NEAT1_2 was observed in patient cohorts and experimental studies suggest that NEAT1_2 fine-tunes the function of multiple neurodegeneration-associated pathways, like mitochondrial signaling and miRNA biogenesis (An et al., 2018).

BACE1-AS is an antisense transcript originating from the BACE1 (β secretase 1) gene coding for a protein which is essential for the generation of β-amyloids. This lncRNA is evolutionarily conserved across vertebrates and was observed to be elevated in subjects with AD and in APP transgenic mice (Faghihi et al., 2008). BACE1-AS has an important role in enhancing the stability of BACE1 mRNA via the formation of a RNA duplex, thus masking miR-485-5p binding sites and leading to an increase of BACE1 protein (Faghihi et al., 2010). The knock-down of this antisense transcript decreases the level of BACE1, reducing amyloid formation and aggregation in the brain. BACE1-AS represents a clear biomarker and potential therapeutic target for the treatment of AD.

Another antisense RNA is UCHL1-AS (ubiquitin carboxy-terminal hydrolase L1-antisense) associated with AD, whose activity depends on the presence of a 5′ overlapping sequence with UCHL1 and an embedded inverted SINEUP (SINEB2 sequence to UP-regulate translation). Thanks to the formation of the RNA-RNA duplex with UCHL1 mRNA, UCHL1-AS enhances Cap-independent UCHL1 protein translation under stress condition (Carrieri et al., 2012). UCHL1 expression is associated with a delay of AD, making UCHL1-AS a fundamental regulator of the disease onset and a promising target for therapeutic intervention. Interestingly, both UCHL1 and UCHL1-AS are also found to be downregulated in Parkinson's disease (PD) (Carrieri et al., 2015).

Another antisense transcript, PINK1-AS, is transcribed from the antisense direction of the PINK1 gene (PTEN phosphatase and tensin homolog deleted on chromosome 10-induced putative kinase 1), a gene abundant in mitochondrial-rich tissues and often mutated in PD (Gispert et al., 2009). PINK1-AS controls *in cis* the expression of a PINK1 splice variant, the svPINK1 transcript, through the formation of a RNA-RNA duplex. The silencing of PINK1-AS results in the reduced expression of svPINK1 in neuronal cells (Scheele et al., 2007). Given that, svPINK1 codes for a homolog of the C-terminus of PINK1, a peptide sequence which regulates PINK1 kinase activity; modulation of PINK1-AS expression may therefore have direct relevance in PD.

Given these examples, it is of no surprise that lncRNAs may play crucial roles in many other molecular processes involved in neurodegeneration. Importantly, except for few reports, most of these molecules are located in proximity or antisense to critical neuronal loci and so the dissection of these specific classes of molecules requires specific attention.

ANTISENSE TRANSCRIPTION OF NUCLEOTIDE REPEAT EXPANSIONS IS INVOLVED IN NEURODEGENERATION

A conspicuous amount of evidence coming from the transcription of nucleotide expansions emphasizes a role of NATs alongside the coding sense strand in the etiology of neurodegenerative diseases (Salta and De Strooper, 2017; Zucchelli et al., 2019). Here, we describe how nucleotide repeat expansion-associated NATs impact on the disease through complex gain- and loss- of-function mechanisms. One well-exemplified case is described for C9ORF72, a gene that harbors a hexanucleotide repeat expansion representing the most common cause of FTD and ALS. In the mutated loci the sense strand codes for an ORF that produces a repetition of six dipeptide proteins (RAN) forming co-aggregates in the cytoplasm of neurons (DeJesus-Hernandez et al., 2011). This locus is also transcribed in the antisense direction and both the sense/antisense RNA transcripts accumulate to form disease-associated nuclear RNA foci, the number of which correlates with the clinical severity of ALS and FTD (DeJesus-Hernandez et al., 2011; Cooper-Knock et al., 2015a). Interestingly, the knock-down of sense expanded C9ORF72 transcripts through the use of antisense single-stranded oligonucleotides (ASOs) in *in vitro* derived motor neurons mitigates defects in nucleocytoplasmic transport and glutamate toxicity phenotype, but is not sufficient to fully revert the molecular signatures derived from the hexanucleotide expansion (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Zhang et al., 2015; Jiang et al., 2016). These findings strongly suggest that the antisense ncRNA and the associated RNA foci contribute to the neurodegenerative phenotype. Notably, antisense C9ORF72 RNA foci hijack RBPs as sense RNA foci but, differently from these, antisense foci are associated with TDP-43 mislocalization in motor neurons from C9ORF72 patients (Cooper-Knock et al., 2014, 2015b). All these findings point to the importance of targeting both sense and antisense expanded C9ORF2 transcripts in order to develop an effective therapeutic approach.

Fragile X mental retardation 1 (FMR1) locus is characterized by the production of multiple non-coding transcripts (FMR5, FMR6, FMR4) in addition to the FMR1 mRNA. Expansion of the CGG triplet in the FMR1 gene (>200 repeats for complete penetrance) is attributed as the main cause of Fragile X syndrome (FXS), and in a pre-mutation state (55–200 repeats) is responsible for Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) (Rajan-Babu et al., 2017). Although the pathogenic relevance of all the FMR1 associated transcripts remains to be fully defined, FMR4 is a lncRNA antisense to FMR1 that spans the repeated region and that was observed to significantly affect human cell proliferation and apoptosis *in vitro*

(Khalil et al., 2008). Peschansky et al. (2016) confirmed the proliferative effect of FMR4 also in human neural precursor cells (hNPCs) and determined that this lncRNA alters the chromatin state of 100s of genes *in trans*, with a significant enrichment for genes involved in neural development and proliferation (Peschansky et al., 2016).

In HD expansion of a CAG-repeat in the huntingtin gene (HTT) results in an elongated polyglutamine stretch and is the main cause of the pathology. From the HTT locus, two ncRNAs are produced: a small CAG containing RNA (sCAG) of around 21 nts with neurotoxic Ago2-dependent activity and an antisense lncRNA (HTT-AS) overlapping with the repeated expansion and observed to be reduced in human HD frontal cortex (Chung et al., 2011; Bañez-Coronel et al., 2012). Additionally, HTT-AS acts as a transcriptional repressor of HTT gene, thus suggesting a protective role of this lncRNA in the penetrance of the disease (Chung et al., 2011).

In spinocerebellular ataxias (SCA) CAG expansions are found in several loci that code for poly-Q SCA proteins. In the SCA8 locus, an antisense transcript (ATXN8-OS/KHL1-AS) that includes the reverse complement of the expansion (CTG) is produced and accumulates in RNA foci in the brain (Moseley et al., 2006); in the SCA7 locus, convergent transcription is also found to produce an antisense SCAANT1 lncRNA. SCAANT1 suppresses SCA7 expression in mice and inversely correlates with SCA7 expression in SCA patients, thus suggesting a loss-of-function mechanism where the lncRNA is involved (Sopher et al., 2011).

Altogether these observations prove that NATs may impact on the penetrance and severity of the clinical symptoms of many neuronal diseases, thus re-centering the attention of the research on new therapeutic strategies and modes of intervention as described in the following paragraphs.

CIRCULAR RNAs IN NEURODEGENERATIVE DISEASES

Recent investigations have suggested that circRNAs not only function in physiological conditions but that they may also play crucial roles in the occurrence and development of neurological diseases (**Table 1**).

The first evidence comes from the observation that deficiency or mutations in proteins involved in circRNA biogenesis are linked to the pathogenesis of neurodegenerative diseases: for instance deficiency of QKI may contribute to the development of inherited ataxia while mutations of the FUS gene as well as the deregulation of ADAR2 expression are linked to the pathogenesis of ALS (Chénard and Richard, 2008; Kwiatkowski et al., 2009; Vance et al., 2009; Hideyama et al., 2012; Aizawa et al., 2016).

The implication of circRNAs in neuronal diseases is further supported by studies of expression profiling performed in cellular or animal model systems and by using patients' specimens. Regardless the different modes of action attributed to circRNAs, their activity as miRNA sponges is the only one explored in all the studies reporting implications of these ncRNAs in neuronal disorders. However, this evidence is far from considering the miRNA sponge activity as a general mode of action of this class of ncRNAs in neurons. Indeed, besides CDR1-AS, most of the circRNAs defined as sponges have only one or a very few binding sites for miRNAs, making the effectiveness of their sponge activity questionable (Li et al., 2019; Ragan et al., 2019). Nevertheless, it has been detected that subsets of circRNAs could act in concert to exert reasonable miRNA sponge function (Ragan et al., 2019).

Huang et al. (2018) have identified more than 300 circRNAs deregulated in the hippocampus of 5- and 10- month-old senescence-accelerated mice P8 (SAMP8), an AD animal model, compared to WT mice. Among them, they characterized mmu_circRNA_017963 circRNA, which might be involved in several cellular processes including apoptosis and synaptic function (Huang et al., 2018). Furthermore, microarray technology combined with RNA-seq analysis allowed to simultaneously characterize circRNA, miRNA and mRNA expression in the hippocampus of an AD rat model in order to build putative regulatory networks linked to AD pathogenesis (Wang et al., 2018b).

These analyses identified two possibly AD-linked networks involving the genes Iodothyronine Deiodinase 2 (Dio2) and the high-mobility group box 2 (HMGB2). In particular, the expression of Dio2, that activates myelination, and of HMGB2 which controls the amyloid-β plaque clearance, is altered in AD (Calza et al., 2002; Gao et al., 2011; Humphries et al., 2015; Yamanaka et al., 2015). The networks identified by *in-silico* analyses have linked the deregulation of these two genes to the aberrant expression of specific circRNAs acting as sponges of mir-122-5p for Dio2 and of let-7-g-3p for HMGB2.

Furthermore, the most expressed and studied circRNA, CDR1-AS, was found reduced in hippocampal CA1 samples from sporadic AD patients compared to controls (Lukiw, 2013). As aforementioned, CDR1-AS has multiple binding sites for miR-7 and its reduction in AD conditions has been hypothesized to increase the levels miR-7 which in turn targets the ubiquitin protein ligase A (UBE2A). Notably, UBE2A plays an essential function for the proteolytic clearance of amyloid-β peptides and its expression is indeed reduced by 2.8 folds in the hippocampal CA1 regions of AD brains (Zhao et al., 2016). Through a similar mechanism CDR1-AS could be also involved in PD since also α-synuclein is a target of miR-7 (Junn et al., 2009). Indeed, it has been demonstrated that the repression effect of miR-7 on α-synuclein expression in human cell lines can be rescued by the concomitant overexpression of CDR1-AS (Hansen et al., 2013).

CircDLGAP4, originally reported to ameliorate ischemic stroke outcomes, is found to be downregulated in both MPTP-induced PD mouse and MPPþ-induced PD cell models (Bai et al., 2018; Feng et al., 2019). Feng et al. (2019) demonstrated that circDLGAP4 participates in PD biological processes regulating miR-134-5p activity. The reduction of CircDLGAP4 expression in PD conditions allows miR-134-5p to repress CREB1 and, as a consequence, the CREB1 target genes including BDNF, Bcl-2 and PGC-1. This would contribute to the development of PD via affecting cell viability, apoptosis, mitochondrial damage and autophagy in human and mouse (Feng et al., 2019).

Deregulation of circRNAs and not of their linear counterparts, has also been reported in *in vitro* derived motor neurons

lacking the FUS gene or carrying FUS mutations linked to a severe form of familial ALS (Errichelli et al., 2017; D'Ambra et al., 2019), suggesting a possible role of circRNAs in the pathogenesis of this disorder. In particular, Errichelli et al. (2017) demonstrated that FUS impacts directly on the biogenesis of specific circRNAs through the binding of intronic regions involved in circularization. Whether circRNAs deregulation is caused by loss or gain of function of FUS mutations still remains to be addressed.

NON-CODING RNA DIAGNOSTICS AND THERAPEUTICS

One of the major challenges for researchers pursuing the understanding and ultimately the treatment of neurological disorders is early diagnosis. By this matter, it is of crucial importance to find suitable molecular markers detectable in patients' specimens obtained through non-invasive methods. In this regards, liquid biopsies are the most applied non-invasive method to measure biomarkers that are soluble in body fluids such as plasma, blood, saliva, and cerebrospinal fluid (CSF). It is believed that circulating molecules potentially reflect the type of disease and can be detected at early stages when other diagnostic tools are not effective (Kelemen et al., 2019).

NcRNAs have been recognized as very important markers in the field of molecular diagnosis since they can be easily detected and quantified in body fluids. LncRNAs and even more circRNAs are high stable while circulating in body fluids, especially when included into extracellular vesicles (**Figure 3I**). Moreover, they may reflect the origin of the disease because of their tissue specificity. There are many examples of lncRNAs and circRNAs already proposed as possible diagnostic and prognostic biomarkers for various illnesses including cancer, diabetes, Crohn's disease, coronary artery disease, and rheumatoid arthritis (Li et al., 2015b; Ouyang et al., 2017; Zhao et al., 2017; Kelemen et al., 2019). Nevertheless, few reports are available on lncRNAs and circRNAs as biomarkers for neurodegenerative diseases. In particular, Feng et al. (2018) studied the potential of selected lncRNAs as biomarkers in AD by analyzing plasma from a population of 88 AD patients vs. 72 control individuals. They found that BACE1 lncRNA may be a potential candidate biomarker to predict AD since it was significantly higher in AD patients than in healthy controls and showed high specificity (88%) for AD (Feng et al., 2018).

In another study the RNA extracted from CSF of a cohort of 27 PD patients and 30 controls was analyzed by RNA-seq: among the differentially expressed transcripts, the lncRNA SCN9 antisense (AC010127.3) and two lncRNAs close to LRRK2 locus (AC079630 and UC001lva.4) have been suggested as potential RNA biomarkers for diagnosis and response to treatment of PD (Hossein-Nezhad et al., 2016).

Moreover, Gagliardi et al. (2018) analyzed lncRNA expression in Peripheral Blood Mononuclear Cells of sporadic ALS and found 293 lncRNAs differentially expressed between normal control and sporadic ALS patients. Among these, NATs of genes which are already linked to neurodegenerative disease (Gagliardi et al., 2018).

As reported by this review, numerous studies have revealed a plethora of lncRNAs and circRNAs differentially expressed and whose activity is altered in disease conditions. This knowledge allows to identify RNA candidates to be used as markers for diagnosis and response to treatments, and even more importantly as potential therapeutic molecules. One interesting example comes from the work on UCHL1-AS and its functional role in up-regulating Uchl1 translation (Carrieri et al., 2015). Based on this study, Zucchelli et al. (2015) have designed synthetic SINEUPs to potentially target any mRNA in the cell. One application has been shown very recently in PD, where an increase of GDNF levels is beneficial for the reduction of the neurodegenerative symptoms. Previous therapeutic strategies to increase GDNF levels have produced side effects due to high ectopic doses of this factor (Kordower and Bjorklund, 2013). Using a PD mouse model and adeno associated viral (AAV) delivery of miniSINEUPs, a twofold increase of GDNF was observed in dopaminergic neurons, thus ameliorating motor deficits of the mice (Espinoza et al., 2020). MiniSINUPs are an encouraging approach for the increase of endogenous GDNF levels in patients and may represent a unique RNA-based therapeutic platform to address many other diseases.

A promising strategy to target ncRNAs takes advantage of the use of ASOs designed to bind perfectly to target transcripts, inducing either their enzymatic degradation or inhibition of the binding of RBPs required for RNA maturation/activity. It is likely that ASOs targeting NATs represents a powerful tool for novel therapeutic strategies, considering that NATs are pervasively associated with coding genes loci and have an impact on the regulation of neuronal genes. For instance, ASOs designed against BACE1-AS and SMN1-AS have been tested in murine and human model systems and have provided proofs of principle that these NATs are clinically relevant novel therapeutic targets for AD and SMA respectively. Notably, the downregulation of BACE1-AS in an AD mouse model lowers the amyloid-β levels and ameliorates adult neurogenesis while reduced levels of SMN1-AS increases the transcription of SMN2 gene in patient-derived cells, in SMA neurons, and in a mouse model of severe SMA (Modarresi et al., 2011; d'Ydewalle et al., 2017). As demonstrated in these two cases, the increase of knowledge concerning the biology of NATs in normal and disease states still represents the most important milestone to achieve in order to develop and design novel therapeutic approaches.

Another example of ncRNA used as potential therapeutic target is the repeat-containing C9orf72 transcript. A new approach that has been employed to knock-down these transcripts is the use of artificial miRNAs (miC). Notably, Martier et al. (2019) proved, *in vivo*, the delivery and efficacy of AAV5-miC in cortex and hippocampal neurons of Tg(C9orf72_3) ALS mouse model thus providing a proof of concept for the use of this strategy in the treatment of ALS and FTD.

Differently to lncRNAs, the field of circRNA research is still in its infancy and even though the use of these RNAs

in the diagnosis and treatment of neurological disorders can be foreseen, we are still far from employing circRNAs in clinical practice.

Indeed, aside from the study by Cui et al. (2016), observing modulation of hsa_circRNA_103636 expression in peripheral blood mononuclear cells of patients with major depressive disorder treated with antidepressants for 8 weeks, the potential use of circRNAs as biomarkers in neurological diseases has not been well-explored yet. Additionally, the study by Cui et al. (2016) suggests that circulating circRNAs can be also used to assess responses to drug treatments.

The potential use of circRNAs in therapy comes from evidence suggesting that the accumulation of ciRNAs in the cytoplasm, caused by the inhibition of debranching enzyme 1 (Dbr1) activity, suppresses the toxicity of TDP-43 aggregates in human neuronal cell line and primary rat neurons (Armakola et al., 2012). ciRNAs might act as decoys for TDP-43 thus avoiding its interaction with other cellular RNAs in the cytoplasm. Since TDP-43 is deposited in protein aggregates in neurons and glia in > 96% of ALS cases, the modulation of ciRNAs biogenesis by targeting Dbr1 might represent a therapeutic strategy for ALS and other related TDP-43 proteinopathies.

Lastly, one important point of discussion arises for the delivery of therapeutic molecules to central nervous system (CNS) since the blood–brain barrier (BBB) and blood spinal cord barrier (BSCB) represents a bottleneck in the development of new therapies to treat CNS diseases. Indeed, in the last decade a great deal of effort has been dedicated to the achievement of an efficient and effective drug delivery to CNS focusing on the types of administration as well as on the design and modification of the potential therapeutic molecules (Krizbai et al., 2016; Kumar et al., 2017; Alexander et al., 2019; Fowler et al., 2020). A promising approach to circumvent the BBB and the BSCB is the delivery of therapeutic molecules directly to CNS through intrathecal injection (IT). Indeed, ASOs or AAV based molecules that have been IT administrated through intracerebroventricular (ICV) injection in rodent models and non-human primates, showed a widespread distribution in brain and spinal cord indicating the feasibility of this approach in targeting tissues mostly affected in neurodegenerative diseases (DeVos and Miller, 2013; Rigo et al., 2014; Biferi et al., 2017; Casaca-Carreira et al., 2017; Schoch and Miller, 2017; Martier et al., 2019). More importantly, pre-clinical and clinical trials involving IT/ICV delivery of ASOs against disease-associated transcripts (SMN, SOD1, and C9ORF72) have demonstrated the effectiveness and tolerability of this approach (Miller et al., 2013; Finkel et al., 2016; Cappella et al., 2019; Neil and Bisaccia, 2019).

PERSPECTIVES

The knowledge derived from the studies on ncRNAs has increased exponentially in the last decade. Advances from international consortia, such as the FANTOM and the ENCODE projects for the functional identification of the whole transcriptome repertoire, have created a clear picture that extended regions of the genome are actively transcribed and contain previously undiscovered functional elements. Ambitious projects for the characterization of novel functions of non-coding transcripts and in particular of lncRNAs and circRNAs have deepened our understanding on the regulatory processes that underlie higher eukaryotes molecular complexity. This is particularly intriguing for the study of the nervous system, where tissue and cellular complexity seem to be evolutionary associated with an increase of non-coding genes number, expression and activity (Qureshi and Mehler, 2012). In this review we have described how lncRNAs and circRNAs are involved in controlling multiple neuronal functions in physiological as well as in pathological conditions. However, in this latter case most of the experimental studies focused on the differential expression of ncRNAs in disease respect to healthy conditions, while only partial information on ncRNA functions is available so far. Surprisingly, ncRNAs act through very diverse modes of action and, except few cases, no common feature is known to predict the function, making the study of each lncRNA or circRNA an incredibly challenging process.

The development of murine and cellular model systems, such as patient derived Induced Plutipotent Stem Cells efficiently differentiated through specific protocols, represent powerful model systems for the study of ncRNA functions in neurodegenerative diseases (Dawson et al., 2018; Wu et al., 2019). Indeed, these systems offer the opportunity to compare healthy with disease conditions providing mechanistic insights into molecular principles of neurodegenerative biology. The study of ncRNAs in these contexts might provide a unique resource for high-throughput functional screenings of non-coding genes involved in neurodegeneration.

Moreover, the use of model systems as well as patient specimens could represent a helpful resource for the identification of candidates having therapeutic potential, particularly in the preclinical stages when the neuronal loss is still minimal leading to a more effective intervention. As described in this review efforts to attain this goal have already started.

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MM and BS wrote and revised the manuscript. SB prepared figures and revised the manuscript.

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REVIEW
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Circular RNAs in Embryogenesis and Cell Differentiation With a Focus on Cancer Development

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In the recent years thousands of non-coding RNAs have been identified, also thanks to highthroughput sequencing technologies. Among them, circular RNAs (circRNAs) are a well-represented class characterized by the high sequence conservation and cell type specific expression in eukaryotes. They are covalently closed loops formed through back-splicing. Recently, circRNAs were shown to regulate a variety of cellular processes functioning as miRNA sponges, RBP binding molecules, transcriptional regulators, scaffold for protein translation, as well as immune regulators. A growing number of studies are showing that deregulated expression of circRNAs plays important and decisive actions during the development of several human diseases, including cancer. The research on their biogenesis and on the various molecular mechanisms in which they are involved is going very fast, however, there are still few studies that address their involvement in embryogenesis and eukaryotic development. This review has the intent to describe the most recent progress in the study of the biogenesis and molecular activities of circRNAs providing insightful information in the field of embryogenesis and cell differentiation. In addition, we describe the latest research on circRNAs as novel promising biomarkers in diverse types of tumors.

Keywords: circRNA, embryogenesis, development, stemness, cancer

INTRODUCTION

With the advent of next-generation sequencing, the list of diverse non-coding RNA species with functional capacity expressed in eukaryotic cells has grown very rapidly and some computational algorithms emerged to predict circRNAs, which were most commonly found at back-splicing junctions (Veneziano et al., 2016; Gao and Zhao, 2018).

Abbreviations: AD, Alzheimer's disease; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukaemia; APL, acute promyelocytic leukemia; BMSCs, bone marrow stem cells; circRNA, Circular RNA; CML, chronic myeloid leukemia; CSCs, cancer stem cells; DMD, Duchenne muscular dystrophy; EMT, epithelial mesenchymal transition; FLT3, FMS-like tyrosine kinase-3; GBM, glioblastoma multiforme; GCs, granulosa cells; HCC, hepatocellular carcinoma; HSCs, hematopoietic stem cells; MIBC, muscle invasive bladder cancer; miRNA, micro RNA; NGS, Next Generation Sequencing; OBs, osteoblasts; OCs, osteoclasts; OS, osteosarcoma; PD, Parkinson's disease; PML/RARα, promyelocytic leukemia-retinoic acid receptor α; RA, Retinoic Acid; RBP, RNA-binding protein; RNA-seq, RNA sequencing; SSC, spermatogonial stem cells; SZ, Schizophrenia; VIM, vimentin.

Circular RNA (circRNA) is a type of single-stranded RNA usually formed by alternative splicing of pre-mRNA where the 5′ upstream splice acceptor is joined to 3′ downstream splice donor in a process named "backsplicing" (**Figure 1**). This event forms covalently closed continuous loops without polyadenylated tails and, as result, circRNAs are insensitive to the majority of exoribonucleases (Kristensen et al., 2019). CircRNAs are classified into three categories: exonic circRNAs (ecircRNAs; with one or more exons) that represent 85% of all circRNAs, exonic-intronic circRNA (EicirRNA) and circularized intronic RNA (ciRNA) (Kristensen et al., 2019; **Figure 1**). A competitive relationship may exist between the linear RNA splicing and the back-splicing events. The two introns flanking the circularized exons, which have been found to be enriched in Alu repeats, usually increase the efficiency of circularization (Zhang et al., 2014).

Initially, circRNAs were occasionally discovered by RT-PCR amplification and sequencing (Nigro et al., 1991; Cocquerelle et al., 1992). Only 20 years later, to find genomic rearrangements in cancers, the expression of circRNAs was discovered through RNA-seq in human pediatric acute lymphoblastic leukemia (Salzman et al., 2012). The authors showed that this phenomenon could be extended to leukocytes from healthy adults as well as to several other cancer and non-cancer cell lines and to mouse brain (Salzman et al., 2012).

The most important features of circRNAs can be summarized as follows: (a) circRNAs are abundant forms of non-coding RNAs that are expressed from thousands of human genes, sometimes even at higher level than their cognate linear isoforms (Salzman et al., 2012; Zhang et al., 2014; Kristensen et al., 2019); (b) circRNAs exhibit cell type-specific expression (Chen, 2016); and (c) circRNAs present a high rate of conservation between mouse and human and are quite stable molecules, with half-lives exceeding 48 h (Jeck et al., 2013).

Circular RNAs could have multiple functions within the cell acting for example as miRNA sponges, by competing for miRNA binding sites and thus decreasing the miRNA activity on the target mRNA, or acting as protein sponges; moreover, circRNA can interact with RNA-binding proteins (RBPs), act as platform for enzimatic reactions or act as a protein platform (**Figure 2**); finally, circRNAs may regulate the transcription and may interact with ribosomes thus affecting protein translation (Kristensen et al., 2019; **Figure 2**).

CircRNAs IN EMBRYOGENESIS

The embryo development begins when the genetic transcription of zygote is activated. Different from the intensely studied mRNAs, circRNAs are still in the opening of this research field. By means of deep sequencing and bioinformatics technologies, the set of circRNA expressed in human pre-implantation embryos have been reported (Fan et al., 2015; Dang et al., 2016). Embryonic stem cells derived from the pluripotent inner cell mass of the blastocyst are clonogenic and have the ability for unlimited self- renewal and pluripotency, leading to all cell types in the human body tissues. That ncRNAs play an important role in the maintenance of pluripotency has been recently established (Fu et al., 2018). Specifically, it has been demonstrated that two circRNAs, namely circBIRC6 and circCORO1C, are functionally connected with the maintenance of pluripotency in human embryonic stem cells and, in particular, circBIRC6 acts as a sponge for miR-34a and miR-145, relieving the suppression of NANOG, OCT4, and SOX2 espression (Yu et al., 2017). In an widely used vertebrate model, aimed at exploring circRNAs with potential functions during early vertebrate development, Liu et al. performed high-throughput sequencing, and applied the circRNA Identifier algorithm, throughout the duration of zebrafish embryo development (Liu et al., 2019); this study provided important information on the dynamic regulation of circRNAs implicated in the control of zebrafish differentiation and described novel specific circRNAs responsible for embryo development.

The possible role and impact of circRNAs in human development have been also recently reported with specific regard to cardiogenesis and neurogenesis (Lee et al., 2019). Of note, circRNAs were shown to be more abundant in the brain than in other tested organs in the adult mouse (You et al., 2015), pig (Veno et al., 2015), and human (Chen B. J. et al., 2019). The expression and roles of circRNAs in brain development and aging as well the implications in CNS diseases have been recently reviewed (Mehta et al., 2020).

CircRNAs IN REPRODUCTIVE SYSTEM AND GERM CELL DEVELOPMENT

During spermatogenesis and oogenesis, a tightly controlled expression of stage-specific genes is crucial for the normal development of gametes. Recently, circRNAs have emerged as a novel class of ncRNAs that regulate gene expression also in gametogenesis, but their role has not been completely clarified yet.

A study focused on the expression levels of circRNAs in brain, liver, heart, lung and testis, indicated that testis produces a huge amount of circRNAs, only second to that in brain (You et al., 2015), suggesting that circRNAs may have an important role in the testis function.

Notably, about 30 years ago the first circRNA was discovered in mouse as a transcript originating from the testis-determining gene Sry (Capel et al., 1993). In male, spermatogonial stem cells (SSCs) undergo self-renewal to ensure at the same time the maintenance of the stem cells pool and the differentiation to spermatocytes and spermatids. SSCs can also dedifferentiate into embryonic stem (ES)-like cells to acquire pluripotency *in vitro* (Conrad et al., 2008) and they are able to be reprogrammed to transdifferentiate to cell lineages of other tissues and for this reason SSCs have relevant applications in treating male infertility (Chen et al., 2017). Distinct circRNA expression profiles in different types of male germ cells indicate an important role exerted by circRNAs in the control of self-renewal and differentiation processes of SSCs (Zhou et al., 2019). By using highthroughput sequencing, circRNAs expression profiles have been identified in mouse male and female germline stem cells: a

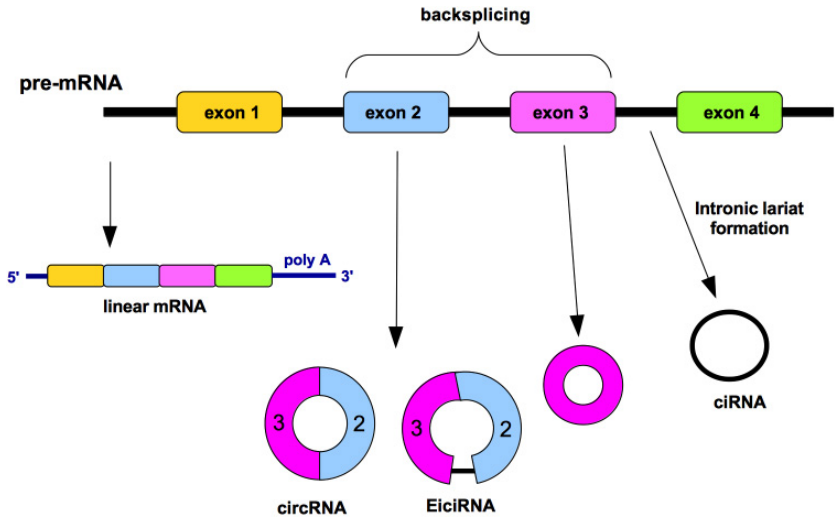

FIGURE 1 | circRNA generation by "backsplicing" mechanism. Diverse circular RNAs (circRNAs) can be formed from a single gene. The non-canonical splicing process named "backsplicing" is the responsible of the circRNA formation. A downstream splice donor is joined to an upstream splice acceptor generating diverse isoforms. Such circRNAs can consist of one or more exons and can even contain unspliced intronic sequences. Circularized intron RNAs are not produced by backsplicing, rather by an inefficient debranching. Colored bars, exons; black lines, introns.

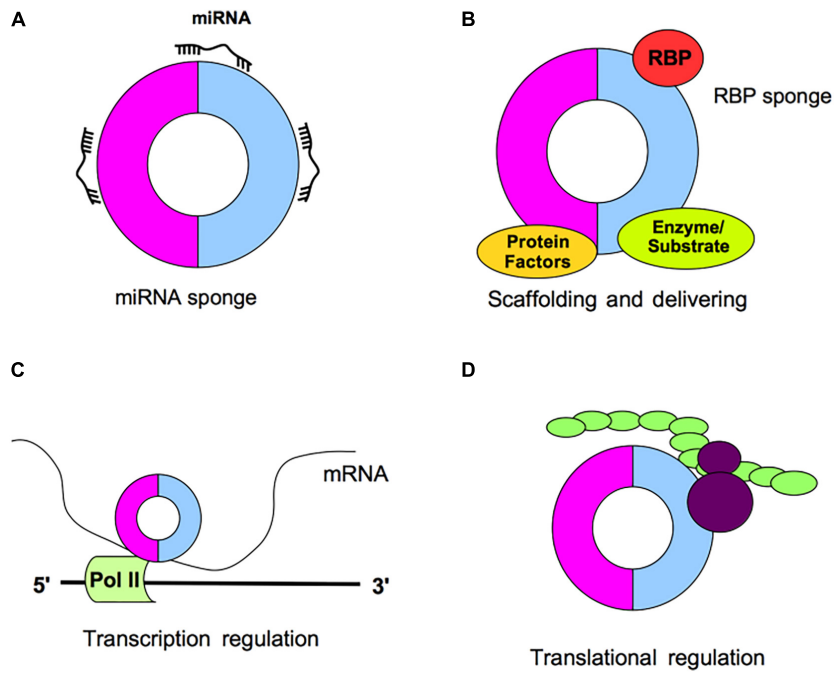

FIGURE 2 | Schematic representation of circRNA functions. **(A)** CircRNAs may act as miRNA sponges by competing for miRNA binding sites, decreasing the miRNA activity on the mRNA targets. **(B)** CircRNAs may act as protein sponges, by binding RNA-binding proteins (RBPs) or acting as platform for enzymatic reactions or acting as a protein platform. **(C)** CircRNAs may regulate the transcription. **(D)** CircRNAs may interact with ribosomes and affect protein translation.

total of 18822 circRNAs were described in the germline stem cells and 921 circRNAs were differentially expressed between the male and female germline stem cells, suggesting that circRNAs could confer sex-specific properties needed for differentiation into gametes between male and female stem cells in mouse (Li X. et al., 2017; Li et al., 2019).

Moreover, testis-derived circRNAs have been detected in human seminal plasma because they are resistant to exonuclease activity due to their circular form which confer them a great potential as liquid biopsy tools for various human diseases (Dong et al., 2016; Cai et al., 2018). Interestingly, in a recent study the expression of eight candidate circRNAs generated

from six linear transcripts (CNR1, LEPR, MTHFR, NAPEPLD, NPC2, and SIRT1) has been profiled in five RNA samples from human and murine spermatozoa. Among them, authors focused on circNAPEPLDiso1, investigating its ability to bind miRNAs; they showed that circNAPEPLDiso1, expressed in mouse and human spermatozoa, specifically interacts with five miRNAs (miR-146a-5p, miR-203a-3p, miR-302c-3p, miR-766-3p, and miR-1260a) involved in the control of cell cycle and, some of them, expressed by the oocyte. This finding suggests a role of circNAPEPLDiso1 as a paternal-derived sponge for miRNAs inside the fertilized oocytes to regulate the first stages of embryo development by increasing levels of miRNA targets (Ragusa et al., 2019; **Figure 3A**).

An exhaustive review has recently described the potential roles of circRNAs in reproduction, particularly by analyzing circRNAs expression pattern in ovary (Quan and Li, 2018). Granulosa cells (GCs), the somatic cells surrounding oocyte, play an important role during oogenesis and early stages of embryo development (Moreno et al., 2015) and the study of circRNAs expressed in the GCs of subjects undergoing *in vitro* fertilization at a young age (less than 30 years) and at an older age (more than 38 years) showed that in older women, the expression of 46 circRNAs was up-regulated, whereas, 11 circRNAs were down-regulated. In particular, a negative correlation between the elevated expression of circRNA_103827 and circRNA_104816 in GCs and the top quality embryo number has been shown, suggesting that both circRNAs were closely related to decreasing ovarian reserve and adverse reproductive outcomes (**Figure 3A**). Therefore, circRNAs pattern of GCs may be used as potential biomarker to predict oocyte developmental capability and consequent assisted reproduction outcome (Cheng et al., 2017).

CircRNAs IN CELL DIFFERENTIATION

Circular RNAs are expressed in several different organs following a spatial- and temporal-specific course, which suggests their potential biofunctions (Chen and Schuman, 2016; Zhao W. et al., 2019). To date, there is a growing number of studies reporting that circRNAs could be involved in the development of mammalian tissues as in neural development (van Rossum et al., 2016; Constantin, 2018), in osteogenic differentiation (Gu et al., 2017; Huang et al., 2019), in skeletal muscle development (Chen et al., 2020) or in hematopoiesis (Bonizzato et al., 2016).

Neuronal CircRNAs

Several recent reports have shown that circRNAs are more enriched in neuronal tissues respect to other tissues (Chen and Schuman, 2016; **Figure 3B**). The reasons may be: (i) the brain has a consistent number of expressed circRNA host genes such as neuronal genes which regulate neurogenesis, neurodevelopment, and neuronal differentiation (Rybak-Wolf et al., 2015); (ii) neuronal genes contain very long introns (>10 kb), and circularized exons are more frequently flanked by long introns with inverted repeated sequences, thereby facilitating formation of circRNAs (Jeck et al., 2013); (iii) as circRNAs haven't 5′ and 3′ ends, they are more stable than linear coding or non-coding RNAs, leading to a relatively longer half-life (Piwecka et al., 2017).

The first study of circRNAs in neuronal development documented their significant enrichment in brain and most of them were derived from host genes that code for synaptic proteins. The authors profiled the mouse circRNA population in the hippocampus over several stages: embryonic (E18), early postnatal (P1), postnatal at the beginning of synapse formation (P10) and late postnatal hippocampus following the establishment of mature neural circuits (P30) (You et al., 2015). They observed that circRNA expression pattern associated with the onset of synaptogenesis at P10. Interestingly, the circRNAs that were induced during hippocampal development were transcribed from the gene loci coding for proteins enriched with synapse-related functions (You et al., 2015). Interestingly, using high resolution *in situ* hybridization, for the first time, this study visualized circRNA punctae in the dendrites of neurons. These data show that circRNA expression levels are regulated by neural plasticity, suggesting their importance in regulating synaptic transmission and/or local translation.

As circRNAs are found to be preferentially expressed along neural genes and in neural tissues, several research groups focused their efforts on the study of the circRNAs involvement as new biomarkers for aging-correlated multiple sclerosis characterized by neurodegeneration, the mental illness schizophrenia and for the neurodegenerative pathologies as Alzheimer's disease (AD), Parkinson's disease (PD) (Ghosal et al., 2013; Lukiw, 2013). AD is the most common cause of dementia worldwide characterized by progressive dysmnesia, cognitive impairment, and psychiatric symptoms. Although the mechanisms of onset and progression of AD remain unknown, the primary clinicopathological characteristics of AD are aggregates of amyloid precursor protein-derived amyloid-β (Aβ) peptides and intraneuronal neurofibrillary tangles in the brain. Targeted molecular therapies for the treatment of AD have recently entered in medical practice (Chang et al., 2018).

Dysregulation of miR7-ciRS-7 interaction has been reported in the hippocampus of AD patients, where the expression of ciRS-7 is low and, therefore, the level of miR-7 is increased, with consequent down-regulation of miR-7 target mRNAs (Lukiw, 2013; Floris et al., 2017; **Figure 3B**). The dysregulation of the interaction between ciRS-7 and miR-7 has been reported to be crucial for other neuronal disorders, including PD, where ciRS-7 has a sponge activity on miR-7 expression (Lu and Xu, 2016; Floris et al., 2017; **Figure 3B**). In fact, miR-7 is highly expressed in cortical neuronal progenitors and its depletion causes microcephaly-like brain defects (Piwecka et al., 2017; **Table 1**).

Parkinson's disease is characterized by loss of dopaminergic neurons in the substantia nigra, which leads to a series of motor function disorders including rest tremor, muscular rigidity, and bradykinesia. The overexpression and aggregation of α-synuclein (SNCA), which is present in Lewy bodies, is a distinctive diagnostic marker in PD (Rodriguez et al., 2015). It has been shown that miR-7 overexpression induced more efficient repression of SNCA in the empty HeLa cell line that did not express ciRS-7, suggesting that ciRS-7 may play a role in

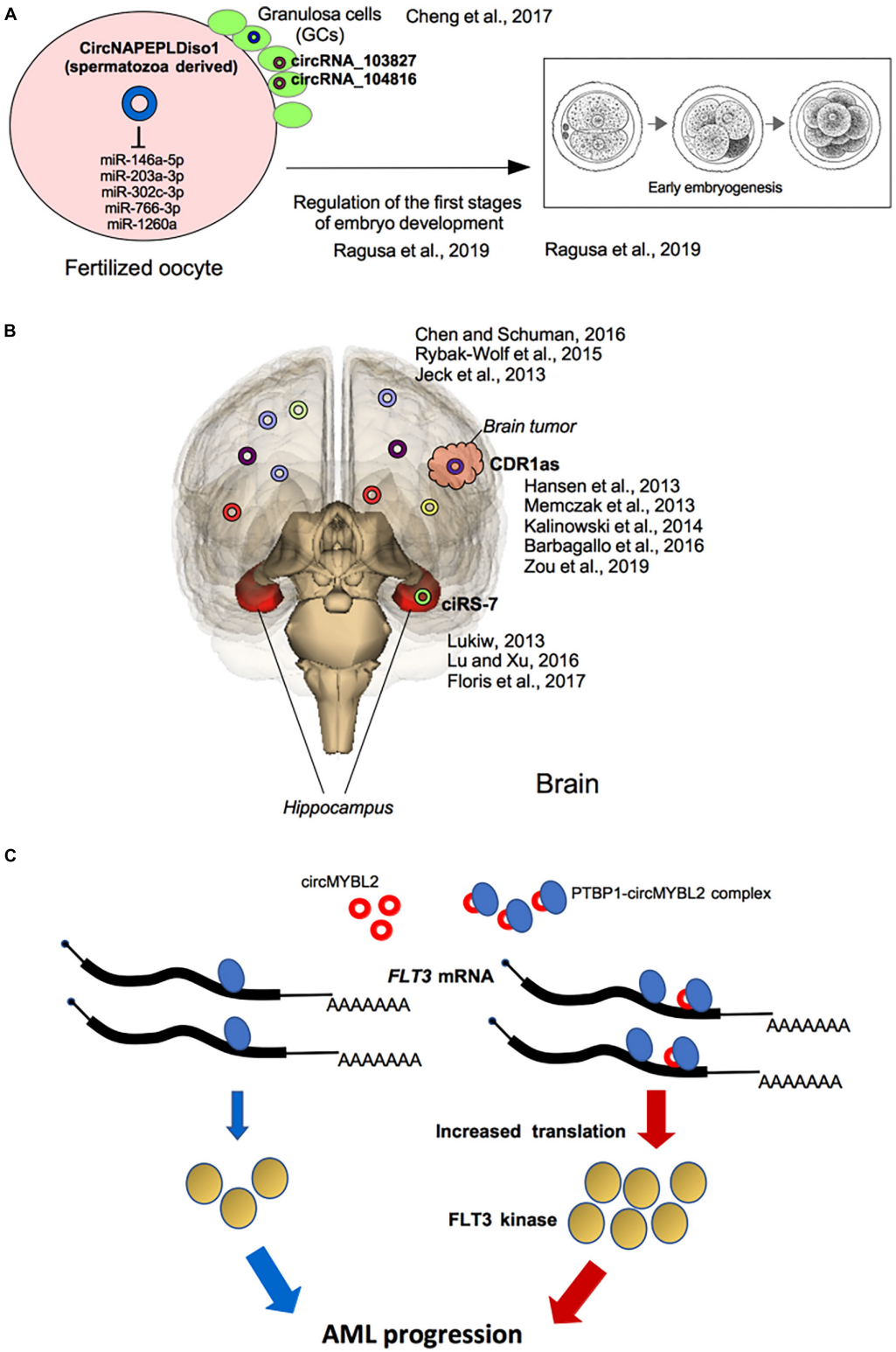

FIGURE 3 | Selected functional effects of circRNAs in development and cancer. **(A)** Potential roles of circRNAs in reproduction: circRNAs expressed in granulosa cells (GCc) and in spermatozoa and involved in the first stages of embryo development into the fertilized oocytes are shown. **(B)** Roles of circRNAs in brain disease: in the Hippocampus, dysregulation of ciRS-7 expression is associated with Alzheimer's disease and, generally, with neuronal-associated diseases. CiRS-7/CDR1as deregulated expression is also involved in brain tumorigenesis. **(C)** The PTBP1-circMYBL2 complex is highly expressed in AML patients with FLT3-ITD mutations where the translation of FLT3 mutated kinase is specifically induced fostering tumor progression.

TABLE 1 | CircRNAs expressed in neuronal tissue and neuronal diseases.

circRNA	Function	References
ciRS-7		Hansen et al., 2013
		Floris et al., 2017
	Sponge activity on miR-7	Lu and Xu, 2016
		Sang et al., 2018
hsa_circRNA_104597	Valuable marker for schizophrenia	Yao et al., 2019
CDR1as		Hansen et al., 2013
		Kalinowski et al., 2014
		Uhr et al., 2018
	Sponge activity on miR-7	Tanaka et al., 2019
		Zhong et al., 2019
circFBXW7	Tumor suppressor	Yang et al., 2018
circSHPRH	Tumor suppressor	Zhang et al., 2018

modulating SNCA through a miR-7-dependent pathway (Hansen et al., 2013). These results also suggested a possible sponge effect between ciRS-7 and miR-7 *in vitro*. Other studies reported that circSNCA can sponge miR-7 thereby up-regulating expression of SNCA mRNA, resulting in reduced autophagy and increased apoptosis in SH-SY5Y cells (Sang et al., 2018).

Schizophrenia (SZ) is a serious neuropsychiatric disorder with high recurrence and disability rates (van Os and Kapur, 2009). The pathogenesis of SZ is not yet fully understood and the most accredited causes seem to be environmental and genetic factors, although the lack of reliable biomarkers hinders early diagnosis and effective treatment of SZ patients (van Os and Kapur, 2009). Very recently, to assess whether expression of circRNAs in peripheral blood mononuclear cells (PBMCs) may be useful as low invasive biomarkers for diagnosis and/or therapeutic response in SZ patients, a research group analyzed circRNA expression profiles in PBMCs from SZ individuals and healthy controls (Yao et al., 2019). The expression of hsa_circRNA_104597 was assessed to be at low level in patients affected by schizophrenia (Yao et al., 2019; **Table 1**). ROC curve analysis showed that hsa_circRNA_104597 alone had a sensitivity of 84.31% and specificity of 86.41% respect to hormones (e.g., cortisol, insulin, leptin, prolactin, and growth hormone), miRNAs, lncRNA,indicating it as diagnostically valuable marker (Yao et al., 2019). In addition, they found that hsa_circRNA_104597 expression level increased after the treatment for 8 weeks with antipsychotic medications confirming it as potential therapeutic biomarker for SZ (Yao et al., 2019).

CircRNAs in Osteogenic Differentiation

Bone remodeling is a dynamic process based on the balanced activities of the bone-forming osteoblasts (OBs), differentiating from bone marrow-derived mesenchymal stem cells (MSCs), and the bone-resorbing osteoclasts (OCs), multinucleated cells deriving from the monocyte/macrophage lineage (Berendsen and Olsen, 2015). Mature OBs produce and secrete proteins, such as alkaline phosphatase and type I collagen, which are necessary for the formation of the bone extracellular matrix, which then undergoes the process of mineralization. While most of the OBs die by apoptosis, some reach quiescence as bone lining cells on bone surfaces or become embedded in the bone matrix as osteocytes (Berendsen and Olsen, 2015). OCs control calcium and phosphate homeostasis, and play the role of mechano-sensors to respond to mechanical effort of the skeleton (Berendsen and Olsen, 2015). The cellular crosstalk of OBs and OCs is important to ensure bone integrity, repair, and calcium homeostasis, and imbalance between OB and OC activities can lead to bone diseases, such as osteoporosis and cancer-associated bone destruction (Berendsen and Olsen, 2015).

Several microRNAs and long non-coding RNAs have been reported as differentially expressed during osteogenesis (Ell and Kang, 2014; Huynh et al., 2017; Xie et al., 2017; Puppo et al., 2019). Their involvement in bone cancer and metastasis is well addressed (Puppo et al., 2019). Nevertheless, little is known about the regulation of the expression and the role of circRNAs in bone development and in bone pathologies.

Circular RNAs are emerging as important molecules that may regulate bone homeostasis. Using gene expression analysis, several circRNAs were found to be differentially expressed in MSCs undergoing OB differentiation, respect to their undifferentiated counterparts (Zhang et al., 2019). Some circRNAs were linked to miRNAs with osteogenic roles, indicating that these circRNAs potentially function in osteogenic differentiation of BMSCs (bone marrow stem cells) (Zhang et al., 2019). The authors identified a crosstalk between miR-199b-5p and circIGSF11 (**Table 2**). Silencing of circIGSF11 promoted osteoblast differentiation and increased the expression of miR-199b-5p (Zhang et al., 2019).

It has been reported that BMP2 promoted the proliferation of osteoblasts *in vitro* (Qian et al., 2017). RNA-seq analysis of BMP2-treated MC3T3-E1 cells has been performed to analyze differential expression of circRNAs during different osteoblast differentiation stages (Qian et al., 2017). 158 circRNAs were differentially expressed and, specifically, the expression of circRNA.5846, circRNA.19142 and circRNA.10042 was increased in the BMP2 treated group (**Table 2**). Circ.19142 and circ.5846 were found to be not only strongly associated with the positive regulation of developmental processes but also related to the fibroblast growth factor, epidermal growth factor, platelet-derived growth factor and Wnt signaling pathways, which are involved in cell growth and differentiation (Qian et al., 2017).

TABLE 2 | CircRNAs in osteogenic differentiation and pathologies.

circRNA	Function	References
circIGSF11	Interaction with miR-199b-5p	Zhang M. et al., 2019
circ19142	Osteoblastic differentiation	Qian et al., 2017
circ5846		
hsa_circ_0006393	Sponge activity on miR-145-5p	Wang Y. et al., 2019
hsa-circ-0016347	Onco-circRNA in osteosarcoma	Jin et al., 2017
circHIPK3		Xiao-Long et al., 2018
circ_001569		
circ-Cdr1as	Tumor suppressor	
circ-Foxo3		

Recently, Wang X.B. et al. (2019) found that the overexpression of hsa_circ_0006393 increased the expression level of genes associated with osteogenesis (**Table 2**). Hsa_circ_0006393 is expressed mainly in the cytoplasm and nucleus of BMSCs. miR-145-5p was shown to be sponged by hsa_circ_0006393, thus increasing the expression levels of osteogenic genes during bone remodeling (Wang X.B. et al. (2019)).

Unfortunately, no functional analysis was carried out. Additional investigations and functional studies are required to address the biological role of circRNAs in bone differentiation and remodeling.

CircRNAs in Skeletal Muscle Development

Recent studies reported that circRNAs are well expressed in skeletal muscle tissue, and their expression levels are regulated during muscle development and aging (Cai et al., 2019; Zhang M. et al., 2019). Skeletal muscle is the largest tissue in the body, playing an important role in locomotion and metabolism (Millay et al., 2013). Skeletal muscle has mature cells that are syncytial and can contain hundreds of nuclei; therefore, correct muscle growth and homeostasis are determinant for human mobility. Conversely, muscular diseases, such as muscular dystrophy, sarcopenia, atrophy, and cachexia, severely affect the everyday life of humans (Millay et al., 2013). Development and growth of muscle mainly rely on the proliferation and differentiation of myogenic stem cells. Most of the myogenic stem cells are derived from the mesodermal cell lineages (Kuang et al., 2007) and Pax3 and Pax7 paired box genes are the principal biomarkers for the myogenic stem cell (Buckingham and Relaix, 2007).

Regarding the expression of circRNAs in the muscle and in its development, many studies have been performed on mouse and chicken embryos (Chen et al., 2018; Shen et al., 2019).

Duchenne muscular dystrophy (DMD), which is characterized by a progressive decrease of muscle function, is caused by frame-shifting deletions or nonsense mutations in the DMD gene (Koenig et al., 1989), being this last among the first genes where circRNAs were identified as RNA circles consisting of exons that were skipped by alternative splicing (Surono et al., 1999). In contrast to the previous idea that circRNAs might be derived from aberrant splicing events (Sharp, 1994), the formation of circRNAs from the DMD gene is not necessarily created by the exon skipping, mainly because no strong correlation has found between the identified spliced transcripts and the circRNAs expected to be produced (Surono et al., 1999).

A recent work showed that most of the circRNAs expressed in myoblast during the growth stage regulated the cell cycle, while the circRNAs expressed in the differentiation stage are related with development activity (Zhang et al., 2018). For examples, circZNF609 showed higher expression in myotubes than in myoblasts and its downregulation reduced myoblast proliferation (Legnini et al., 2017; **Table 3**). In the mouse model a proposed mechanism implies that circZNF609 inhibits myoblasts differentiation by sponging miR-194-5p and upregulating BCLAF1 (Wang Y. et al., 2019).

CircLMO7, derived from LMO7 gene, was highly expressed in skeletal muscle tissue. High levels of circLMO7 significantly decreased the expression of MyoD and myogenin (MyoG), suggesting that circLMO7 inhibited myoblast differentiation (**Table 3**). On the other side, circLMO7 overexpression increased myoblasts proliferation (Wei et al., 2016). From a mechanistic point of view, circLMO7 interacted with miR-378a-3p that targeted HDAC4 expression (Wei et al., 2016). CircLMO7 may serve as a decoy for miR-378a-3p, resulting in higher expression of HDAC4 thus decreasing the transcription of MEF2A and repressing myoblast differentiation (Miska et al., 1999).

Conversely, circSNX29 expression is correlated with myoblasts differentiation. It was reported that the expression level of circSNX29 was much higher in embryonic skeletal muscle than adult skeletal muscle and it was principally enriched in the cytoplasm (Peng et al., 2019; **Table 3**). CircSNX29 acts as a miR-744 sponge and increases Wnt5a and CaMKIId expression resulting in the activation of non-canonical Wnt pathways and myoblasts differentiation. Other groups have documented a similar "sponge" mechanism for other circRNAs, by sequestering miRNAs that regulate the expression of genes positively or negatively implicated in myoblasts differentiation (Li H. et al., 2018; Ouyang et al., 2018).

Many evidences of circRNAs dysregulation in muscle diseases are emerging. As previously mentioned, circRNAs produced by transcripts spliced from the DMD gene were among the first to be identified in skeletal muscles, mostly generated at the 5′ end of the transcript (Surono et al., 1999). Recently, a region spanning exon 45 to exon 55 of the DMD gene that represents a deletion hotspot in 63% of DMD patients was characterized (Suzuki et al., 2016). The authors reported that multiple exon skipping, targeting exon 45-55, was related with the increase of the symptoms in the patients identifying the favorite splice-sites involved in both circRNA formation and in multiple exon skipping of exon 45-55. The data confirm a circRNA-generation model in which the interaction between upstream and downstream introns triggers multiple exons skipping and creates circRNAs (Suzuki et al., 2016).

CircRNAs in Hematopoiesis

Next Generation Sequencing (NGS) recently showed a conspicuous circRNA expression in human hematopoietic progenitors, and in differentiated lymphoid and myeloid cells (Nicolet et al., 2018). In the hematopoietic compartment, circRNAs are significantly enriched and secreted in vesicles named exosomes derived from platelets, where circRNAs

TABLE 3 | CircRNAs in skeletal muscle development and diseases.

circRNA	Function	References
circZNF609	Sponge activity on miR-194-5p	Legnini et al., 2017; Wang Y. et al., 2019
circLMO7	Sponge activity on miR-378a-3p	Miska et al., 1999; Wei et al., 2016
circSNX29	Sponge activity on miR-744	Peng et al., 2019
circLPAR1	Biomarker for the prognosis of MIBC	Lin et al., 2019

resulted to be more abundant and widely expressed compared with other cell types (Preußer et al., 2018).

In the hematopoietic system, circRNA expression is cell-type specific, and it increases upon cell maturation. CircRNA splicing variants can also be cell-type specific (Nicolet et al., 2018). In the bone marrow, the hematopoietic stem cells (HSCs) differentiate into various progenitor cells, which in turn generate many types of myeloid and lymphoid cells requiring a tight regulation of gene expression of transcription factors and non-coding RNAs (Goode et al., 2016).

In a cutting-edge article dating back 20 years, Caldas et al. (1998) documented in the hematopoietic tissue the expression of circRNA isoforms of key genes such as MLL although the work was not very successful at the time, probably because the expression of circular isoforms was lower than mRNAs encoding the key transcriptional regulators.

CircRNAs IN CANCER: NOVEL DIAGNOSTIC AND THERAPEUTIC BIOMARKERS

One of the peculiar characteristics of circRNAs is that they are more resistant to the enzymatic activity of RNase R than linear mRNA, bypassing common RNA turnover steps (Kristensen et al., 2019). This feature has been exploited by liquid biopsies in the context of clinical trials conducted on various pathologies including cancer. Thanks to the stability of their structure in the longtime, it has been easy to trace the circRNAs in human tissues and fluids as serum and urine (Zhang et al., 2018). Their presence or absence in the fluids is emerging as an attractive diagnostic and prognostic tool and for this reason, in the field of translational medicine, they are becoming potent non-invasive biomarkers (Meng et al., 2017; Zhang et al., 2018).

The list of circRNAs involved in carcinogenesis continues to grow, however, the functional relevance of the vast majority is yet to be discovered. Some circRNAs can act as oncogenes and sustain proliferative signaling in cancer progression, while others may behave as tumor suppressors (Kristensen et al., 2018). Therefore, the cancer-specific expression status and functional mode of circRNAs may be used in cancer diagnosis and precise treatment in the future.

CircRNAs in Hematological Disease

Hematological cancers are characterized by the aberrant growth of oligoclones of hematopoietic cells, which are able to invade the bone marrow and the blood, leading to severe anemia and immunodeficiency (Handschuh, 2019). Recently, using NGS technology, Salzman et al. (2012) addressed new cancer-specific fusion transcripts in hyperdiploid B-lineage acute lymphoblastic leukemia (ALL). They sequenced several RNA transcripts with numerous combinations in which the exons could merge ("scrambled exons") and identified circularized RNAs. In five samples of hyperdiploid B-cell precursor-acute lymphoblastic leukemia they observed hundreds of circRNA transcripts with >700 circular isoforms where more than 10% of all transcripts derived by a comparable number of genes (Salzman et al., 2012). This study showed that many genes could produce scrambled exons (*ESYT2*, *FBXW4*, *CAMSAP1*, *KIAA0368*, *CLNS1A*, *FAM120A*, *MAP3K1*, *ZKSCAN1*, *MANBA*, *ZBTB46*, *NUP54*, *RARS*, and *MGA*) but they were expressed both in normal and blood cancer cells, not providing a more specific and useful interpretation of circRNA relevance for hematopoietic cell functions and pathology (Salzman et al., 2012). In a more recent study, RNA-seq analysis from whole-blood samples reported a very high number of expressed circRNAs, comparable to the brain (Memczak et al., 2015). Also in this case the functional aspect of circRNAs expression was not investigated but it was observed that hundreds of circRNAs were much higher expressed than corresponding linear mRNAs (Memczak et al., 2015).

If little is documented about the function of circRNAs in the development of hematopoietic tissue, much has been documented on their role in leukemias (Mei et al., 2019). Acute myeloid leukaemia (AML) represents the clonal expansion of hematopoietic precursors blocked at different stages of differentiation. The pathogenesis of AMLs is correlated to the presence of genetic alterations and the transcription factors regulating myelopoiesis are consistently involved in chromosomal translocation (Tenen et al., 1997). In AML the molecular event related to the transfation ability of chromosomal translocation-generated AML fusion proteins (such as PML/RARa, PLZF/RARa, AML1-ETO, MLL/AF9) is strictly dependent on their capacity to induce abnormal epigenetic modification on genes relevant to the transformation process (Grignani et al., 1998; Di Croce et al., 2002; Fazi et al., 2007). Of note, recently, fusion-circRNAs (f-circRNAs) derived from transcribed exons of chimeric proteins as MLL/AF9 and PML-RARA, generated by leukemia-associated chromosomal translocation, were discovered and demonstrated to be oncogenic by *in vitro* and *in vivo* experiments (Guarnerio et al., 2016; **Table 4**). Guarnerio et al. (2016) showed that *f-circPR* and *f-circM9* expression in leukemic cells increases cell proliferation and clonogenicity and that f-circRNA silencing reverted the phenotype, demonstrating that these f-circRNAs are biologically active and play pro-proliferative and pro-oncogenic activities. Recently it was reported that circ-VIM expression level in *de novo* AML patients [non-Acute Promyelocytic Leukemia (APL) patients with normal karyotype] was significantly upregulated compared with that in healthy controls (Yi et al., 2019; **Table 4**).

TABLE 4 | CircRNAs in hematopoiesis and cancer.

circRNA	Function	References
f-circPR f-circM9	Pro-oncogenic activities in leukemia	Guarnerio et al., 2016
circ-VIM	Up-regulated in AML	Yi et al., 2019
circ-HIPK2	Biomarker in APL	Li et al., 2018
circMYBL2	Required for FLT3-dependent leukemia progression	Sun et al., 2019
circ-PVT1	Pro-oncogenic activities in AML	Hu et al., 2018
hsa_circ_0004277	Biomarker in AML	Li et al., 2018
circ-BA9.3	Pro-oncogenic activities in CML	Kaleem et al., 2015; Pan et al., 2018

Vimentin (VIM) is a component of type III intermediate filament protein, involved in the regulation of lymphocyte adhesion and transcellular migration, and is associated with poor clinical outcome in older patients with AML (Wu S. et al., 2018). Collectively, these results make circ-VIM as a promising diagnostic biomarker and treatment target in AML (Yi et al., 2019).

Mutations within the FMS-like tyrosine kinase-3 (FLT3) gene, resulting in the internal tandem duplication (ITD; FLT3-ITD) or in the tyrosine kinase domain mutation (TKD; FLT3-TKD), occur in approximately 30% of AML patients. Recently, it has been shown that circMYBL2, a circRNA generated from the circularization of the cell-cycle checkpoint gene MYBL2, is crucial for FLT3-dependent leukemia progression. Mechanistically circMYBL2, by interacting with the polypyrimidine tract-binding protein 1 (PTBP1) to FLT3 messenger RNA significantly increases the protein level of mutant FLT3 kinase contributing to the AML progression. The depletion of this circRNA significantly impairs tumorigenicity of FLT3-ITD AML cells, highlighting this circRNA as putative relevant therapeutic target in this AML subtype (Sun et al., 2019; **Figure 3C**).

Of note, APL is a subtype of AML, characterized by the presence of the promyelocytic leukemia-retinoic acid receptor α (PML/RARα) fusion protein, which induces an oncogenic transcriptional silencing of the Retinoic Acid (RA) signaling pathway and causes the block of differentiation at the promyelocytic stage and neoplastic transformation of APL blasts (Grignani et al., 1998; Di Croce et al., 2002). Of note, the treatment of APL blasts with pharmacological doses of RA can overcome this repression and induce terminal differentiation *in vitro* and *in vivo* (Fazi et al., 2005). Gene expression analysis by RNA-seq showed 4,313 APL-expressed circRNAs in NB4 cells (Li et al., 2018). Furthermore, 508 circRNAs were expressed during all-*trans* retinoic acid treatment. The expression of circ-HIPK2 was lower in AML cells compared with APL cells, and overexpression of circ-HIPK2 increased differentiation in NB4 cells (APL cells with PML-RARA) (Li et al., 2018). Furthermore circ-HIPK2 had lower expression in APL samples of patients respect to that in healthy control samples and other subtypes of AML cases. The expression level of circ-HIPK2 significantly increased when APL patients achieved complete remission. This may suggest that circ-HIPK2 could act as a biomarker in APL cells.

Other circRNAs have been identified in hematopoietic malignancies as for example: circ-PVT1 in AML and in head and neck squamous cell carcinoma, where its expression is significantly associated with mutant p53 (Verduci et al., 2017; Hu et al., 2018; **Table 4**); hsa_circ_0004277, significantly lower in the AML than in healthy controls and patients who entered complete remission after treatment (Li W. et al., 2017; **Table 4**); circ-BA9.3 in chronic myeloid leukemia (CML), which is a stem cell disorder of uncontrolled myeloid proliferation characterized by the reciprocal translocation t(9;22) (q34; q11.2) resulting in the BCR-ABL1 fusion (Kaleem et al., 2015; Pan et al., 2018; **Table 4**).

Different cirRNA expression profiles correlate with different types of leukemia and clinical features, including tumor stage and recurrence, supported by recent RNA-seq studies. From all these studies it is clear that circRNA dysregulation signatures in cancers (in tissue- and development stage-specific manner), their tumor suppressive/oncogenic roles and stability and abundance in body fluids make them attractive non-invasive biomarkers in liquid biopsies.

CircRNAs in Brain Tumors

Many articles about the involvement of circRNAs in brain tumors are emerging due to their versatility and hypothetical use for liquid biopsy-based diagnosis and prognosis. Glioma is a common type of central nervous system tumor where diffuse glioma (glioma cells exhibiting extensive invasive growth into the surrounding central nervous system) is the most frequent tumor especially in adults (Wesseling and Capper, 2018). Song et al. (2016) conducted a study that included seven oligodendrogliomas, 20 glioblastomas and 19 normal brain specimens to explore the expression level of circRNAs using high-throughput sequencing. To analyze the great number of raw data, the authors developed a sofisticated computational pipeline named UROBORUS. They found that the total number of detected circRNAs in GBM was significantly lower than that in normal brain tissue showing that eight highly expressed GBM-specific circRNAs might be good GBM-specific biomarker candidates (Song et al., 2016).

Among the first circRNAs identified and studied in brain cancer there is the one originating from the antisense transcript of the cerebellar degeneration-related protein 1 gene (CDR1as, also known as ciRS-7, previously described) that many reports have considered to have miRNA sponge activity (Hansen et al., 2013; Memczak et al., 2013; Kalinowski et al., 2014; Barbagallo et al., 2016; Zou et al., 2019; **Table 1** and **Figure 3B**).

Mature ciRS-7 as is principally expressed in the cytoplasm, it has 74 miR-7 binding sites that can specifically bind to miR-7 molecules. As a result, the miR-7 target mRNAs are released from binding to miR-7 (Hansen et al., 2013; Kalinowski et al., 2014). The same seizure mechanism of ciRS-7 as toward miR-7 has been reported in development of nasopharyngeal carcinoma (Zhong et al., 2019), in colon cancer (Tanaka et al., 2019), in breast cancer (Uhr et al., 2018) and others.

Interestingly, Pamudurti et al. published that some circRNAs are associated with ribosomes suggesting a possible translation into proteins (Pamudurti et al., 2017). About this, it has been reported that circFBXW7 has coding potential and that translated peptide could be bound by an antibody targeted to related sequences holding potential prognostic implications in brain cancer (Yang et al., 2018). CircFBXW7 expression can significantly inhibit cell progression, migration, and tumor formation *in vivo* acting as tumor suppressor (Yang et al., 2018; **Table 1**). In this study FBXW7-185aa reduced the half-life of c-Myc by antagonizing USP28-induced c-Myc stabilization. An *in situ* GBM mouse model revealed the tumor suppressing effect of FBXW7-185aa but not of circ-FBXW7 circRNA with an IRES mutation. Also circSHPRH has coding abilities and leads to the formation of a peptide (Zhang M. et al., 2018). SHPRH-146aa is downregulated in GBM compared with para-cancerous tissues

and overexpression of SHPRH-146aa significantly inhibited glioma growth in xenograft mouse models (Zhang M. et al., 2018; **Table 1**).

Altogether these data highlighted that circRNAs in brain tumors could be excellent biomarkers for their diagnosis, prognosis and classification. According to this idea, some groups reported that the knockdown of oncogenic circRNAs might be a reasonable approach for the treatment of glioma in the future. On the contrary, circRNAs that play tumor suppressor activity might be used in overexpression therapies. Novel approaches to express proteins acting as potent tumor suppressors in brain tumor models by engineering a circRNA vector have been also recently published, suggesting a circRNA-based treatment of glioma in the near future (Meganck et al., 2018; Wesselhoeft et al., 2018).

CircRNAs in Osteosarcoma

Osteosarcoma (OS), a primary bone tumor arising from mesenchymal cells, has the highest fatality rate of all cancers among children and adolescents and many patients suffer from disease recurrence due to existing or potential distant metastasis (Ritter and Bielack, 2010). Patients with OS may benefit novel non-conventional therapies, such as small molecule-targeted drugs, but these strategies often lead to severe side effects and have failed in clinical trials (Otoukesh et al., 2018), therefore therapies focusing on complex gene regulation axes or networks are urgently needed. An increasing number of circRNAs that act as tumor suppressor or onco-circRNAs deregulated in bone cancer have been published. Below we describe the most significant studies in terms of inclusion of exploitation of patients' cohorts and *in vitro* models.

Circular RNA hsa-circ-0016347 that derives from the *KCNH1* oncogene was found to have high expression levels in osteosarcoma tissue samples and in cell lines and to promote proliferation, invasion and metastasis *in vitro* and *in vivo* (Jin et al., 2017; **Table 2**). Zhang et al. (2017) found a circRNA from *UBAP2* gene to be the most upregulated circRNA in osteosarcoma patient samples respect to adjacent non-tumoral tissues. High expression of circUBAP2 correlated with lower overall survival of the patients, promoted osteosarcoma growth and inhibited apoptosis both *in vitro* and *in vivo*.

In OS cell lines, tissues and plasma circHIPK3 was demonstrated to be down-regulated together with circ_001569, ciRS-7 and circ-Foxo3 (Xiao-Long et al., 2018; **Table 2**). Moreover, the authors showed that patients with lower expression of circHIPK3 had shorter overall survival time than those with higher circHIPK3 expression. Furthermore, they showed that circHIPK3 expression was associated with several clinicopathological features of patients with OS (Xiao-Long et al., 2018; **Table 2**). CircHIPK3 levels were associated to Enneking stage and lung metastasis other than age, gender and tumor location according to statistical analysis. These results showed that lung metastasis and advanced cancer were associated with lower expression levels of circHIPK3 and suggested that circHIPK3 may be used as a biomarker for diagnosis and prognosis prediction of osteosarcoma.

CircRNAs in Muscle-Invasive Cancers

Bladder cancer is the most common malignancy of the urinary system and it can be classified into two types according to the depth of cancer invasion: non-muscle invasive tumor (70–80%) and muscle-invasive tumor (20–30%). Patients with muscle-invasive bladder cancer (MIBC) present high incidence of metastasis and poor prognosis. Therefore, for MIBC patients it is urgent to find biomarkers for early diagnosis and to follow the progression of the disease during active surveillance by liquid biopsy (de Kruijff et al., 2020). Very recently a novel circRNA, circ lysophosphatidic acid receptor 1 (LPAR1) (hsa_circ_0087960), derived from two exons of 226 base pairs in length, has been identified in MIBC tissues (**Table 3**). circLPAR1 was found to be expressed at low level in a cohort of 125 cases of MIBC tissues. Furthermore, it predicted a worse disease-specific survival time than patients with high circLPAR1 expression (Lin et al., 2019). The authors showed the circLPAR1 may function as a potential novel biomarker for the prognosis of MIBC and may be associated with invasion and metastasis (Lin et al., 2019).

For an overview of the various circRNAs that have been found expressed in the various solid tumors as bladder cancer, breast cancer, colorectal carcinoma, esophageal squamous cell carcinoma, hepatocellular carcinoma, gastric cancer, lung and ovarian cancer, skin cancer, see the comprehensive review by Kristensen et al. (2018).

CircRNAs IN CANCER STEM CELLS

cancer stem cells are a small proportion of cells considered the driving force of tumor initiation, progression, chemoresistance, relapse, and are also responsible for metastatic dissemination and therapeutic failure. By definition, both CSCs and normal tissue stem cells possess self-renewal capacity, however, in CSCs self-renewal is typically deregulated. Several lines of evidence have suggested that circRNAs might contribute to the stemness of cancer and to the resistance of cancer cells to chemotherapy. Recently, many studies have demonstrated that circRNAs may be stably expressed and present in relatively high quantities in human body fluids, such as saliva, plasma, serum and exosomes, which also makes circRNAs ideal candidates as non-invasive liquid biopsy biomarkers for cancer (Su et al., 2019).

Increasing evidence obtained analyzing circRNA/miRNA network by bioinformatics approaches has established that specific circRNAs are implicated in stemness via serving as miRNA sponges in different cancers, such as breast cancer (Yan et al., 2017), laryngeal cancer (Wu Y. et al., 2018), gastric cancer (Zhao X. et al., 2020), and hepatocellular carcinoma (Zhu et al., 2019). As for glioma, since circPTN was shown to sponge miR-145-5p, which is a negative regulator of stemness (Zhao X. et al., 2020) and self-renewal (Rani et al., 2013) and since circPTN expression was more than 10-fold higher in glioma cells compared with normal glial cells, Chen J. et al. (2019) hypothesized that circPTN may be a positive regulator of stemness. As expected, tumor sphere formation assays determined that circPTN promoted increased levels of stemness markers, such as Nestin, CD133, SOX2, and SOX9,

and tumor growth *in vitro* and *in vivo*, primarily via sponging miR-145-5p/miR-330-5p (Chen J. et al., 2019).

Interestingly, a recent study showed that exosomes from CD133+ cells carrying circ-ABCC1 mediate cell stemness and metastasis in colorectal cancer, revealing that circ-ABCC1 may be used as a biomarker in CRC studies (Zhao H. et al., 2020).

In addition, the stem transcription factors upregulated by specific circRNAs can acts as positive regulators of circRNAs themselves, as revealed for SNAIL. It is a key transcription factor regulating many processes in tumor biology, such as the epithelial mesenchymal transition (EMT) and the induction and regulation of cancer stem cells. SNAIL is regulated by signaling networks involving plenty of ncRNAs, including circRNAs, which usually act as sponges for miRNAs targeting SNAIL. For example, in hepatocellular carcinoma (HCC), circ-ZNF652 is significantly upregulated and linked to highly metastatic features and poor prognosis, it physically interacts with miR-203 and miR-502-5p as a sponge to increase the expression of SNAIL. Interestingly, Snail, in turn, may also regulate circ-ZNF652 through physically binding to the E-box motif on the promoter of circ-ZNF652 to increase its expression. This loop thus forms a positive feedback that perpetuates the circ-ZNF652/miR-203/502-5p/Snail signaling axis (Guo et al., 2019; Skrzypek and Majka, 2020).

CONCLUSION

Circular RNAs are emerging as an extremely relevant class of endogenous RNAs expressed abundantly by the transcriptome. They are characterized by a covalently closed loop structure, resulting in RNA molecules that are more stable than linear RNAs. Thanks to these molecular structural features, they are suitable to be considered excellent biomarkers for the diagnosis and prognosis in cancer disease using liquid biopsy techniques.

High-throughput sequencing technologies are revealing an increasing number of circRNAs in all human organs and systems, the deregulation of which is a very important source of knowledge because it can be exploited for the early diagnosis and/or for the prediction of the outcome in some tumors. The implementation of specific bioinformatic tools makes possible to predict the cellular functions of circRNAs in physiological conditions and in diseases.

The circRNAs-microRNA code, in particular, is emerging to have great impact on the regulation of gene expression during development and differentiation, as well as in diverse pathologies and cancer. The molecular stability of circRNAs provides a great potential which renders them good tools for therapeutic innovative strategies. Engineered circRNA delivery to cells via exosomes could be among the most important issues in the next years to improve the precision therapy against several diseases, including cancer.

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All authors listed have contributed to collecting the data and to writing, improving, and finalizing the manuscript.

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REVIEW
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The Non-coding Side of Medulloblastoma

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Medulloblastoma (MB) is the most common pediatric brain tumor and a primary cause of cancer-related death in children. Until a few years ago, only clinical and histological features were exploited for MB pathological classification and outcome prognosis. In the past decade, the advancement of high-throughput molecular analyses that integrate genetic, epigenetic, and expression data, together with the availability of increasing wealth of patient samples, revealed the existence of four molecularly distinct MB subgroups. Their further classification into 12 subtypes not only reduced the well-characterized intertumoral heterogeneity, but also provided new opportunities for the design of targets for precision oncology. Moreover, the identification of tumorigenic and self-renewing subpopulations of cancer stem cells in MB has increased our knowledge of its biology. Despite these advancements, the origin of MB is still debated, and its molecular bases are poorly characterized. A major goal in the field is to identify the key genes that drive tumor growth and the mechanisms through which they are able to promote tumorigenesis. So far, only protein-coding genes acting as oncogenic drivers have been characterized in each MB subgroup. The contribution of the non-coding side of the genome, which produces a plethora of transcripts that control fundamental biological processes, as the cell choice between proliferation and differentiation, is still unappreciated. This review wants to fill this major gap by summarizing the recent findings on the impact of non-coding RNAs in MB initiation and progression. Furthermore, their potential role as specific MB biomarkers and novel therapeutic targets is also highlighted.

Keywords: non-coding RNA, microRNA, long non-coding RNA, neuronal differentiation, pediatric tumors, medulloblastoma, tumor subgroups

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INTRODUCTION

Medulloblastoma (MB) is an aggressive tumor arising in the cerebellum, and one of the most frequent malignant central nervous system (CNS) cancers in childhood. Accounting for about 20% of all brain primary tumors in children younger than 14 years (Gajjar et al., 2004; Juraschka and Taylor, 2019), it represents one of the leading causes of pediatric tumor-related death with an overall annual incidence of about 5 cases per 1 million in the pediatric population (Siegel et al., 2013; Rusert et al., 2014; Huang et al., 2016; Ostrom et al., 2018). Medulloblastoma is considered a pediatric tumor since its incidence in adulthood is far rarer, with 0.05 cases per 100,000 population (Ostrom et al., 2018). Over the years, the consideration of this very heterogeneous tumor substantially changed, allowing the achievement of a better clinical risk stratification and

therapeutic treatments, which have improved the overall survival rate of MB patients from 20% to approximately 80% in the last 35 years (Gottardo et al., 2014). This has been mainly due to the development of large-scale sequencing technologies that allowed the classification of MB into molecular subgroups and additional subtypes, each considered as a distinct disease. As a direct consequence, this categorization has opened the way to the development of molecular-based diagnoses and targeted therapeutic approaches. It is noteworthy that, so far, only protein-coding genes aberrantly expressed in distinct MB subgroups have been considered as potential tumor drivers, cancer biomarkers, and/or therapeutic targets. The implication of the predominant portion of the genome encoding for RNAs without coding potential, referred to as non-coding RNAs (ncRNAs), is only now coming into view.

NON-CODING RNAs: THEIR ROLE IN PHYSIOLOGY AND PATHOLOGY

It is now well established that protein-coding genes represent only a small portion of the mammalian genome, the non-coding genes representing the vast majority. It has been established that transcription is pervasive, because more than 75% of the genome is transcribed (Djebali et al., 2012). The consequence is the production of a huge number of transcripts without coding potential, the ncRNAs. Initially regarded as transcriptional noise, ncRNAs were then reconsidered as crucial regulators of gene expression (The Encode Project Consortium, 2012). These findings, together with the discovery that the proportion of non-coding genes strongly increases with the eukaryotic complexity, suggest that in complex organisms the non-coding sequences contain a large amount of regulatory information, much of which is managed by RNA.

Several classes of ncRNAs have been described. They differ in length, structure, biogenesis and maturation, and in their mechanism of action (Laneve et al., 2019). Nevertheless, their common theme is the ability to regulate gene expression by sequestering from or delivering to specific targets other nucleic acids and/or protein factors. Through these mechanisms, ncRNAs control almost every step of gene expression, from epigenetic modifications on chromatin (Lee, 2012; Mercer and Mattick, 2013) to transcription and splicing in the nucleus and RNA stability (Faghihi and Wahlestedt, 2009) and translation (Huarte, 2013; Bartel, 2018) in the cytoplasm.

The first parameter used to classify ncRNAs was their nucleotide (nt) length. A threshold of 200 nt distinguishes long ncRNAs (lncRNAs) from short ncRNAs (Kapranov et al., 2007; Wang and Chang, 2011), which include several classes of transcripts, as the well characterized microRNAs (miRNAs) and the nuclear (snRNA) and nucleolar RNAs.

Long ncRNAs represent a loosely classified group of long transcripts that have recently attracted increasing attention for their unique versatility. Exploiting their large size, they may work as flexible modular scaffolds endowed with discrete domains for protein interactions and with sequences for selecting RNA and DNA targets (**Figure 1**). They comprise a heterogeneous class of transcripts, both intergenic and intragenic, as well as enhancer RNAs (eRNAs), which enhance the expression of their coding counterparts, and circular RNAs (circRNAs), covalently closed molecules derived from non-canonical splicing events (Memczak et al., 2013) (**Figure 1**). Long ncRNAs have been proposed to participate in relevant biological processes as proliferation, differentiation and development, by regulating gene expression at the epigenetic, transcriptional, or posttranscriptional levels. Furthermore, they may act *in cis*, affecting the expression of their nearby genes, or *in trans*, influencing genes very far away. In line with their function, genome-wide studies of tumor specimens have shown that a large number of lncRNAs are associated with various types of cancer and have established that their mutations, as well as their deregulated expression, promote tumor initiation and progression (Vitiello et al., 2015; Bhan et al., 2017). A major feature of lncRNAs is their tissue-specific expression, which is often higher than messenger RNAs (mRNAs; Djebali et al., 2012). In line with this, a growing body of evidence suggests that lncRNAs are promising candidates as diagnostic biomarkers or therapeutic targets for disease (Huarte, 2015).

MicroRNAs are a conserved class of endogenous small ncRNAs (20–23 nt long) that are processed from stem-loop regions of long transcripts. They mediate posttranscriptional control of gene expression, acting as negative regulators. In particular, they function as "guide RNAs" that deliver the silencing machinery to specific mRNA targets. By binding through base-pairing to response sequences generally distributed in the 3′ untranslated regions (3′ UTRs) of mRNAs, they promote degradation or translational repression of their target genes (**Figure 1**). Even if the effect of an individual miRNA on a target's protein level is of fine-tuning, usually less than twofold (Baek et al., 2008), the combinatorial activity of different miRNAs on the same target strongly increases their repressive effect. Another salient feature of miRNAs is their pleiotropic activity. It means that an individual miRNA may target hundreds of target genes, which is relevant to canalize the regulatory programs. Through their activity, miRNAs influence gene programs underlying crucial biological processes as cell growth, proliferation, and differentiation, contributing to homeostasis and development (Jonas and Izaurralde, 2015; Bartel, 2018). In agreement with their biological importance, alterations in miRNA expression have been associated with tumorigenesis (Calin and Croce, 2006). Accordingly, miRNAs have been proposed as promising cancer biomarkers and potential therapeutic targets.

NON-CODING RNAs AND CEREBELLUM

Cerebellum has been considered for a long time as responsible for the acquisition of motor skills (Ramnani, 2006). However, over the past few decades, the advancements in neuroimaging studies, together with the development of computational model systems, have extended its contribution also to non-motor functions. Thanks to the connections with the cerebral cortex, the cerebellum is actively engaged in cognition and emotional activities (Ramnani, 2006; Koziol et al., 2014).

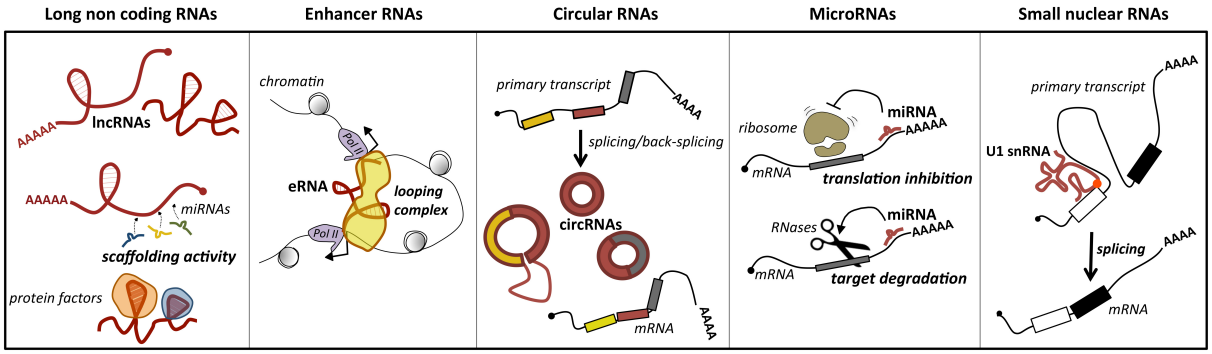

FIGURE 1 | Main classes of ncRNAs implicated in MB. For each class, the predominant activity is depicted: lncRNAs act as scaffolds for microRNAs (ceRNA function) or protein factors, which affects their activity; enhancer RNAs (eRNAs) may have a pivotal role in promoting mRNA transcription by facilitating enhancer–promoter interaction; circular RNAs (circRNAs), for their biogenesis from a non-canonical splicing event, may have a role in the control of mature mRNA levels, or additional functions as miRNA or protein decoys; miRNAs by direct pairing with their mRNA targets trigger their translation inhibition or degradation; small nuclear RNAs, as components of the ribonucleoprotein machinery operating the splicing, may control this reaction underlying gene expression. The position of U1 snRNA mutation identified in MB is indicated by a red dot.

It is a bilaterally symmetric structure originating from the dorsal part of the most anterior hindbrain, lying adjacent to the embryonic midbrain (Hibi and Shimizu, 2011; Butts et al., 2014). In human CNS, the cerebellum represents 10% of total brain mass and is the most architecturally complex region containing 80% of all neurons (Azevedo et al., 2009; Butts et al., 2012). It can be divided, along the mediolateral axis, into a midline vermis and two lateral hemispheres (**Figure 2A**; Constantin, 2017).

At a variance with this complexity, cerebellum is a very simple structure from a histological point of view (**Figure 2B**). Cerebellar cortex contains eight neuronal populations that are organized in three layers. The intermediate layer (Purkinje cell layer) contains the soma of Purkinje cells and candelabrum cells and is squeezed between a superficial (molecular) layer, containing a network of neuronal processes and interneurons, and an internal (granular) layer, composed of granule cells, Lugaro cells, interneurons, and unipolar brush cells (Constantin, 2017). Additionally, inside the Purkinje cell layer, a population of unipolar astrocyte, the Bergmann glia, is present, extending its radial fibers into the superficial layer.

In humans, cerebellar development occurs in the third trimester and continues beyond birth. During development, its surface increases significantly because of the formation of lobules allocating in a small area the large number of neurons, mainly granule cells (Scott et al., 2012). The territory from which the cerebellum originates is close to the hindbrain–midbrain boundary (the "isthmus"), an organizing center in the vertebrate neural tube required for the development of the midbrain–hindbrain domain. Recently, the involvement of miRNAs in the regulatory circuits that guarantee the establishment and the maintenance of hindbrain–midbrain boundary has been highlighted (**Figure 2C**). Mir-10 has been shown to downregulate key midbrain markers as *Otx2* and to upregulate hindbrain markers caudal to mid–hindbrain boundary as *Gbx2* in human neural progenitor cells (NPCs) (Jonsson et al., 2015). Loss of miR-10 expands the Gbx2 domain affecting the cerebellar development (Katahira et al., 2000). In zebrafish, miR-9, which is expressed adjacent to the midbrain–hindbrain boundary, reduced the boundary size by targeting components of the Fgf signaling pathway (Leucht et al., 2008), whereas in frog it promotes neurogenesis in the hindbrain by modifying the onset of the antineurogenic bHLH transcription factor (TF) program (Bonev et al., 2011).

MicroRNA profile analyses, knockout of the miRNA biosynthetic factor Dicer, and specific miRNA manipulations have revealed the pattern of miRNA expression in individual cerebellar cell types and have clarified miRNA implication in cell development or function (**Figure 2D**). In Bergmann glia, miRNAs, among which miR-9, establish specific transcriptional signatures ensuring proper cerebellar morphology (Tao et al., 2011; Kuang et al., 2012). In Purkinje cells, the expressed miRNAs (Pieczora et al., 2017) protect neurons from degeneration (Schaefer et al., 2007). Finally, in granule cells, Dicer-dependent pathways sustain cell development through the SHH signaling (Constantin and Wainwright, 2015) and the DNA damage response (Swahari et al., 2016).

MEDULLOBLASTOMA: FROM THE INITIAL DISCOVERY TO THE PRESENT CLASSIFICATION

In 1910, James Homer Wright described for the first time MB as a distinct CNS tumor and proposed that it may derive from restricted neuronal precursor cells, referred to as "neuroblasts" (Wright, 1910). Later, in 1926, Bailey and Cushing (1926) formulated a new theory on the origin of MB. They postulated that it is a posterior fossa brain tumor derived from primitive embryonic neuroepithelial cells, termed "medulloblasts," that reside in the primitive neural tube (Rutka and Hoffman, 1996; Kunschner, 2002; Millard and De Braganca, 2016). Taking advantage of his activity as a neurosurgeon, Cushing described the salient features of MB as a disease occurring mainly in preadolescents, with

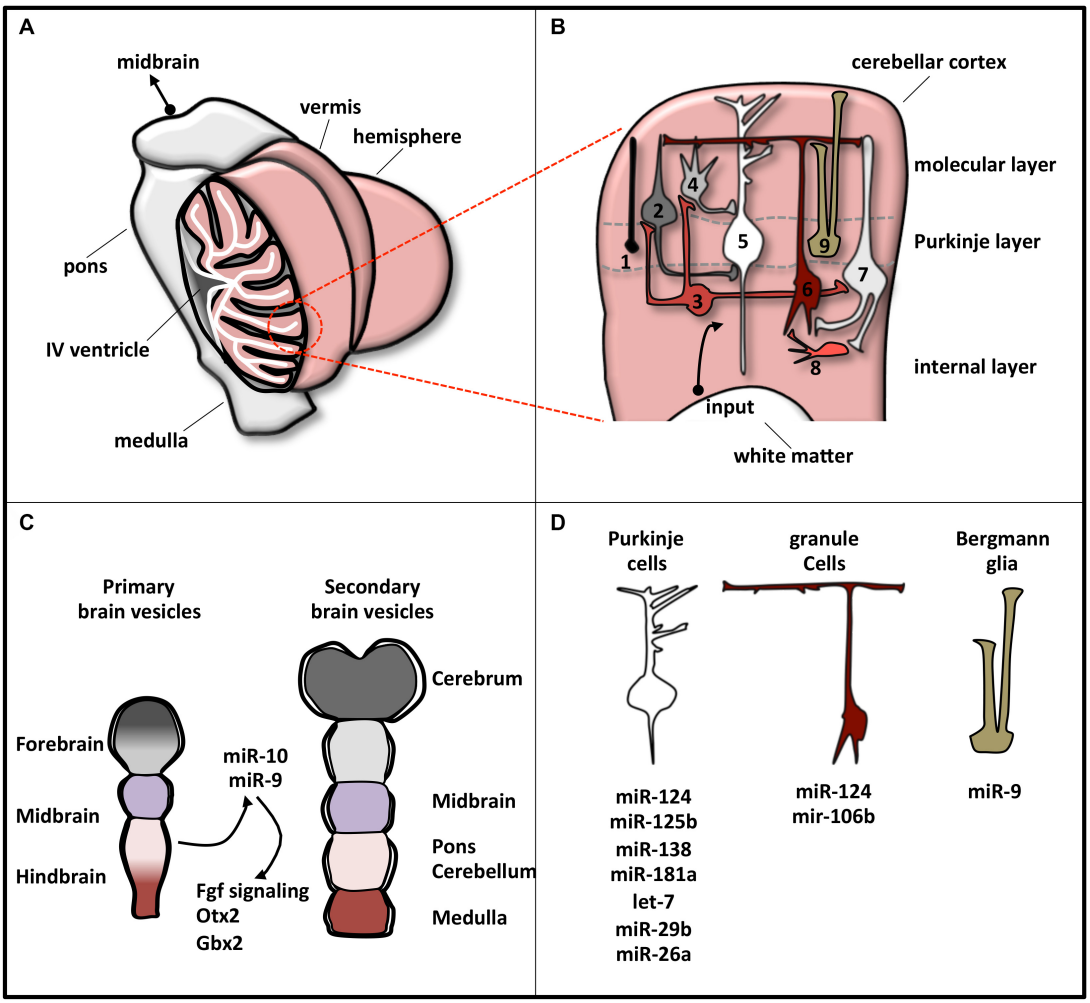

FIGURE 2 | Schematic representation of cerebellar structure. **(A)** The cerebellum consists of two lateral hemispheres connected by a narrow midline area (vermis). In the picture, the left hemisphere is sectioned to show the IV ventricle (which separates the cerebellum from the pons) and the lobular structure of the cortex, made of convoluted folia of gray matter supported by branching central medulla of white matter. **(B)** Magnification of cerebellum cortex. The eight neuronal cerebellar cell types are numbered as follows: (1) candelabrum cells; (2) basket cells; (3) Lugaro cells; (4) stellate cells; (5) Purkinje cells; (6) granule cells; (7) Golgi cells; (8) unipolar brush cell. Bergmann glia is indicated as 9. These cell types compose the three layers, indicated on the right. Input pathways from white matter include mossy and climbing fibers. **(C)** Brain early embryonic development. Primary and secondary vesicles identify boundaries between the prospective brain regions. miR-9 and miR-10 are expressed in the cerebellar anlage and specify the midbrain–hindbrain boundary. **(D)** Specific cerebellar cells express a plethora of miRNAs that regulate their differentiation or function. The most representative miRNAs expressed in each cell type are reported.

a relatively short history of symptoms and signs and the tendency to originate from the cerebellum vermis (Cushing, 1930). A further step toward the comprehension of the disease occurred in 1973, when MB was classified as a primitive neuroectodermal tumor for its histological features. At the same time, its origin from undifferentiated cells in the subependymal zone was hypothesized (Hart and Earle, 1973). However, only with the rise of the molecular era this hypothesis was overtaken, and MB started to be considered as a molecularly distinct brain tumor, arising from cerebellar granule cells (Pomeroy et al., 2002).

In 2007, the World Health Organization (WHO) published a classification of CNS tumors, primarily based on histopathological features. Accordingly, MBs were assigned to one of the four entities: classic (CMB), desmoplastic/nodular (DNMB), extensive nodular (MBEN), or anaplastic/large cell (LC/A) MB (Louis et al., 2007). The CMB variant is the most common histological subtype and is characterized by prototypical sheets of repetitive small cells with round nuclei and a high nuclear-to-cytoplasmic ratio (Northcott et al., 2012a). The MBEN variant occurs predominantly in infants. It is related to DNMB but differs for the presence of a markedly expanded lobular architecture, due to the presence of large zones, rich in neuropil-like tissues, containing a population of small cells similar to those of a central neurocytoma. A further difference between MBEN and DNMB relates to the internodular reticulin-rich component, which is reduced in MBEN, whereas it dominates the DNMB variant (Louis et al., 2007). The LC/A

type displays high levels of atypia and is characterized by marked nuclear pleomorphism, cell–cell wrapping, and high mitotic activity. This variant shows cytological overlap with the highly malignant LC MB, which is characterized by spherical cells, open chromatin, and prominent central nucleoli (Louis et al., 2007).

Over the past two decades, the rapid advances in genomics, epigenomics, and transcriptomics studies have tremendously accelerated the process of identification of genes, pathways, and biological processes underpinning MB onset. The -omics analyses were conducted by several international consortia, as the International Cancer Genome Consortium (Jones et al., 2012; Rausch et al., 2012; Kool et al., 2014; Northcott et al., 2014), the Pediatric Cancer Genome Project (Robinson et al., 2012), and the Medulloblastoma Advanced Genomics Consortium (Northcott et al., 2012c; Morrissy et al., 2016), and led to a new interpretation of MB as a collection of distinct diseases.

Genomics data, through the identification of frequently mutated new cancer genes, revealed the occurrence of distinct MB subgroups. Epigenomics and transcriptomics approaches in the postgenomic era disclosed typical epigenetic and transcriptional signatures that definitely converged on four MB subgroups. They are the better described Wingless (WNT) and Sonic Hedgehog (SHH) subgroups and the less characterized groups 3 and 4 (Northcott et al., 2011a,b, 2012a; Taylor et al., 2012). These new MB entities, allowing the shift from a tumor classification based on purely histological parameters to a new molecularly oriented categorization, were incorporated into the current 2016 WHO Classification of CNS tumors (Louis et al., 2016).

Recently, further studies investigated the proteomic landscape of MBs through quantitative mass spectrometry carried out on primary tumors (Rivero-Hinojosa et al., 2018). These analyses, while confirming the classification of MBs into the consensus subgroups, revealed a poor correlation between mRNA and protein expression, highlighting the crucial role of posttranscriptional mechanisms in MB etiology.

Moreover, studies making use of DNA methylation genome-wide approach, carried out on a large cohort of primary MB samples, produced new data that were integrated with gene expression profiles. These combined efforts provided a more comprehensive view of MB, further refining the molecular and clinical heterogeneity. Exploiting the similarity network fusion, a method of integrative clustering of multiple heterogeneous data sources (Wang et al., 2014), 12 new MB subtypes have been described. Even if not yet deeply characterized, the MB subtypes display distinct somatic copy-number aberrations, specific transcriptional signatures, differentially activated pathways, and different clinical outcomes (Cavalli et al., 2017). This important achievement will be helpful to resolve the molecular mechanisms and oncogenic drivers underlying the etiology of each subtype. This, in turn, will eventually shape driver events that are typical of a specific subtype, even if uncommon at a subgroup level.

WNT Subgroup and Subtypes

Among MBs, the best known subgroup is WNT, which occurs primarily in children from 4 years to early adulthood (Kool et al., 2012; Northcott et al., 2012b; Hovestadt et al., 2020) and accounts for about 10% of all MBs. WNT-MBs are usually of classic histology, are associated with most favorable prognosis, with 95% survival at 5 years in pediatric patients, are rarely metastatic at diagnosis (5–10% of cases), and rarely recur (Juraschka and Taylor, 2019; Hovestadt et al., 2020). WNT tumors are typically located midline with involvement of the brainstem or are positioned in the cerebellar peduncle and cerebellopontine angle cistern (Perreault et al., 2014). The hallmark feature of WNT-driven MB, found in about 85% of patients, are somatic mutations in *Ctnnb1* gene, which encodes β-catenin. The increased stability of the β-catenin protein induces the constitutive activation of WNT pathway and the consequent activation of WNT-responsive genes that promote cell proliferation (Northcott et al., 2017; Wang et al., 2018). The majority of WNT tumors lacking *Ctnnb1* mutations contain mutations in the *Apc* tumor-suppressor gene (Taylor et al., 2012; Waszak et al., 2018). Other genes recurrently mutated in these tumors are *Ddx3x* (36%), encoding a putative RNA helicase involved in chromosome segregation and cell cycle progression (Jones et al., 2012; Pugh et al., 2012; Robinson et al., 2012), and the genes encoding epigenetic factors as S*marca4* (19%) and C*rebbp*, suggesting that deregulation of the epigenome may be relevant for MB tumorigenesis (Robinson et al., 2012). This subgroup rarely displays copy number aberration except for loss of one copy of chromosome 6 (monosomy 6) occurring in 86% of patients (Northcott et al., 2012c, 2017).

The existence of two WNT subtypes—WNTα and WNTβ—has also been described (Cavalli et al., 2017). They differ in several features: WNTα occurs in 70% of cases, primarily affects children (median age of 10 years), and is characterized by monosomy 6. WNTβ has a lower incidence (30% of cases) and occurs in older children and adults, who infrequently have monosomy 6.

SHH Subgroup and Subtypes

Approximately 30% of all MBs are classified as SHH tumors. They display CMB and DNMB histologies occurring at similar frequency (by about 40%), whereas the remaining cases are of LC/A histology (Hovestadt et al., 2020). SHH-MBs are characterized by an intermediate prognosis with overall survival rates ranging from 60 to 80% (Cho et al., 2011; Northcott et al., 2011a,b; Kool et al., 2012; Taylor et al., 2012). SHH-MBs typically arise in the cerebellar hemispheres, and most often occur in infants and adults, with a minority of cases described in childhood (Perreault et al., 2014). Typical alterations of this subgroup include germline or somatic mutations or copy number alterations of components of the SHH pathway that results constitutively activated. Among mutated or deleted genes, there are *Ptch1* (43%) and *Sufu* (10%), encoding negative regulators of the SHH pathway (Johnson et al., 1996; Taylor et al., 2002; Brugières et al., 2010). Amplifications of SHH target genes as *MycN* (7%) and *Gli1* or *Gli2* (9%) (Gibson et al., 2010; Perreault et al., 2014; Raybaud et al., 2015) are frequently observed.

Other recurrent mutations, occurring in 30% of childhood SHH-MB, concern the *Tp53* gene and are associated with poor outcomes (Zhukova et al., 2013; Louis et al., 2016; Ramaswamy et al., 2016). Differently from WNT subgroup, frequent cytogenetic events in SHH tumors include loss of

chromosome 9q (causing loss of heterozygosity of *Pitch1*) and 10q (loss of *Sufu*) (Northcott et al., 2017).

Four subtypes, SHHα, SHHβ, SHHγ, and SHH*δ* have been described (Cavalli et al., 2017). SHHα tumors primarily affect children aged 3–16 years, have the worst prognosis, and are characterized by frequent *Tp53* mutations and *Myc/Gli2* amplifications. SHHβ tumors occur in infants and are frequently metastatic; they harbor focal *Pten* deletions (25% of cases) and have multiple focal amplifications. SHHγ occurs in infants and displays extensive nodularity histology. The SHHδ subtype, typical of adulthood, is enriched for *Tert* promoter mutations.

Group 3 Subgroup and Subtypes

Group 3 MB occurs almost exclusively in infants and young children, with a male predominance and a prevalent LC/A histology. Anatomically, these tumors have a midline vermian location adjacent to the fourth ventricle (Taylor et al., 2012; Perreault et al., 2014; Hovestadt et al., 2020). Group 3 accounts for approximately 25% of all MBs and is the most aggressive of the four subgroups, with the worst survival outcomes (<60% at 5 years) and the highest rates of metastasis at diagnosis (40–45%). Nevertheless, it is still considered an enigmatic tumor, because a common driver pathway has not yet been identified (Taylor et al., 2012). The most common genetic aberration is the amplification of *MYC* oncogene (17–20% of patients), which represents the major group 3 signature. *MYC* amplification frequently co-occurs with *PVT1-MYC* fusion, where PVT1 is a lncRNA supposed to stabilize the MYC protein (Northcott et al., 2012c; Tseng et al., 2014).

Gene mutations are rare in this group, and only four genes, namely, *Smarca4*, *Kbtbd4*, *Ctdneo1*, and *Kmt2d*, are mutated in 5% of cases (Northcott et al., 2017). Small fraction of group 3 tumors is associated with amplification of *Mycn* (5%) and of the TF *Otx2* (3%) (Northcott et al., 2017). Also, enhancer activation of *Gfi1* and *Gfi1b* expression has been observed in 40–50% of cases (Northcott et al., 2014). Moreover, this subgroup is characterized by genomic instability with gains of chromosome 1q,7 and deletions of 10q, 11, 16q, and 17p (Northcott et al., 2012a).

Two subtype classifications have been proposed for group 3 MB. The first one is based on methylation data and identifies as an high-risk subtype that displaying *Myc* amplification and a hypomethylation phenotype (Schwalbe et al., 2017). The second classification identifies three subtypes: 3α, occurring in infants, frequently metastatic but associated with a favorable prognosis; 3β, displaying a high frequency of activation of the oncogenes *Gfi1* and *Gfi1b* and of *Otx2* and rarely metastatic; and 3γ occurring in infants, associated with *Myc* amplification and displaying the worst prognosis (Cavalli et al., 2017).

Group 4 Subgroup and Subtypes

Group 4 is the least understood and the most common subgroup among MBs. It accounts for 35–40% of all MB diagnoses and typically occurs in childhood and adolescence with a higher frequency in males (3:1 sex ratio) (Taylor et al., 2012). The outcome of group 4 patients is intermediate even if metastases are often present at diagnosis. In approximately 6–9% of cases, common mutations in *Kdm6a*, *Zmym3*, *Ktm2c*, and *Kbtbd4* genes have been described, together with the amplification of *Mycn* and *Cdk6* genes and the overexpression of *Prdm6* gene, which is frequently associated with *Sncaip* duplication events (Northcott et al., 2017). Group 4 MB has been further subdivided into three subtypes: group 4α characterized by *MycN* and *Cdk6* amplifications and strongly enriched for 8p loss, group 4β displaying *Sncaip* duplication, and group 4γ enriched for focal *Cdk6* amplification and for 8p loss (Cavalli et al., 2017).

MEDULLOBLASTOMA ORIGIN

Besides the genome, epigenome, and transcriptome alterations, the heterogeneity of the four MB subgroups may be partially due to their different developmental origins. Identification of the specific cell types these tumors originate from may be very informative for both the understanding of the malignancy and the development of appropriate treatments. Medulloblastoma tumors are thought to originate in the cerebellum, except for the WNT subgroups that arise outside the cerebellum and are distributed within the fourth ventricle and infiltrated the dorsal surface of the brainstem (Gibson et al., 2010). Dorsal brainstem progenitor cells of cochlear, mossy fiber, and climbing fiber neurons are regarded as the potential source of these tumors (Gibson et al., 2010), following the activation of *Ctnnb1* and the concurrent *Trp53* deletion (Lu et al., 2019).

Differently, all available SHH-subtype tumors were localized away from the brainstem, within the cerebellar hemispheres. They are thought to originate from cerebellar neural stem cells (NSCs) or committed granule neuron precursor cells (GNPCs) following aberrant activation of SHH (Gibson et al., 2010; Lu et al., 2019). The group 3 MB tumors are often positioned near the fourth ventricle, pointing to cerebellar stem/progenitor cells or GNPCs as potential sources (Kawauchi et al., 2012; Pei et al., 2012; Lu et al., 2019). The source of group 4 MB is still debated. Based on the spatiotemporal activity of a subset of group 4 master TFs, deep cerebellar nuclei, residing in the cerebellar nuclear transitory zone, or their earlier precursors deriving from the upper rhombic lip, are considered their putative cells of origin (Lin et al., 2016).

A major shift in our understanding of MB origin occurred very recently, thanks to the use of large-scale single-cell RNA sequencing (RNA-Seq), a powerful technique to identify cellular populations that are biologically distinct on the basis of gene activity. This transcriptome analysis, carried out on RNA from murine cerebellum at specific time points of development, allowed the identification of specific neural cell types and subtypes and their anatomical location and putative developmental origin, and to draw pseudotime trajectories for the various cerebellum lineages (Carter et al., 2018; Vladiou et al., 2019). By applying single-cell analysis on a cohort of primary MBs, two independent research groups revealed the high transcriptional heterogeneity of MB subgroups (Hovestadt et al., 2019; Vladiou et al., 2019).

Following comparison between the transcriptomes of lineage-restricted cell populations during cerebellum development and of MB cells, Vladiou et al. (2019) were able to demonstrate that SHH-MB includes a variety of cell types with various levels of differentiation and growth capacity mirroring the temporal evolution of the developing GNPC hierarchy. In particular, SHH-MBs better match to the GNPCs in the early postnatal period. RNA-Seq from Group 3 MB revealed the presence of highly divergent lines of differentiation that mirror normal development along the GNPC, unipolar Brusch cell, Purkinje cell, and GABAergic interneuron lineages. This indicates an origin from uncommitted cerebellar stem cells, followed by differentiation of transformed cells along diverse developmental lineages. The same analysis carried out on group 4, whose cell of origin is unknown, revealed that these tumors display a better match with the transcriptomes of the UBC lineage at several time points during UBC development. However, the observation that group 4 MB cells simultaneously express both GNPC and UBC marker genes suggests that group 4 arises from a population of bipotential progenitor cells that are able to generate cells of both the GNPC and UBC lineages.

MB CANCER STEM CELLS

An additional cause of intratumor heterogeneity is ascribed to cancer stem cells (CSCs), which are responsible for triggering tumor initiation, maintenance, and progression *in vivo*. Cancer stem cells result from the accumulation of transforming mutations and maintain two abilities: self-renewal, which allows the expansion of CSC pool, and differentiation, through which they generate the heterogeneous cell lineages that constitute the bulk of the tumor (Huang et al., 2016).

The CSC model, proposed to explain the intratumor heterogeneity, derives from studies carried out on different tumors including brain tumors (Singh et al., 2003). Medulloblastoma stem cells (MBSCs) have been initially identified and sorted using specific stem cell biomarkers, as CD133 and CD15, and different approaches, such as flow cytometry, xenograft models, and lineage tracing. The first studies revealed that $CD133^+$/$Nestin^+$ cells isolated from MB tissue were able to proliferate, self-renew, and differentiate *in vitro* (Singh et al., 2003). However, further studies concluded that also $CD133^-$ MB cells possessed the same properties (Srivastava and Nalbantoglu, 2008; Read et al., 2009), highlighting the troubles in isolating MBSCs. A significant step forward in the identification of MBSCs was done exploiting the genetically engineered mouse (GEM) models established on the basis of MB molecular classification. In 2009, a tumor-propagating $CD15^+$ cell was isolated from a *Patched* haploinsufficient ($Ptch^{+/-}$) MB model (Read et al., 2009). These cells showed stem-like and tumor-initiating capacity (Ward et al., 2009). It was also demonstrated that $CD15^+$ MBSCs express aberrant levels of the SHH target genes *Gli1* and *CyclinD1*, which in turn suggests that their increased proliferative capacity is related with increased activation of SHH pathway (Vanner et al., 2014). In addition, the findings that Gli1/2, the main effectors of the SHH pathway, interact with the stemness factors Nanog (Po et al., 2010) and MycN (Marino, 2005) and with the polycomb protein Bmi-1 (Leung et al., 2004; Wang et al., 2012) in the self-renewal regulation of MBSCs indicate that the SHH pathway has a pivotal role in MBSC maintenance and activity. A role in MBSC self-renewal has also been unveiled for the Notch pathway that is involved in the regulation of stem cells both in physiological and pathological conditions. In particular, the main effector of the pathway Hes1 was found upregulated in $CD133^+$ MB cells, and its downregulation largely reduced the $CD133^+$ cell fraction (Fan et al., 2006).

More recently, it was shown that quiescent $Sox2^+$ MB cells from postirradiated $Ptch^{+/-}$ mice were tumorigenic and showed a greater self-renewal capacity than their proliferating progeny (Vanner et al., 2014). Further studies revealed a tight association of oncogenes as *Myc* (Venkataraman et al., 2014) and *MycN* (Ahmad et al., 2015), which are aberrantly amplified in MB, with MBSC stemness. Accordingly, inhibition of Myc-regulated transcription program causes the suppression of stem cell–associated signaling in MB cells and the inhibition of MB tumor cell self-renewal (Venkataraman et al., 2014). Similarly, it was shown that depletion of *MycN* in tumor-derived neurosphere cell line, derived from a GEM model of MycN-driven MB, negatively affects the expansion of cells expressing markers of NSCs and/or progenitors associated with MB tumorigenesis (Ahmad et al., 2015).

Increasing evidence indicates that also the non-coding portion of the genome participates in the regulation of cancer cell stemness and in the maintenance of CSC population (see section "MiRNAs and MB Cancer Stem Cells").

MiRNAs IN MB

A number of studies aimed to identify miRNAs engaged in MB tumorigenesis, in order to define novel markers for accurate diagnosis and regulatory modules as prospective targets for therapeutic interventions. As a consequence, miRNAs have been largely associated with MB, in which their aberrant expression or mutations (Lu et al., 2009; Lv et al., 2012) underpin oncogenic or oncosuppressive functions.

These research lines took advantage of high-throughput methods, candidate-oriented approaches, or even combined strategies.

High-Throughput Analyses of miRNAs in MB: Pioneering Studies

To globally investigate the involvement of miRNAs in MB carcinogenesis, Ferretti et al. (2009) performed the first high-throughput miRNA expression profile in human MB specimens. This study: (1) highlighted that miRNAs were predominantly downregulated in MB, suggesting a general function as tumor suppressors; (2) allowed the identification of specific miRNA signatures, which distinguished tumors from healthy tissues, which recognized distinct MB histotypes or subsets, and which correlated with disease risk and (3) identified single miRNA

candidates for functional analyses. Among them, miR-9 and miR-125a, previously shown to inhibit proliferation of cells of neuroblastoma (NB) (Laneve et al., 2007), a pediatric tumor of the sympathetic NS, were confirmed to arrest MB cell growth, through both reduction of cell proliferation and increase of apoptosis. MiR-9 and miR-125a: (1) target the truncated isoform of the tyrosine kinase receptor C (tTRKC), overexpressed in many tumors; (2) balance the ratio with the full-length isoform (Kim et al., 1999; Grotzer et al., 2000) and (3) are associated with a favorable prognosis. This research group also analyzed the role of miRNAs in SHH pathway, whose constitutive activation makes GNPCs susceptible of malignant transformation into MB (Ruiz et al., 2002; Kimura et al., 2005). MicroRNA expression profiling was performed in two subsets of human primary MBs (Ferretti et al., 2008), showing high or low SHH signaling strength (i.e., high or low Gli levels). A signature of 30 downregulated miRNAs was identified in Gli^{High} tumors, suggesting that loss of specific miRNAs may be associated with SHH signaling alteration in MB. Among the deregulated species, miR-125b, miR-326, and miR-324-5p were found to control the expression of positive members of the pathway, the activator Smo, and the effector Gli1. This miRNA-mediated circuitry antagonizes SHH activity during cerebellar GNPC differentiation, whereas its abrogation during neuronal development promotes brain tumorigenesis. Interestingly, the loss of miR-324-5p is caused by chromosome 17p deletion, a hallmark of approximately 40% of MBs. Later on, the same authors demonstrated that miR-326 and its host gene Arrb1, encoding for an adaptor and scaffold protein regulating several signaling pathways involved in cell development and cancer (DeWire et al., 2007), are both downregulated in MBSCs derived from SHH-MB, where they act as negative regulators of self-renewal at the posttranscriptional and posttranslational level (Miele et al., 2017).

Finally, Birks et al. (2011) were among the first to apply microarray-based determination of miRNA expression to pediatrics brain cancers, among which MB. Interestingly, besides specific signatures of overexpressed or downregulated miRNAs in each tumor group, differential expression of miR-129, miR-142-5p, and miR-25 in all tumor types, compared to normal tissues, was unveiled.

MiRNA Profiling as a Tool for MB Diagnosis and Stratification

In the following decade, great emphasis was devoted to the study of small regulatory RNAs. MicroRNA profiling was largely exploited to identify useful biomarkers for MB diagnosis (Dai et al., 2017; Tantawy et al., 2018), classification, and clinical management.

The detection of miRNAs in biological liquids (Chen et al., 2008; Mitchell et al., 2008), including cerebrospinal fluid (CSF) (Baraniskin et al., 2011; Teplyuk et al., 2012; Shalaby and Grotzer, 2015), coupled to miRNA high stability and easy detectability, makes them ideal diagnostic and prognostic markers for brain tumors. Croce's group utilized for the first time NanoString, a variation of microarray technology, to identify a CSF miRNA signature that distinguishes among CNS malignancies (Drusco et al., 2015). More than 80 samples were collected from 34 patients with CNS benign and malignant tumors, including MB. MiR-451, -711, -935, -223, and -125b were found differentially expressed between and among groups. These miRNAs were validated, by reverse transcriptase–polymerase chain reaction (RT-PCR) and *in situ* hybridization, as promising cancer CSF markers for accurate diagnosis of CNS neoplasms. The identification of candidate circulating biomarkers remains a primary goal in cancer research. Analysis of the proteome and miRNome of extracellular microvesicles (MVs), released by highly aggressive stem-like MB cells, detected 10 miRNAs exclusively present in MVs of cells overexpressing the pluripotent factor *Oct4A* and revealed ERK, PI3K/AKT/mTOR, and EGF/EGFR as the primary altered oncogenic signaling pathways in these cells (Kaid et al., 2020).

Other studies used extensive miRNA analysis for classifying primary MBs (Cho et al., 2011), also in the rare adult cases (Kaur et al., 2016). By low-density array, Gokhale et al. (2010) identified 216 MB-expressed miRNAs that segregated into subgroups closely matching those identified by protein-coding gene profiling. The most robust miRNA signature was found in WNT-MB. Among the 16 differentially expressed miRNAs in the WNT subgroup, the two most upregulated species, miR-193a and miR-224, were functionally validated in MB cell lines. They were able to reduce proliferation, to increase radiation sensitivity, and to inhibit anchorage-independent cell growth, indicating their contribution to the lower metastatic potential and better response to radiation therapy of WNT-MB. In 2013, the same authors confirmed the effectiveness of miRNA-based molecular subgrouping on additional 103 MBs, including 59 formalin-fixed paraffin-embedded tissues (Kunder et al., 2013). Later on, miR-449a (Li et al., 2016) and miR-148a (Yogi et al., 2015) were reported as candidate tumor-suppressor genes and potential diagnostic markers or therapeutic targets in WNT-MBs.

Large-scale miRNA evaluations also allowed inferring MB bioclinical features (Pezuk et al., 2017). MicroRNA microarrays in pediatric samples of CNS neoplasms (MB and atypical teratoid/rhabdoid tumors), together with meta-analyses based on available miRNA datasets, identified and validated miRNA candidates whose differential expression was associated with MB prognosis: age at diagnosis, disease progression, and clinical outcome (Braoudaki et al., 2014). In particular, miR-34a was found as upregulated in all the samples tested.

Recently, high-throughput sequencing on tissues from different subgroups of MB revealed miRNA signatures distinguishing between groups 3 and 4 (Gershanov et al., 2018), whose discrimination is still challenging due to the paucity of available biomarkers. Of 783 expressed miRNAs in at least 1 sample, 462 were common to all subgroups, and 19 were differentially expressed between group 4 and the others. Three miRNAs, miR-20a-5p, 181a-2-3p, and 224-5p, were identified as specific group 4 markers, able to distinguish between group 4 and group 3. Integrated miRNA–mRNA analysis revealed the anticorrelation between group 4 differentially expressed miRNAs and their predicted/validated mRNA targets, identifying at least

five inversely expressed miRNAs–mRNAs couples involved in pathways as cellular development, cellular growth and proliferation, and connective tissue development and function.

Taking advantage of integrated analyses downstream to comprehensive RNA profiling in primary MBs, additional reports highlighted the miRNA–mRNA anticorrelation in MB, revealing coding and ncRNA pathways possibly relevant in this malignancy (Kumar et al., 2018). Overall, these findings support the potential of miRNAs as biomarkers for MB classification. In addition, the identification of miRNA–mRNA regulatory networks underscores specific genes or gene pathways as candidate targets for MB molecular therapies.

MiRNA and mRNA Integrated Analyses in MB Progression

The categorization of MB into distinct subgroups with specific mutational and expression profiles fueled miRNA/mRNA comparative studies to clarify the cellular and molecular bases of MB origin and maintenance.

Genovesi et al. (2011) assessed miRNA expression in primary human MB specimens, MB cell lines, and, notably, in $CD133^+$ NSCs compared to $CD133^-$ NPCs. Approximately 30 miRNAs were differentially expressed in primary MB samples compared to $CD133^+$ NSCs, many of the upregulated or downregulated species mapping to identical chromosomal regions. By integrating sample-matched miRNA expression profiles with mRNA gene expression data, several putative regulatory networks in MB were identified. An enrichment analysis focused on deregulated mRNA targets revealed overrepresentation of pathways associated with neuronal migration and NS development or with cell proliferation and programmed cell death. The most significantly downregulated miRNA, miR-935, was deeply analyzed, and several putative anticorrelated targets were validated in MB cells.

Along this line, miRNA and mRNA expression in MBSCs derived from $Ptch^{+/-}$ mice (SHH-MB, Po et al., 2010) was compared to equivalent profiles from cerebellar NSCs (Po et al., 2018). Medulloblastoma SCs were characterized by 35 upregulated and 133 downregulated miRNAs. The intersection between the gene pathways enriched in the SHH-MBSC miRNAome and transcriptome revealed common gene networks (such as PI3k-Akt pathway and protein processing in endoplasmic reticulum pathway), including putative targets of the differentially expressed miRNAs, reported to play a role in cancer cell growth and maintenance.

MicroRNA and mRNA expression profiling were also paralleled in murine highly tumorigenic MBSCs (from $Ptch^{+/-}$; $p53^{-/-}$ mice) and low-tumorigenic CSCs ($Ptch^{+/-}$; $p53^{+/+}$) or normal NSCs (Hemmesi et al., 2015). The expression of miR-135a was inversely correlated with the tumor-initiating potential of MBSCs and with the expression of its target Arhgef6, a factor implicated in the formation of focal adhesion structures essential for cell motility (Rosenberger et al., 2005; Rosenberger and Kutsche, 2006). MiR-135a restoration in MBSCs inhibits tumor progression *in vivo*, and its expression was significantly downregulated in all the histological variants of human MBs.

Taking advantage of analogous model systems, Tanno et al. (2016) explored the miRNome alterations in radiation-induced MB tumorigenesis. MicroRNA expression analysis by NGS in *ex vivo* unirradiated or radiation-treated WT and $Ptch^{+/-}$ GNPCs identified a subset of miRNAs controlling different biological functions, as inferred by mRNA network analysis. MicroRNAs were also proposed as putative epigenetic transgenerational messengers responsible for MB susceptibility in the progeny of irradiated male mice (Paris et al., 2015).

Altogether, these and other studies (Catanzaro et al., 2016) confirm the potential of integrated miRNA/mRNA expression analyses to provide insights into MB biology and to identify candidate genes with a functional role in MB progression.

MiRNA-MEDIATED GENE PATHWAYS IN MB

Dozens of miRNAs have been specifically linked to MB and already largely reviewed. Four historically relevant and deeply characterized examples of miRNA-regulated molecular pathways in MB are detailed below (**Figure 3**).

MiR-124 and Cell Cycle Regulation

MiR-124 is a highly conserved and brain-enriched miRNA, whose role in neural development has been extensively described (Cheng et al., 2009; Yoo et al., 2011). It was the first miRNA linked to MB pathogenesis through targeted analyses.

The observation that overexpression in MB of the adverse prognostic marker Cdk6 (Mendrzyk et al., 2005) cannot be completely explained through its genomic amplification raised the hypothesis of a miRNA-based control of its levels. Due to its preferential expression in differentiating and mature neurons (Gao, 2010), its potential binding to *Cdk6* 3′ UTR and its significant downregulation in both MB cell lines and primary cells (Pierson et al., 2008), Pierson and colleagues focused their attention on miR-124. It was found to be downregulated in patient tumor samples, where miR-124 and *Cdk6* expression levels were inversely correlated. Mir-124 was demonstrated to target *Cdk6* gene and to impact on cell growth when re-expressed in MB. These findings enforced the idea that miR-124 may play a role in MB pathogenesis as a tumor suppressor, controlling *Cdk6* that is crucially implicated in cell proliferation and differentiation (Grossel and Hinds, 2006). Consistently, low levels of miR-124 may explain why MB cells have a less differentiated phenotype than the adult cerebellar tissues. The regulatory axis between mir-124 and *Cdk6* was substantially corroborated in 2013, by Silber et al. (2013). They showed: (1) an opposite trend of expression between the two transcripts in primary MBs; (2) a Cdk6-dependent cell cycle arrest and inhibition of MB cell proliferation through miR-124 ectopic expression and (3) MB growth arrest *in vivo* by intracerebellar or subcutaneous transplantation of miR-124 over-expressing cells in immunosuppressed mice.

Independent research lines further strengthen the idea of miR-124 as a tumor suppressor and cell cycle regulator in MB (Li et al., 2009a; Tenga et al., 2016). Overall, these studies demonstrate a deregulation of miR-124 in brain tumor tissues

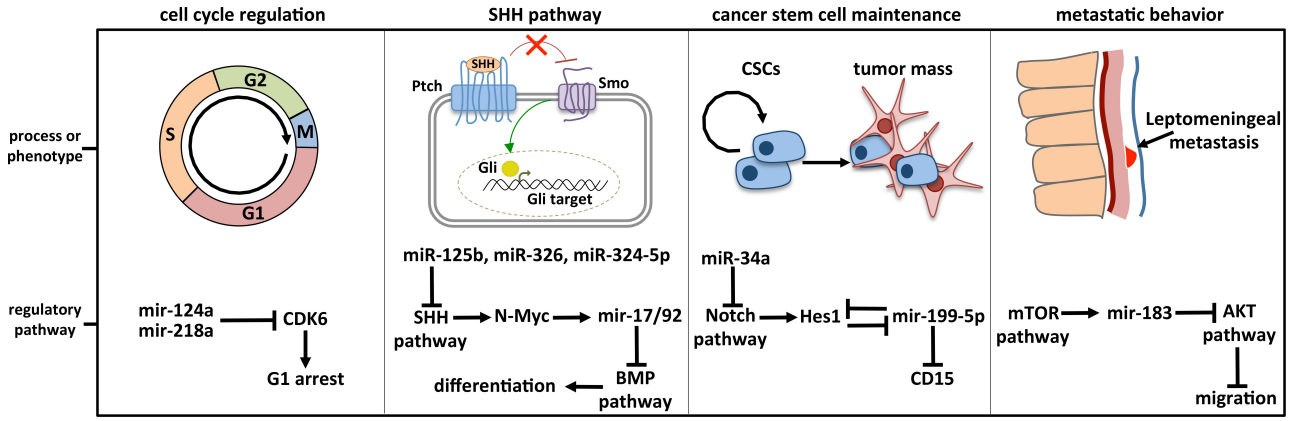

FIGURE 3 | Deeply characterized cellular processes affected by miRNAs in MB. The brain-enriched miR-124a and miR-218a participate in cell cycle control by modulating the expression of CDK6, the cyclin-dependent kinase 6 crucially implicated in cell cycle progression. MiR-125b, miR-326, miR-324-5p, and MiR-17/92 cluster is included in the regulatory axis between the SHH and the BMP signaling, participating in the cell choice between proliferation and differentiation. MiR-125b, miR-326, and miR-324-5p guide the SHH pathway, modulating Smo and Gli1, respectively activator and effector of the cascade. MiR-199-5p is involved in CSC maintenance by modulating the CSC marker CD15 and Hes1, the main Notch downstream effector with which a negative regulative feedback loop is established. Notch signaling is also controlled by miR-34a, at the level of the ligand Dll1. The retinal miR-183 is part of the regulative axis between mTOR pathway, from which it is activated, and AKT pathway involved in cell migration.

and cells and suggest the possibility that its altered expression in differentiating NPCs may contribute to brain malignancies. This link is underscored by the finding that REST, the global transcriptional repressor of neuronal differentiation, is one of the most relevant regulators of miR-124 (Conaco et al., 2006). Its abnormal expression in cerebellar NPCs causes the arrest of neuronal differentiation *in vitro* (Su et al., 2004) and the formation of histologically MB-like tumors in implanted mice (Su et al., 2006).

Subsequently, a role for miR-218 in the control of cell cycle in MB was also described, as an additional modulator of *Cdk6* expression (Venkataraman et al., 2013). Consistently with the findings that: (1) Dicer supports proper cerebellar development (Constantin et al., 2013) and curbs MB formation (Zindy et al., 2015) and (2) its ablation impairs the expression of cell cycle regulator genes in MB (Liu et al., 2017), a plethora of miRNAs were described as modulators of cell homeostasis in MB. They mainly affect cell proliferation, differentiation, and apoptosis (Li K. K. et al., 2013; Li et al., 2015; Jin et al., 2014; Xu et al., 2014; Pal and Greene, 2015; Panwalkar et al., 2015; Salm et al., 2015; Senfter et al., 2019; Yang et al., 2019), as well as senescence (Venkataraman et al., 2010) and autophagy (Singh et al., 2017).

MiR-17/92 Cluster and the SHH Pathway

The miR-17/92 polycistron was one of the first miRNA clusters to be validated as oncogenic (He et al., 2005). Its implication in MB was discovered in 2009 (Uziel et al., 2009), upon deep sequencing and comparative expression analyses of both cerebellar tissues and purified GNPCs from wild-type and MB mouse models. Within the 26 identified miRNAs, putatively acting as oncogenic miRNAs due to lower expression in wild-type samples, as many as nine species were encoded by the miR-17/92 cluster and its paralogues. Upregulated miR-17/92 cluster expression was also confirmed in human MB showing an activated SHH pathway, which suggested a correlation between SHH signaling and miR-17/92 levels.

The association between miR-17/92 and MB was assessed in parallel by Northcott et al. (2009), through single-nucleotide polymorphism arrays aimed to define recurrent copy number aberrations. They identified both amplification and higher expression of miR-17/92 locus in SHH-MB samples compared to normal tissues. Functionally, this study demonstrated that MycN, a TF known to regulate miR-17/92 in other systems together with Myc (O'Donnell et al., 2005; Schulte et al., 2008), was abundantly expressed in tumors with high levels of miR-17/92, and it was able to drive miR-17/92 transcription in GNPCs. This finding, together with the concepts that *MycN* is a downstream target of SHH signaling and drives GNPC proliferation (Kenney et al., 2003; Oliver et al., 2003), shed new light on the regulatory axis through which aberrant SHH signaling drives tumorigenic pathway in MB. Phenotypically, enforced expression of the miR-17/92 cluster in purified primary mouse GNPCs increased the penetrance and accelerated the development of tumors in orthotopically transplanted mice (Uziel et al., 2009). It is noteworthy that this occurred specifically in *Ptch1*$^{+/-}$ GNPCs, where the SHH cascade is aberrantly activated (Evangelista et al., 2006), which definitively demonstrates the functional link between the SHH pathway and the miRNA cluster. Indeed, even though dispensable for cerebella development, miR-17/92 cluster is necessary for SHH-MB tumor formation (Zindy et al., 2014). To clarify the underlying control circuitry, reverse genetics approaches were undertaken (Murphy et al., 2013). LNA-anti-miR–mediated miR-17 and miR-19a silencing in primary cells from SHH-MB led to suppression of cell growth *in vitro* due to cell cycle arrest and to inhibition of secondary tumor progression in allograft mice. MiR-17 silencing also reduced the progression of tumors derived from intracranial

transplants and increased survival. These effects may be explained by the finding that Brmp2, a member of the bone morphogenetic protein (BMP) signaling pathway that induces the differentiation of SHH-MB (Zhao et al., 2008), is a target of miR-17 and miR-19.

MiRNAs and MB Cancer Stem Cells

The Notch signaling plays an essential role in the regulation of cellular processes during embryonic and postnatal development (Artavanis-Tsakonas et al., 1999). In the cerebellum, it regulates the differentiation of GNPCs (Solecki et al., 2001). Notch cascade is also involved in the maintenance of MBSCs. Consistently, pathway components such as the receptor Notch2 and the TF Hes1, the main Notch downstream effector, are deregulated in MB (Fan et al., 2004). *In silico* inspections of miRNA databases predicted miR-199-5p as a putative modulator of *Hes1*, and this regulative axis was confirmed by reverse-genetics approaches (Garzia et al., 2009). Cellular and molecular assays demonstrated that the stable expression of miR-199-5p in several MB cell lines reduced their proliferation rates through cell-cycle alterations, increased the expression of differentiation markers, and impaired clonogenic potential. Interestingly, clones stably expressing miR-199-5p were depleted of a $CD133^+$ cancer stem-like cell side-population, whereas restoration of *Hes1* expression partially rescued cell cycle block, decreased cell differentiation, and recovered side-population cells. This demonstrates that *Hes1* downregulation was directly correlated with these phenotypes. The potentiality of miR-199b-5p as a tumor suppressor was validated *in vivo*, in xenografts derived from stably expressing miR-199-5p clones. On the other hand, miR-199b-5p was downregulated in metastatic MB samples where Hes1 protein is upregulated, thus confirming their participation in the same regulatory module.

The molecular circuit underlying miR-199-5p expression was deeply unraveled by Zollo's group, who revealed an exciting negative feedback loop between the repressor Hes1 and the miRNA (Andolfo et al., 2012). *Hes1* inhibition or silencing caused a significant increase of miR-199-5p, mediated by the presence of Hes1 binding sites on miR-199b-5p promoter. Furthermore, an additional layer of regulation was highlighted, dependent on the methylation status of a CpG island in the region upstream to miR-199-5p promoter. Functionally, the tumor-propagating cell marker CD15 was demonstrated to be a direct target of miR-199-5p, which also alters the expression and phosphorylation of the major proteins of AKT and ERK networks, involved in cancer metastasis and cancer stem-cell maintenance.

The control of Notch pathway in MB is also contributed by other miRNAs, such as the key regulator of neuronal differentiation miR-9 (Fiaschetti et al., 2014) and the p53-dependent miR-34a (He et al., 2007). MiR-34a was deeply analyzed in MB. It was found to affect Notch signaling as a negative regulator of the major ligand of Notch receptor, Delta-like 1 (Dll1) (de Antonellis et al., 2011), which contributes to the maintenance of the undifferentiated state of neural progenitors in CNS (Kawaguchi et al., 2008). MiR-34a–sustained expression induced apoptosis and inhibited proliferation of $CD15^+/CD133^+$ MBSCs *in vitro*, promoting neural differentiation. *In vivo*, miR-34a inhibits tumor growth in orthotopically and heterotopically transplanted nude mice through inhibition of AKT/PI3K/PTEN signaling, which is responsible for the maintenance and propagation of this cell population.

The pleiotropic role of miR-34 as a tumor suppressor is paradigmatic. In MB, it potently inhibits c-Met, a tyrosine kinase receptor activating a range of signaling pathways in development and cancer (Li et al., 2005), and suppresses numerous malignancy parameters *in vitro* (Li et al., 2009b). Its deficiency in MB mouse models accelerates the incidence and timing of tumor formation *in vivo* (Thor et al., 2015), possibly affecting multiple targets, among which MycN, frequently amplified in SHH-MB and known to drive GNPC proliferation during medulloblastomagenesis. A body of evidence indicates that miR-34a may represent a potential therapeutic agent in MB, by modulating at multiple levels p53 tumor suppressor pathway (Fan et al., 2014) and conferring sensitivity to chemotherapeutic treatments. In particular, the identification of targets, among which miRNAs (Lee et al., 2014; Abdelfattah et al., 2018; Ray et al., 2018), serving as effective and low neurotoxic adjuvants to treat MB, is of paramount importance. This is due to the high risk of MB relapses and to the severity of side effects related to therapeutic regimens. The implication of miRNAs in MBSC biology (Lee et al., 2014; Kaid et al., 2015; Besharat et al., 2018) may help for this search.

MiRNAs and MB Metastases

The spread of cancerous cells to the spinal and intracranial leptomeninges (leptomeningeal dissemination) is the principal hallmark of MB unfavorable outcome, due to fatal tumor recurrence (Rossi et al., 2008). This explains the deep interest toward the comprehension of the molecular mechanisms responsible for MB metastatic behavior. A miRNA screening of 32 MB samples indicated that the retinal miRNAs miR-182, miR-183, and miR-96, members of the oncogenic miR-183/96/182 cluster (Xu et al., 2007), were significantly overexpressed in non-SHH-MBs (Bai et al., 2012). Two candidates, miR-182 and miR-183, were further phenotypically characterized, showing an increased migratory effect upon overexpression in MB cells and a decreased motility propensity upon their knockdown. *In vivo*, orthotopic xenograft experiments revealed that miR-182 overexpressing tumors extensively infiltrated in the surrounding normal tissue compared to control samples and, importantly, showed local leptomeningeal metastases.

An independent study further investigated the function of miR-183/96/182 cluster in the maintenance, survival, and dissemination of tumor cells in the aggressive *Myc*-amplified subgroup, where the miRNA cluster was upregulated (Bai et al., 2012). LNA-mediated knockdown of miRNA cluster components in MB cell lines revealed decreased cell proliferation and viability, cell cycle arrest, and increased senescence and expression of apoptotic markers. Gene expression and

proteomic profiling upon miR-183/96/182 knockdown allowed identifying enrichment of gene sets associated with several biological pathways. Authors functionally linked the increase of invasion/migration signature with the enrichment of genes associated with PI3K/AKT/mTOR pathways (Irie et al., 2005) and demonstrated that mTOR is required for both miR-183 cluster activation and metastatic activity in MB. MiR-183 cluster was recently described as highly expressed also in mouse models for SHH-MB subgroup inactivated for the tumor suppressor *Pten*, which alters tumor histology and accelerates tumorigenesis. In this condition, miR-183/96/182 cluster promotes MB development by controlling GNPC proliferation (Zhang et al., 2013).

Microarray profiling of seeding *versus* non-seeding MBs revealed 12 differentially expressed miRNAs (Yang et al., 2015), among which miR-192, known to regulate epithelial–mesenchymal transition (Wang et al., 2010), a transdifferentiation process favoring cancer cells migration from the primary tumor site, invasion of surrounding tissues, and eventually metastasis generation (Brabletz et al., 2005). MiR-192 inhibited the anchoring ability of MB cells *in vitro* and *in vivo* by targeting several integrins and integrin-related proteins, central regulators of focal adhesion dynamics. Therefore, miR-192 downregulation in MB may promote leptomeningeal dissemination through cancer cell adhesion to secondary sites.

MiR-21 is associated to the metastatic behavior of several neoplasms, including brain tumors (Chan et al., 2005). Its expression levels were found upregulated in 29 MB samples and inversely correlated to the levels of its target *Pdcd4*, a gene suppressing metastasis in human cancer cells through inhibition of invasion-related proteins (Grunder et al., 2011). In MB, this decreased the activity of factors implicated in cell migration/invasion (such as AKT, c-JUN, and ERK), or in solid cancer dissemination/metastases, such as Timp-2, uPAR, E-cadherin, and integrin. Consistently, inhibition of miR-21 reduced MB cell motility and invasiveness.

Besides the instances collected above, additional miRNAs are potentially linked to MB dissemination, based on their involvement in cancer cell migration, invasivity, and metastatic behavior (Gao et al., 2015; Yogi et al., 2015; Pan et al., 2017).

LncRNAs IN MB, FROM PAST TO PRESENT

Similarly to miRNAs, a large number of lncRNAs have been characterized as potential oncogenes or oncosuppressors in cancer, including CNS tumors (**Table 1**). Long ncRNAs are considered as promising biomarkers and therapeutic targets in oncology; nonetheless, our comprehension of their involvement in MB is still largely incomplete.

The first lncRNA analyzed in MB was H19 (Albrecht et al., 1996). H19 gene was already known to code for a small spliced and polyadenylated RNA not containing any long, conserved ORF. Even though its function was not clarified yet, it was known that H19 transfection in cell lines suppressed tumorigenesis (Hao et al., 1993) and that, due to genomic imprinting of the paternal promoter, H19 was expressed from the maternal (non-imprinted) allele (Zhang et al., 1993). Because biallelic gene expression [i.e., loss of imprinting (LOI)] of *IGF2*, a maternally imprinted gene regulated by H19 (Leighton et al., 1995), occurs in tumors (Ogawa et al., 1993; Rainier et al., 1993), Albrecht et al. (1996) examined, by allele-specific RT-PCR, IGF2 and H19 imprinting in fetal cerebella, MB samples, and cell lines. They found only a partial H19 LOI in 50% of MBs analyzed, independently of *IGF2* imprinting, indicating that, in MB, expression and imprinting of the two genes are individually controlled.

Over years, high-throughput investigations dramatically entered the scene in molecular oncology. Even though -omic and integrative approaches provide a valuable source of data for identifying lncRNAs and may potentially reveal intriguing aspects of MB, less than 20 lncRNAs have been associated to this malignancy to date. In some cases, MB lncRNAs emerged by *de novo* sequencing experiments or dataset interrogations (see PVT1 and TP73-AS1, next section), whereas the vast majority of lncRNAs characterized in MB derived from previous studies, carried out in different pathological model systems. Nevertheless, only for a few of them the molecular mechanisms have been thoroughly elucidated. Based on the idea that similar mechanisms of action underlie different diseases with overlapping phenotype associations, Xu et al. (2017), through construction of gene and lncRNA coexpression networks, generated a computational approach to systematically prioritize and identify candidate disease risk lncRNAs in 14 cancer types, including MB. This method may help associating identified lncRNA-based mechanisms to human cancers. Along the same line, preliminary reports indicate that genome-wide data reanalyses of lncRNA expression profiles in MB may be useful for identification of lncRNA signatures with a diagnostic, prognostic, or functional value (Joshi and Perera, 2019 preliminary report).

LncRNAs AS MB ONCOGENES

PVT1 was the first lncRNA gene identified in human cancer translocations (Graham and Adams, 1986; Shtivelman et al., 1989). Its role in cancer is still debated: PVT1 has an oncogenic potential, by stabilizing Myc protein (Tseng et al., 2014), whereas its promoter functions as a tumor suppressor DNA element that limits *Myc* oncogene expression and activity (Cho et al., 2018). PVT1 locus has been suggested to be a fragile site (Colombo et al., 2015), and its gene is frequently amplified together with *Myc* in group 3 MB (Northcott et al., 2012c). Accordingly, RNA-Seq of group 3 MB revealed a gene fusion involving the 5′ end of PVT1 and the coding region of *Myc*. This represents the first identified MB group 3–specific gene fusion, which occurs through events of chromosome fragmentation due to erroneous DNA repair events (chromothripsis).

PVT1 is the host transcript of 4 putative oncogenic miRNAs (miR-1204, miR-1205, miR-1206, and miR-1207, Beck-Engeser et al., 2008). MiR-1204, whose sequence is comprised in the rearranged genomic region, was upregulated in group 3 PVT1-*Myc* fusion(+) tumors, where it contributes to the malignant phenotype. Indeed, inhibition of miR-1204 reduced cell proliferation. In turn, *Myc* knockdown resulted in diminished expression of miR-1204, which is in line with the observation that the PVT1 promoter contains non-canonical Myc responsive E-boxes. These observations suggest a positive feedback loop

TABLE 1 | Summary of the features and function of lncRNAs, circRNAs, and snRNAs involved in MB.

Class	Name	Alteration	System	Observed phenotypes	Mechanism/target	References
Long ncRNA						
Oncogene	ANRIL	Genetic variant	Primary tumors	Predisposition to MB	/	Chen et al., 2018
	ANRIL	Upregulation	MB cells	Cell viability migration, apoptosis	Decoy of miR-323; increase of BRI3; induction of MAPK, AKT, WNT pathways	Zhang et al., 2017a
	PVT1	Rearrangement	Group 3	Cell proliferation	miR-1204 upregulation	Northcott et al., 2012c
	Linc-Ned125	Upregulation	Group 4	Cell proliferation, migration invasivity	Decoy of miR-19a-3p, miR19b-3p, mir-106a5p; increase of Group 4 driver genes	Laneve et al., 2017
	CCAT1	Upregulation	Primary tumors	Cell proliferation, migration invasivity, tumor development	Post-translational modification of AKT pathway	Gao et al., 2018b
	LOXL1-AS1	Upregulation	Primary tumors	Cell proliferation, apoptosis, clonogenic potential, cell cycle, migration; EM transition; tumor size and weight	Activation of PI3K/AKT pathway	Gao et al., 2018a
	TP73-AS1	Upregulation	SHH	Cell viability, proliferation migration; tumor survival, growth, aggressiveness	Decoy of miR-494-3p upregulation of EIF5A2	Li et al., 2019; Varon et al., 2019
	HOTAIR	Upregulation	Primary tumors	Cell viability, colony formation, apoptosis, migration and invasion, tumor growth	Decoy of miR-1 and miR-206; upregulation of YY1	Zhang et al., 2020
	UCA1	Upregulation	Primary tumors	Cell cycle, migration, proliferation, aggregation, and apoptosis		Zhengyuan et al., 2017
	CRNDE	Upregulation	Primary tumors	Cell viability, proliferation, colony formation, apoptosis, migration, invasion, chemosensitivity tumor growth	Decoy of miR-29c-3p	Song et al., 2016 Sun et al., 2020
	SPRY4-IT1	Upregulation	MB cells	Cell proliferation, invasion, migration		Shi et al., 2017
	EVF-2	Upregulation	Primary tumors			Bonfim-Silva et al., 2013
Oncosuppressor	Nkx2-2as	Downregulation	SHH	Cell proliferation, apoptosis invasion, colony formation tumor growth	Decoy of miR-103a/107 and miR-548; downregulation of Btg2, Lats1, Lats2	Zhang et al., 2018
	HOTAIR	Downregulation	Primary tumors		Upregulation of Hoxd8 and Hoxd10	Chakravadhanula et al., 2014
circRNA	Circ-SKA3 circ-DTL	Upregulation	Primary tumors	Cell proliferation, migration, invasion	Upregulation of host transcripts	Lv et al., 2018
snRNAs	U1snRNA	Mutation	SHH	Patient survival	Dysregulation of oncogene and oncosuppressor splicing	Suzuki et al., 2019

through which Myc reinforces its own expression and increases the levels of the pathological miR-1024 in PVT1-*Myc* fusion(+) tumors (Northcott et al., 2012c).

A different mechanism altering miRNA activity in a lncRNA-dependent manner is the miRNA sequestration (decoy), which emerged as a relevant pathogenic pathway in several cancers (Tay et al., 2014). In this case, lncRNAs function as competing endogenous RNAs (ceRNA), able to sponge miRNAs by base annealing, inhibiting their activity and causing translational derepression of their target mRNAs.

Several examples of lncRNAs acting as miRNA sponges have been described in MB (**Figure 4**). The lncRNA linc-NeD125 was the first ceRNA described in brain tumors. This transcript was identified in NB-derived cells, as the primary transcript for miR-125-b1 (Bevilacqua et al., 2015), a neuronal miRNA (Smirnova et al., 2005) involved in neural differentiation and function (Boissart et al., 2012; Akerblom et al., 2014) and in cancer cell proliferation (Laneve et al., 2007; Ferretti et al., 2009). In an *in vitro* neuronal differentiation model, linc-NeD125 was shown to be induced upon differentiation stimulus and to act synergistically to miR-125 to promote neuronal differentiation (Bevilacqua et al., 2015). Linc-NeD125 was then revealed as a novel potential biomarker and therapeutic target in MB (Laneve et al., 2017). By RT-PCR analyses, linc-NeD125 was found to be highly expressed in group 4 MB, whereas RNA pull-down and CLIP experiments highlighted its capacity to recruit a pool of miRNAs (miR-19a-3p, miR-19b-3p, and miR-106a-5p), able to alter MB cell tumorigenic properties. Consistently, these miRNAs were validated as regulators of at least four group 4 MB driver genes (*Kdm6A, MycN, Cdk6*, and *Sncaip*). Importantly, alterations of linc-NeD125 expression impinge on the same gene network and biological phenotypes *in vitro*, pointing to this transcript as a non-coding contributor of group 4 MB tumorigenesis or cancer cell maintenance.

An additional transcript acting as a ceRNA in MB is the lncRNA ANRIL, already implicated in several malignancies where it functions as a *cis*-acting epigenetic silencer of the tumor suppressor *Ink4B* (Yap et al., 2010; Kotake et al., 2011). Its levels are enhanced in MB cells (Zhang et al., 2017a), and its repression affects the expression of multiple apoptotic factors, such as *Bcl-2*, *Bax*, cleaved/procaspase-3, and cleaved/procaspase-9, and impacts on cell viability and migration. Mechanistically, ANRIL is able to sequester miR-323, a neuronal miRNA acting as a tumor suppressor in brain cancer (Lavon et al., 2010; Qiu et al., 2013). Notably, the ANRIL/miR-323 regulatory module controls the expression of the brain-specific factor *Bri3* (Vidal et al., 2001), which induces MB-associated pathways, such as MAPK, AKT, and WNT, through both posttranscriptional and posttranslational mechanisms.

Exploiting genome-wide association studies on brain tumors, which have identified multiple disease susceptibility loci (Kinnersley et al., 2015), Chen et al. (2018) tested the hypothesis that some glioma-risk single-nucleotide polymorphisms may contribute to MB predisposition. They found that the rs2157719 T > C genetic variant of ANRIL was significantly associated with an increased MB risk in the Chinese population, supporting the idea that brain tumors might partially share genetic risk factors and etiological pathways.

A paradigmatic example of oncogenic lncRNA is CCAT1 (Nissan et al., 2012), linked to several tumors ranging from hepatocellular carcinoma to breast cancer, where it stimulates malignant phenotypes such as proliferation, invasion, migration, and chemoresistance (Chen et al., 2016; Ma et al., 2017; Zhang et al., 2017b). CCAT1 is overexpressed in MB primary tumors and cell lines (Gao et al., 2018b). *In vitro*, its downregulation causes the reduction of MB cell proliferation, migration, and invasion, whereas its depletion *in vivo* reduces the development of subcutaneously transplanted tumors. Similarly to ANRIL described above, also CCAT1 may play its oncogenic function by altering posttranslational modifications of the tumorigenic MAPK pathway components. However, parallel studies revealed its activity as a miRNA decoy in different tumor model systems, raising the hypothesis of multiple modes of action.

Differently from the previous cases, the tumorigenic role of the LOXL1-antisense RNA (LOXL1-AS1) was addresses for the first time in MB (Gao et al., 2018a). Its expression was higher in 36 out of 50 MB tissues, compared to paired non-cancerous resections. LOXL1-AS1 depletion in two MB cell lines inhibited cell proliferation and clonogenic potential, arrested cell cycle progression at the S phase, slowed cell migration, promoted cell apoptosis rate, and reverted epithelial–mesenchymal transition. Furthermore, LOXL1-AS1 knockdown in xenograft mouse models caused a significant reduction of tumor size and weight. The decreased level of PI3K and AKT phosphorylation observed upon LOXL1-AS1 RNAi suggested that LOXL1-AS1 may activate the PI3K/AKT pathway in MB.

The latest oncogenic lncRNA characterized in MB was TP73-AS1 (Varon et al., 2019), a transcript antisense to p73 mRNA that encodes for a member of the p53 TF family (Dötsch et al., 2010). p73 is implicated in brain development (Niklison-Chirou et al., 2013) and cancer (Flores et al., 2005), including MB (Niklison-Chirou et al., 2017). Similarly to was what observed in a subgroup of glioblastoma with better diagnosis (Sturm et al., 2012), comparative expression analyses of TP73-AS1, using the R2 platform[1] and the Cavalli cohort (Cavalli et al., 2017), revealed its upregulation in SHH-MB, where TP73-AS1 promoter is significantly hypomethylated (according to Schwalbe dataset for CpG island methylation, Schwalbe et al., 2017). In SHH-MB cells, TP73-AS1 supports cell survival and division, proliferation, viability, and migration, irrespective from TP53 status (wild type or mutant). Supporting the genetic independency of TP73-AS1 and P53 family, TP73 knockdown did not impact the levels of TP73-AS1 and *vice versa*. *In vivo* experiments indicate that TP73-AS1 supports MB tumor survival, growth, and aggressiveness, both in cancer tissues and in explanted cell cultures. The mechanism of action of TP73-AS1 as a miRNA decoy was clarified very recently (Li et al., 2019). TP73-AS1 was demonstrated to promote MB growth *in vitro* and *in vivo* and to modulate the expression of the eukaryotic translation initiation factor *EIF5A2*, an oncogene upregulated in several cancers including MB (Yang et al., 2019) by sponging miR-494-3p, previously reported as a putative oncogenic miRNA (Lin et al., 2018).

[1] https://hgserver1.amc.nl/cgi-bin/r2/main.cgi

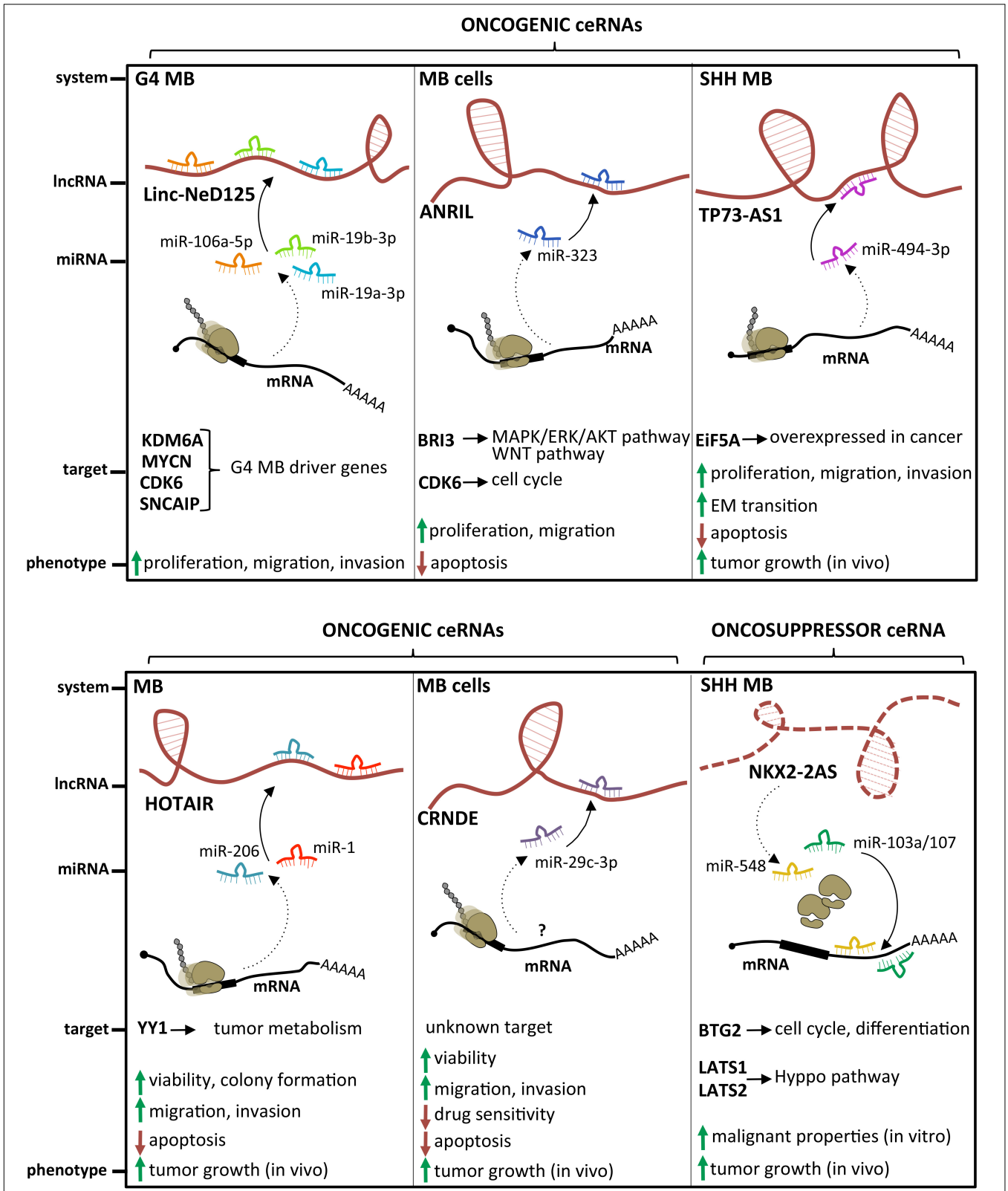

FIGURE 4 | State of the art on lncRNAs functioning as oncogenes or oncosuppressors through their competing endogenous RNA (ceRNA) activity. A handful of lncRNAs, such as Linc-NeD125, ANRIL, TP73-AS1, HOTAIR, and CRNDE, may function as oncogenes by sponging specific miRNAs and leading to derepression of their target genes. Differently, only NKX2-2AS has been described as a lncRNA endowed with oncosuppressor activity.

The role of the prototypical lncRNA HOTAIR (Rinn et al., 2007) in MB is still controversial. One study described it as underexpressed in MB samples, where its target genes *Hoxd8* and *Hoxd10* were upregulated (Chakravadhanula et al., 2014). Another report (Zhang et al., 2020) found it expressed at high levels in MB tissues and cell lines, where it interacts with and modulates miR-1 and miR-206, which in turn directly target the oncogenic TF YY1. Rescue assays in MB confirmed the occurrence of this further ceRNA network, which controls cell viability, ability to form colonies, apoptosis, migration, and invasion *in vitro*, as well as tumor growth in xenografted mice.

LncRNAs as MB Tumor Suppressors

Only one lncRNA has been described as an oncosuppressor in MB. Microarray analysis performed in SHH-MB–derived cells and GNPCs indicated the lncRNA Nkx2-2as as the gene showing the highest degree of downregulation, which is dependent on aberrant SHH signaling (Zhang et al., 2018). Specifically, Nkx2-2as expression in SHH-MB cells is suppressed by the repressor Foxd1, which is induced by the SHH-responsive TF Gli2. Gli2/Foxd1-mediated Nkx2-2as downregulation contributes to the pathogenesis of SHH subgroup. Nkx2-2as ectopic expression in MB cell lines impaired cell growth, colony formation, and invasion, while increasing apoptosis; its knockdown in CGNPs promoted their proliferation. Consistently with *in vitro* results, Nkx2-2as overexpression delayed tumor growth in xenograft mouse models. Rather than through its antisense transcript Nkx2-2 (Tochitani and Hayashizaki, 2008), Nkx2-2as functions as miRNA decoy in MB. It sequesters the oncogenic miRNAs miR-103a/107 and miR-548, whose cellular targets, the cell cycle factor *Btg2* and the Hippo pathway regulators *Lats1* and *Lats2*, function as tumor suppressors in MB (Farioli-Vecchioli et al., 2012; Rao et al., 2016).

Other LncRNAs in MB

A handful of studies suggest a role in MB for some lncRNAs whose molecular mechanism has not yet been entirely clarified. This is the case for the lncRNAs UCA1 (Xiao et al., 2016), which is upregulated in MB samples. It has been demonstrated that it is able to alter MB cell properties, as cell cycle progression, migration, proliferation, aggregation, and apoptosis when downregulated *in vitro* (Zhengyuan et al., 2017). Similar findings have been described for SPRY4-IT1 (Shi et al., 2017) and for CRNDE, a lncRNA already characterized in MB (Song et al., 2016) and other tumors. A molecular interaction was found between CRNDE and miR-29c-3p, whose altered expression affected tumor growth *in vivo* and tumorigenic cell features *in vitro* (Sun et al., 2020). Interestingly, expression studies in MB cell lines exposed to cisplatin treatment revealed that elevated levels of CRNDE are associated with resistance to chemotherapeutics and, consistently, functional studies demonstrated an increased cell chemosensitivity upon miR-29c-3p overexpression. The molecular target of this regulative axis is still unknown.

Finally, one report indicates that the lncRNA EVF-2 is overexpressed in MB cell lines and tissues, compared to control samples (Bonfim-Silva et al., 2013).

OTHER CLASSES OF ncRNAs IN MB: circRNAs, eRNAs, AND snRNAs

Brand-new molecular classes of ncRNAs have been discovered in the last few years, as the results of deep explorations of genome transcriptional outputs.

Circular RNAs are covalently closed continuous loops instead of canonical linear forms. They derive from the joining of a 5′ splice site and a 3′ splice site as the result of back splicing. They are characterized by the lack of 5′ cap and 3′ tail and, due to their unique structure, by high stability and relatively long half-life. Previously considered as aberrant by-products of linear transcript splicing, they are becoming the focus of research interest for their relative abundance, their conservation and tissue specificity, and their enrichment in NS. Recent studies have highlighted the implication of these still enigmatic transcripts in processes as diverse as differentiation and development and their activities as epigenetic and transcriptional regulators, miRNA sponges, and RNA-binding protein decoys (Memczak et al., 2013; Zhang et al., 2016; Kristensen et al., 2019).

As a consequence of their pervasive activity, altered expression of circRNAs occurs in several pathologies, including brain cancers (Bhan et al., 2017). To investigate the expression profile and function of circRNAs in MB, NGS was performed on RNA from 4 human MBs, revealing more than 15,000 distinct circRNAs with more than 1 back-spliced read (Lv et al., 2018). Notably, 36 differentially expressed circRNAs were identified in MB *versus* normal tissues, most of them being downregulated. Two upregulated candidates, circ-SKA3 and circ-DTL (**Table 1**), putatively acting as circular oncogenes, were functionally characterized. Loss-of-function assays revealed that the two species were able to regulate the expression of their host transcripts, previously demonstrated to be important in cancer progression (Kobayashi et al., 2015; Chuang et al., 2016). Parallel rescue experiments with mRNA-overexpressing plasmids indicate that circRNA-dependent dysregulation of the linear counterpart impacts on MB cell proliferation, migration capacity, and invasion ability *in vitro*.

Enhancer RNAs are *cis*-acting transcripts synthetized at active enhancers (De Santa et al., 2010; Kim et al., 2010). They contribute to the transcriptional regulation of target genes by recruiting key TFs or chromatin-associated complexes and by forming high-dimensional DNA structures between enhancer, super-enhancers (clustered enhancers), and promoters or by stimulating RNA polymerase II elongation (de Lara et al., 2019). Although the studies of their biological roles are still at infancy, the eRNA aberrant regulation seems to be closely related to tumorigenesis (Li W. et al., 2013).

Lin et al. (2016) profiled the enhancer landscape in 28 MB primary tumors by ChIP-Seq analysis for H3K27ac and Brd4, which are marks of active enhancers. Through combined analyses aimed at: (1) matching differentially active enhancers and target

genes within the same topologically associated domain and (2) clustering spatially colocalized enhancer domains (super-enhancers), authors sought to clarify MB subgroup identities and origin from a novel perspective. Specifically, they reconstructed core regulatory circuitries in MB subgroups, focusing on master transcriptional regulators. They identified novel TFs implicated in MB development and traced their expression along cerebellar formation, which led to propose the deep cerebellar nuclei in the nuclear transitory zone as the putative cells of origin for G4 MB.

Small nuclear RNA (snRNA) implication in splicing dynamics and regulation is a longstanding dogma of gene expression (Lerner et al., 1980). By combining extensive inspections of MB whole-genome sequencing data with allele-specific PCR analyses, Suzuki et al. (2019) recently revealed in a large fraction of SHH-MBs (97% of adult SHHδ cases and 25% of SHHα adolescent cases) a recurrent hotspot mutation of the snRNA U1 gene (**Figure 1** and **Table 1**), which is associated with an extremely poor prognosis when combined with *TP53* mutation. U1 snRNA mutation (r.5A > G) mapped in the highly conserved 5′ splice-site recognition sequence of the snRNA. In line with this finding, computational and experimental approaches indicate that the mutation does affect splicing. Intron-centric alternative splicing analysis proved that mutant U1 snRNA variants display a threefold increase of alternative 5′ cryptic splicing events, whereas complementary exon-centric alternative splicing analysis revealed a higher incidence of cassette exons events and intron retention. Mutant U1-mediated aberrant alternative splicing inactivates tumor-suppressor genes (*Ptch1, Pax5*) and activates oncogenes (*Gli2* and *Ccnd2*) or affects genes linked to SHH pathway (*Pax6*) or to other tumors (*Tox4*). This study reveals how, besides identifying novel species involved in cerebellar cancer, genome-wide explorations with a careful focus on non-coding loci allow better understanding the contributions of well-known non-coding RNAs to MB. Specifically, it indicates that single-nucleotide variants are not limited to proteinogenic genes, reveals the need of prioritized interventions for SHH patients carrying U1 snRNA mutation, highlights the relevance of aberrant posttranscriptional (de)regulation in MB, and suggests opportunities for novel targeted therapeutic approaches.

CONCLUDING REMARKS

In the last decades, our point of view on molecular oncology has tremendously changed, especially thanks to deep studies on RNA biology.

Since 2000s, the progresses in deep sequencing resolution and bioinformatics analyses have enriched our interpretation of the genomic output, providing the notion that mammalian genomes are pervasively transcribed and enlarging the catalog of regulatory transcripts. This promoted the identification of a previously unsuspected layer of gene expression modulation, mediated by ncRNAs. The extensive implication of ncRNAs in cell physiopathology represents one of the foremost revolutions in the recent molecular biology.

At the same time, our comprehension of cancer biology has greatly improved, and MB represents a paradigmatic example of such conceptual advancements. The reclassification of MB in diverse genetic/histological subgroups and subtypes, mainly based on integrative -omics analyses together with the characterization of MB cells of origin, contributes to resolve the conundrum of the tumor intrinsic heterogeneity and to highlight causative developmental aberrations. This propelled further genetic and gene expression analyses aimed at precisely identifying the tumor-driving molecular alterations, as a source for possible biomarkers and targets for therapy. On the other hand, reshaping our notion of MB has triggered, and still requires, the rising of novel *in vitro*, *ex vivo*, and *in vivo* preclinical models, from cell lines to reprogrammed cells, tumor grafts, engineered mice, and, more recently, organoids. Finally, the identification of MBSCs has shed new light on the contribution of cell stemness to tumor maintenance/propagation and to the establishment of malignant phenotypes, such as the resistance to chemoradiotherapeutic treatments and the tendency to dissemination and relapses.

Importantly, the combination of identification of ncRNA-based circuits, as novel theranostic agents, and improved knowledge of MB molecular and cellular biology has favored the development of MB precision oncology.

As largely discussed, short and long regulatory RNAs are broadly engaged in all the aspects of MB and can therefore be considered as powerful diagnostic, prognostic, and therapeutic targets or adjuvants. The possibility to easily detect stable species (miRNAs and circRNAs) in biopsies, body fluids, or preserved samples may support patient stratification, individual diagnosis, and clinical interventions. Furthermore, the function of ncRNAs as oncogenes or oncosuppressors is particularly suitable for translational applications in tumor therapy. This is true both for miRNAs, whose pleiotropic and combinatorial regulatory activity enhances the efficient targeting of complex downstream gene cascades, and for lncRNAs, whose expression is highly tissue-specific and whose function as ribonucleoprotein scaffolds could be blocked through small inhibitory drugs. In addition, the characterization of specific pathological ncRNA-driven gene networks offers novel opportunities for the treatment of diseases implying the alteration of undruggable genes, factors, or pathways, which is a crucial aspect in pediatric tumors.

Despite the potentialities of ncRNA-dependent personalized medicine, no any clinical trial for ncRNA therapeutics is currently ongoing in MB patients. This is mainly due to technical issues, related to the stability of the targeting molecules, and the specificity of delivery. The evolution of reverse genetics approaches supported by modified nucleic acids (for antisense/mimic or RNA silencing strategies), the exploitation of viral carriers–based constructs or gene editing, and the accurate design of novel biomaterials and rationales for nanotechnologies promise to facilitate the administration of targeting species through the plasma membranes and the blood–brain barrier, which remains a major challenge in the field of brain cancer treatment.

AUTHOR CONTRIBUTIONS

PL and EC reviewed the literature and wrote 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.

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MicroRNAs: From Mechanism to Organism

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MicroRNAs (miRNAs) are short, regulatory RNAs that act as post-transcriptional repressors of gene expression in diverse biological contexts. The emergence of small RNA-mediated gene silencing preceded the onset of multicellularity and was followed by a drastic expansion of the miRNA repertoire in conjunction with the evolution of complexity in the plant and animal kingdoms. Along this process, miRNAs became an essential feature of animal development, as no higher metazoan lineage tolerated loss of miRNAs or their associated protein machinery. In fact, ablation of the miRNA biogenesis machinery or the effector silencing factors results in severe embryogenesis defects in every animal studied. In this review, we summarize recent mechanistic insight into miRNA biogenesis and function, while emphasizing features that have enabled multicellular organisms to harness the potential of this broad class of repressors. We first discuss how different mechanisms of regulation of miRNA biogenesis are used, not only to generate spatio-temporal specificity of miRNA production within an animal, but also to achieve the necessary levels and dynamics of expression. We then explore how evolution of the mechanism for small RNA-mediated repression resulted in a diversity of silencing complexes that cause different molecular effects on their targets. Multicellular organisms have taken advantage of this variability in the outcome of miRNA-mediated repression, with differential use in particular cell types or even distinct subcellular compartments. Finally, we present an overview of how the animal miRNA repertoire has evolved and diversified, emphasizing the emergence of miRNA families and the biological implications of miRNA sequence diversification. Overall, focusing on selected animal models and through the lens of evolution, we highlight canonical mechanisms in miRNA biology and their variations, providing updated insight that will ultimately help us understand the contribution of miRNAs to the development and physiology of multicellular organisms.

Keywords: miRNA, evolution, development, Argonaute, Drosha, Dicer, biogenesis, silencing

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INTRODUCTION

The Emergence of Small RNA-Guided Effector Systems

Regulation of gene expression by small RNAs emerged as an ancient feature of cellular biology and is found in all three domains of life (bacteria, archaea and eukarya). Having evolved primarily as a means of defense against foreign nucleic acids (Shabalina and Koonin, 2008; Obbard et al., 2009) the principle of small RNAs specifically guiding effector proteins to selected

nucleic acids via antisense-complementarity is a recurrent theme in biology. At their core, these RNA-based interference (RNAi) systems consist of two components, a nucleic acid allowing for sequence-specific target recognition, and an effector protein that mediates downstream effects with varying outcomes.

In eukaryotes, the effector proteins that mediate the silencing of target nucleic acids are part of the Argonaute protein family. After their origin in prokaryotes (Makarova et al., 2009; Olovnikov et al., 2013; Swarts et al., 2014a,b; Willkomm et al., 2015), Argonautes diversified into a versatile class of effector proteins, forming the core of various multiprotein regulatory systems or RNA-Induced Silencing Complexes (RISC). They all share common structural elements and the ability to bind short, single stranded RNAs in a conformation that enables base pairing with target RNAs (Steiner et al., 2007; Farazi et al., 2008; Takeda et al., 2008; Czech and Hannon, 2010). The other critical protein component of eukaryotic RNA-induced silencing pathways are nucleases that process precursor RNAs into small RNAs that can be loaded onto Argonaut proteins. A major player in multiple RNAi pathways is Dicer, an RNAse III type endonuclease that cleaves double-stranded RNA molecules to generate targeting-competent small RNAs that guide the effector machinery. Although no prokaryotic homolog of Dicer has been found to date, the origins of individual domains can be traced back to prokaryotes (Shabalina and Koonin, 2008). Together, RNase III type endonucleases and Argonaute proteins lie at the heart of diverse small RNA based pathways involved in multifaceted aspects of molecular biology.

The different modules that constitute the eukaryotic RNAi systems likely originated in archaea, bacteria, and bacteriophages (Koonin, 2017). This core structure has diversified, specialized, and acquired new functions in the course of eukaryotic evolution. A key innovation in diverse lineages was the ability to not only load and target foreign or parasitic RNAs, but also to feed endogenously produced RNAs into the existing RNAi pathways to achieve gene regulation. This formed the basis for the emergence and expansion of the endogenous class of small RNAs called microRNAs (miRNAs), whose regulatory potential has been harnessed in animals, plants, and other eukaryotic lineages for the establishment of elaborate gene regulatory networks that control development and physiology (**Figure 1**).

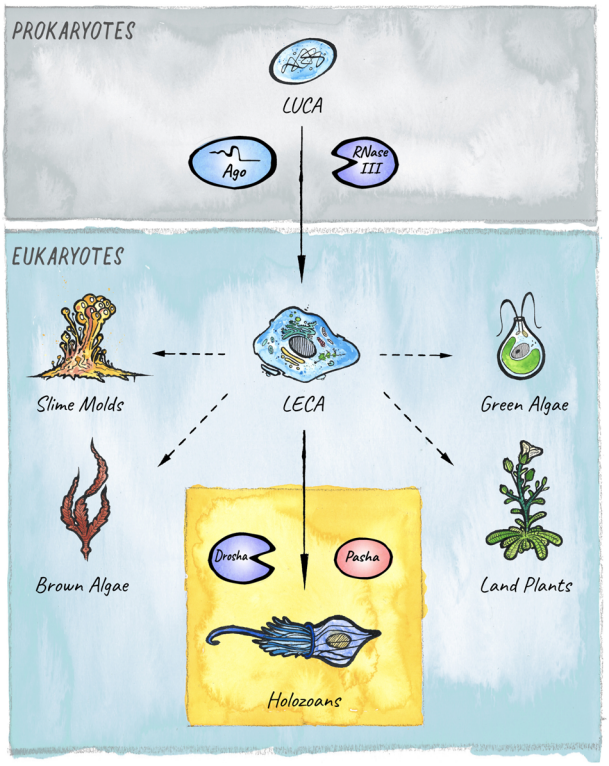

FIGURE 1 | The evolutionary origins of miRNAs in eukaryotes. Two key players in small RNA-mediated silencing, Argonaute proteins and RNase III like enzymes, originated in prokaryotes. The miRNA pathway in animals emerged with the birth of the Microprocessor, composed of Drosha and Pasha, in unicellular holozoans (Bråte et al., 2018). Diverse lineages that branched from the last eukaryotic common ancestor (LECA) also evolved miRNA-like pathways. However, it is still under debate whether these evolved independently in four additional clades: Slime molds, Green algae, Brown Algae, and Land plants (Kruse et al., 2016; Valli et al., 2016; Cock et al., 2017; Bråte et al., 2018); or if the pathway was already present in the last common ancestor (Moran et al., 2017).

What Is the Purpose of This Review?

The complexity of multicellular organisms relies on the segregation of functions across many distinct cell types, which requires intricate gene regulatory mechanisms. As versatile repressors of gene expression, miRNAs are thought to facilitate the generation of different cell types with highly specialized physiology (Alberti and Cochella, 2017). It comes as no surprise that the evolution of multicellular organisms has been accompanied by an increase in complexity of the miRNA pathway and the miRNA repertoires (Hertel et al., 2006; Heimberg et al., 2008; Wheeler et al., 2009; Berezikov, 2011; Guerra-Assunção and Enright, 2012; Hertel and Stadler, 2015). The use of this increased diversity of repressors has been exploited by the addition of multiple mechanisms that control which miRNAs are produced at specific times and in specific cell types; at what levels these accumulate to act effectively; and what the molecular consequences of their repression are.

A comprehensive understanding of the contributions of miRNAs thus requires bridging biochemical and mechanistic insights with the organismal level. When and where does miRNA-mediated regulation take place? How is the production of a specific miRNA controlled in space and time to achieve that? And how do miRNAs integrate into cellular gene regulatory networks that control development and physiology? Recent technological advances have enabled more quantitative assessment of the kinetics of miRNA production and turnover, and context-dependent differences in composition of miRISC during distinct developmental stages. This allows a more nuanced view of when, where and how miRNAs are utilized within animal development and post-developmental processes. Moreover, the sequencing of an increasing number of genomes has upgraded our ability to place miRNAs and the associated protein machinery into an evolutionary context. These layers of understanding will lead

us to broader concepts of what miRNAs contribute to the phenotypic complexity observed in present-day organisms.

We do not attempt to provide a comprehensive survey of all the miRNAs that have been studied and their functions in different animals, for which we suggest the following starting points (Chen et al., 2014; Alberti and Cochella, 2017; Bartel, 2018). Instead, we emphasize general concepts from diverse mechanistic studies to understand how multicellular organisms have exploited the potential of this broad class of repressors. We first focus on how miRNA biogenesis is controlled and discuss how the different modes of regulation can affect miRNA function within an animal. Next, we discuss how different composition of the RISC complex and different cellular contexts can result in distinct outcomes of miRNA-mediated repression. Finally, we present an updated overview of how the miRNA repertoire has evolved and what this teaches us so far about how miRNAs have acquired functionality during evolution.

Evolutionary Origins of the miRNA Pathway

In 1993, the product of the *Caenorhabditis elegans* gene *lin-4* was surprisingly found to give rise to a small non-coding RNA, processed from a longer hairpin precursor, which functions by repressing protein production from the *lin-14* mRNA via an antisense RNA–RNA interaction (Lee et al., 1993; Wightman et al., 1993). First thought of as a nematode peculiarity, it became clear in the early 2000s that such endogenous small RNAs like *lin-4*, in particular *let-7*, play a fundamental role across eukaryotic lineages (Pasquinelli et al., 2000; Reinhart et al., 2000). The discovery of *lin-4* and *let-7*, followed by the identification of hundreds of endogenous small RNAs derived from hairpin precursors in animal genomes, laid foundation to the miRNA field (Lee and Ambros, 2001; Lau et al., 2001; Lagos-Quintana et al., 2003; Aravin et al., 2003; Lim et al., 2003). Not only is the protein machinery associated with miRNAs highly conserved, but so are many miRNAs – with >30 miRNAs shared across all bilaterian animals, and hundreds of others conserved within specific clades. Moreover, at least 37% of *Drosophila* and 60% of human protein-coding transcripts are subjected to selective pressure to retain miRNA binding sites (Friedman et al., 2008; Agarwal et al., 2018), underscoring the biological significance of miRNA-mediated gene silencing.

miRNAs are 21–23 nt small RNAs that elicit post-transcriptional downregulation of protein output from their target mRNAs. This is typically achieved through a combination of translational inhibition and promotion of mRNA decay (Jonas and Izaurralde, 2015; Bartel, 2018). The origin of miRNA-biology lies in the evolution of a mechanism that allowed for processing of long endogenous transcripts into short RNA duplexes that are further recognized and cleaved by Dicer. In animals this is achieved by the Microprocessor complex, composed of the RNase III endonuclease Drosha and its co-factor Pasha (Partner of Drosha, or DGCR8 in vertebrates). Cleavage of a primary miRNA transcript by the Microprocessor in the nucleus releases a hairpin precursor, that upon export to the cytoplasm is further cleaved by Dicer to give rise to a short RNA duplex with characteristic 2-nt 3′ overhangs. From this duplex, one strand is preferentially loaded into an Argonaute protein to generate a functional miRNA-induced silencing complex (miRISC) (**Figure 2**).

Drosha and Pasha/DGCR8 originated in unicellular holozoans after fungi branched from the pre-metazoan lineage (Bråte et al., 2018), placing the appearance of miRNAs before the onset of multicellularity. However, plants do not encode a homolog of Drosha or Pasha but process primary miRNA transcripts via the Dicer-homolog DCL1 (Dicer like 1), which generates mature miRNAs according to a similar principle involving two sequential endonucleolytic cleavage events (Voinnet, 2009). This led to the initial hypothesis that miRNAs evolved independently in animals and land plants. However, the possibility that the last common ancestor of these lineages might have already employed a common miRNA-like pathway for post-transcriptional regulation is not to be dismissed (Moran et al., 2017). Nevertheless, after their introduction, the number of miRNAs continuously expanded during the course of evolution, providing multicellular organisms with a diversified toolset for regulation of gene expression.

miRNAs Are Essential for Development

Regulation by miRNAs evolved into a key feature of multicellular organisms. As general repressors of gene expression, miRNAs have been incorporated into gene regulatory networks that control diverse developmental and post-developmental processes. No higher metazoan lineage has tolerated the loss of essential miRNA pathway components. In fact, defects in the miRNA biogenesis machinery or the effector silencing factors results in severe embryogenesis defects in every animal studied.

In *C. elegans*, removal of the miRNA-specific Argonautes *alg-1* and *alg-2* causes embryonic lethality with a predominant arrest in the morphogenetic process of elongation (Vasquez-Rifo et al., 2012). Disruption of *ago1* in *Drosophila* causes embryonic lethality with notable nervous system abnormalities (Kataoka et al., 2001). Loss of Dicer in Zebrafish results in abnormal gastrulation causing severe defects in brain development and organogenesis. Notably, injection of a miR-430 duplex into embryos considerably rescued neuronal defects, indicating an outstanding role of a single miRNA in early fish development (Giraldez et al., 2005). Mouse embryos lacking *Dicer1* or *Ago2* fail to undergo gastrulation, accompanied by a malformation of germ layers (Bernstein et al., 2003; Alisch et al., 2007). Moreover, this phenotype is recapitulated in animals bearing a deletion of *Dgcr8* (Wang et al., 2007). In plants, disruption of DICER-LIKE1 leads to embryonic arrest early in development, likely caused by a failure to prevent precocious differentiation events (Nodine and Bartel, 2010). Interpretation of these experiments should consider possible miRNA-independent functions of disrupted miRNA-pathway factors, as such roles have been demonstrated for Drosha, Pasha and Dicer (Chong et al., 2010; Macias et al., 2012; Gromak et al., 2013; Luhur et al., 2014; Rybak-Wolf et al., 2014; Cirera-Salinas et al., 2017; Kim et al., 2017; Marinaro et al., 2017). However, given that removal of different components in the miRNA-pathway causes highly similar phenotypes, it

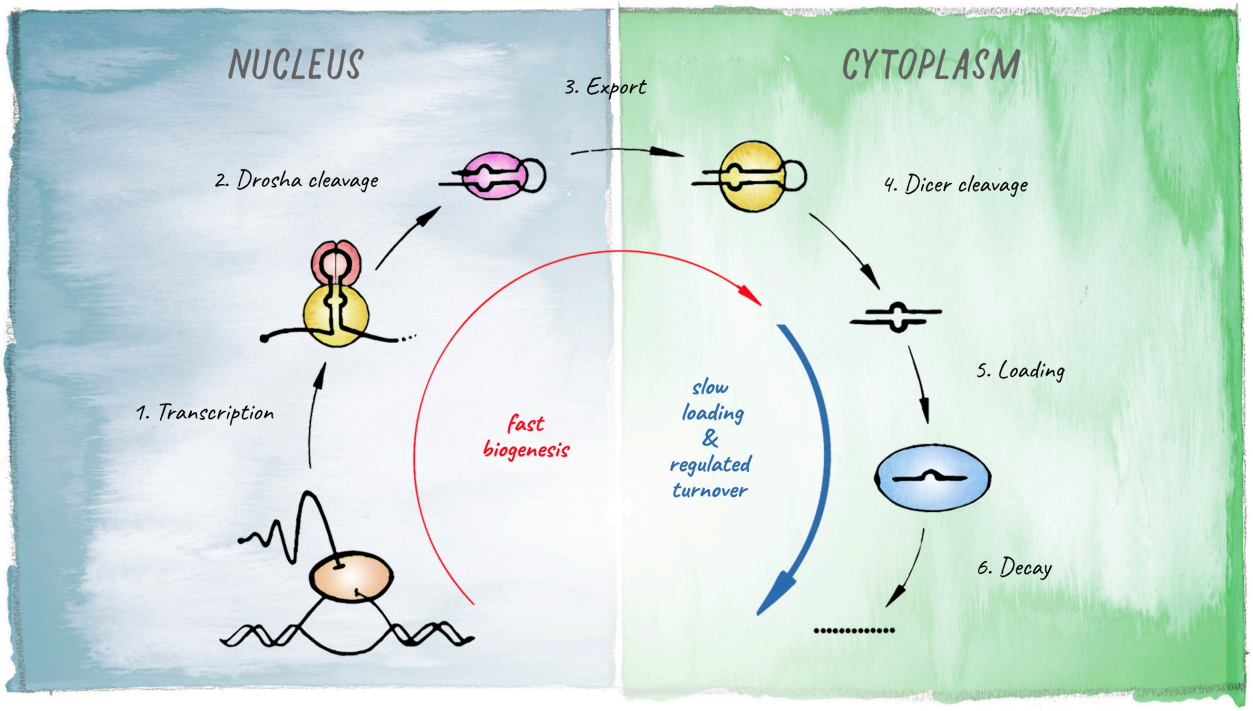

FIGURE 2 | Schematic of the miRNA biogenesis pathway. The different steps leading to production of a mature miRNA are shown. Kinetic studies in *Drosophila* revealed that biogenesis is fast for most miRNAs but loading into Argonaute represents the rate-limiting step (based on Reichholf et al., 2019).

is strongly suggested that miRNA-deficiency underlies the observed defects.

Whereas miRNAs are collectively essential in animals, uncovering the functions of many individual miRNAs has been challenging (Miska et al., 2007; Park et al., 2012; Chen et al., 2014). This is in part due to redundancy (Ge et al., 2012), most notably among miRNAs that fall into so-called families and share targeting specificity (Alvarez-Saavedra and Horvitz, 2010), but also redundancy with other repressive mechanisms that contribute to the output of gene regulatory networks (acting either at the RNA or protein levels). For example, in *Drosophila*, the near-complete loss of miRNA-mediated repression is tolerated to a large extent if general metabolism is simultaneously slowed down (Cassidy et al., 2019). This suggests that under conditions of slow developmental tempo, the timely downregulation of targets or the establishment of the right protein levels, to which miRNAs contribute, might be achieved through compensatory mechanisms. In a number of cases, the contribution of miRNAs has been revealed only in sensitized genetic backgrounds or upon environmental perturbation, such as exposure to extreme temperatures or pathogen stress. This suggests that the activity of some miRNAs is redundant with other factors that are influenced by environmental conditions (Hornstein and Shomron, 2006; Brenner et al., 2010; Ebert and Sharp, 2012; Cassidy et al., 2013, 2016, 2019; Siciliano et al., 2013; Ren and Ambros, 2015; Ilbay and Ambros, 2019). It is also likely that the function of many miRNAs has not been uncovered, even if they play non-redundant roles, because they are often expressed with very high spatial and temporal specificity and may function predominantly in specialized cell types within complex multicellular organisms (Alberti and Cochella, 2017).

miRNA PRODUCTION AND ITS REGULATION

Multicellular organisms have taken advantage of the potential of miRNA-mediated regulation employing multiple mechanisms to control the production of miRNAs in distinct cell-types, under varying conditions, or during different developmental stages. miRNA biogenesis begins with transcription of a primary transcript by RNA polymerase II (**Figure 2**). Transcriptional regulation will thus determine whether a miRNA will be produced in the first place, and in general miRNA abundance correlates well with the rates of primary-miRNA transcription (Reichholf et al., 2019). At the organismal level, there is evidence that for most miRNAs, transcriptional control is the main determinant of cell-type specificity (Alberti et al., 2018). However, the maturation of primary miRNAs, initially by the Microprocessor in the nucleus and later by Dicer in the cytoplasm (**Figure 2**), can also be regulated, contributing significantly to the dynamics of accumulation and steady-state abundance of different miRNAs (Conrad et al., 2014). Moreover, it became evident that regulation of miRNA decay plays an important role in determining the functional levels of individual miRNAs (Zhou et al., 2018; Reichholf et al., 2019).

Transcription of each pri-miRNA is under the control of specific transcription factors and enhancers that outline their expression patterns. For example, an elaborate transcriptional mechanism determines the expression of the miRNA *lsy-6* in a single neuron in *C. elegans* (Cochella and Hobert, 2012). In contrast, post-transcriptional regulation of miRNA biogenesis is achieved at two different levels: (i) globally, by controlling the shared core machinery that produces most miRNAs, e.g., modulating levels or activity of the Microprocessor, and (ii) at the level of individual miRNAs, exploiting unique sequence and structural features of diverse miRNA precursors which impact interaction with the biogenesis machinery. Such interactions are often facilitated by various RNA binding proteins (RBPs).

Recent advances in the use of metabolic labeling of RNA have enabled kinetic studies of different steps during the life of a miRNA, and provided insight into how the rates of these steps vary for distinct miRNAs (Reichholf et al., 2019). An in-depth analysis in *Drosophila* S2 cells revealed that the rates of mature miRNA biogenesis range from 17 to >200 molecules/minute/cell. However, the loading into Argonaute is significantly slower and in fact represents a kinetic bottleneck in the production of functional miRISC. As a consequence, a large fraction of the miRNA duplexes produced by Dicer (approximately 40%) is degraded before loading. Other reports found only <10% of cellular miRNAs are bound by Argonaute (Janas et al., 2012; Stalder et al., 2013). This seemingly wasteful strategy ensures specific loading of Argonaute with miRNAs, which compete against other abundant duplex RNAs originating from tRNAs, rRNAs, snRNAs, or snoRNAs (Reichholf et al., 2019). Consistently, Argonaute binding is a better indicator of the inhibitory potential of a miRNA than its overall concentration (Flores et al., 2014). For this specificity mechanism to be effective, Argonaute levels need to be limited. This is achieved in large part through the relatively short half-life of empty Argonaute, which is efficiently ubiquitinated and degraded (Diederichs and Haber, 2007; Derrien et al., 2012; Janas et al., 2012; Martinez and Gregory, 2013; Smibert et al., 2013; Kobayashi et al., 2019; Reichholf et al., 2019; Bose et al., 2020).

Another important contributor to miRNA abundance is their stability. While overall miRNAs are among the most stable cellular RNAs, individual species can range in stability from minutes to more than a day (Reichholf et al., 2019). Rates of decay can be modulated by external stimuli to remodel the miRNome, as is the case for light-regulated retinal miRNAs (Krol et al., 2010). In general, neuronal miRNAs in mammals appear to have a high turnover rate compared to other cell types, and their abundance can be rapidly regulated in connection with neuronal activity (Krol et al., 2010). Together with the biogenesis rates, the different decay rates determine the steady-state abundance of miRNAs, such that two miRNAs can reach the same abundance through contrasting strategies: a miRNA with slow biogenesis and slow decay can accumulate to the same concentration as one with fast biogenesis and fast decay. The first case will result in a stable concentration that is less likely to change upon small perturbations, while the second is energetically more costly but provides potential for dynamic regulation. Such different strategies for accumulation have important consequences for miRNA function, and further investigation of how these different rates come about will provide critical insight. Nevertheless, this quantitative understanding already provides a framework for determining the ability of miRNAs to reach the necessary cellular concentrations to execute their repressive functions.

Effective repression by miRISC requires a high concentration of a miRNA relative to its target (Ameres and Zamore, 2013). To achieve this, synthesis of a number of miRNAs begins long before the onset of their repressive function. For example, the miRNA *lsy-6* in *C. elegans*, which functions in a sensory neuron by repressing the transcription factor COG-1 (Johnston and Hobert, 2003), is produced in the mother of the sensory neuron (Cochella and Hobert, 2012). Transcription of *cog-1* begins in the postmitotic neuron several hours later in a cell that has high concentration of *lsy-6* (Cochella and Hobert, 2012). This likely contributes to the ability of *lsy-6* to act as a genetic switch by completely preventing COG-1 expression (Johnston and Hobert, 2003; Cochella and Hobert, 2012). Another example of this is the essential *C. elegans* miRNA *let-7*, which is required for transition of the last larval stage to adulthood, yet starts being transcribed in the first larval stage (Martinez et al., 2008; Van Wynsberghe et al., 2011). Taking into account the relative dynamics of accumulation of a given miRNA and its target/s offers helpful insight for understanding the contribution of that miRNA to specific cellular processes.

These are the general forces that determine whether a miRNA is made and if so, to what level it accumulates relative to its targets. The core mechanisms that control each of these steps have been studied in great detail in different cell-based systems. Multicellular organisms take advantage of various ways to adjust these mechanisms to generate the necessary spatio-temporal specificity and the dynamics of miRNA expression that support development and homeostasis.

Drosha/DGCR8 and Dicer: The Gatekeepers of miRNA Production

Processing of primary-miRNA transcripts (pri-miRNAs) initiates co-transcriptionally (Lee et al., 2003; Lee Y. et al., 2004; Morlando et al., 2008; Ballarino et al., 2009). In *Drosophila*, Pasha/DGCR8 can associate directly with RNA pol II via its phosphorylated C-terminal domain, linking transcription with the first step of miRNA maturation (Church et al., 2017). Interaction of DGCR8 and Drosha with pri-miRNAs occurs via recognition of a hairpin secondary structure flanked by single-stranded RNA and leads to cleavage by Drosha to release a precursor hairpin (Lee et al., 2003; Han et al., 2004; Sohn et al., 2007). The efficiency and the accuracy of this reaction are crucial determinants of miRNA abundance and targeting specificity, respectively. Because miRNAs bind their targets primarily through nucleotides 2–8 relative to the 5′ end of the miRNA (the "seed" sequence), a change in cleavage site that affects the 5′ end of a miRNA even by a single nucleotide can drastically alter target specificity of that miRNA. The choice of cleavage site by Drosha is crucial for setting the 5′ end of both miRNA arms: the 5p arm directly and the 3p indirectly by determining the register for the subsequent cleavage by Dicer (Auyeung et al., 2013; Partin et al., 2017; Kwon et al., 2019).

Interestingly, recent mapping of Drosha cleavage sites with single nucleotide resolution in human cell lines (HEK293T and HeLa cells), revealed that some miRNAs undergo alternative processing resulting in different 5′ and 3′ ends (Kim et al., 2017). This has the potential to further diversify the miRNA-repertoire.

Once a pri-miRNA is processed to a precursor hairpin (pre-miRNA) in the nucleus, it will be exported and further cleaved in the cytoplasm by Dicer (Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Lee et al., 2002). Consistent with its evolutionary origin before the appearance of miRNAs (Mukherjee et al., 2013), Dicer has a wider range of substrates than the Microprocessor – it can cleave RNA duplexes of any length, as long as they have a 2-nt 3′ overhang (and in some cases even blunt-ended duplex RNA). The domain structure of Dicer serves as a molecular "ruler," such that the two RNAse III active sites are positioned at a defined length from the 3′ overhang of the pre-miRNA and determine the length of the mature small RNA (MacRae et al., 2006, 2007). Dicer cooperates with other RNA binding proteins, PACT or TRBP in humans, and Loquacious in *Drosophila* (Treiber et al., 2019). This interaction can affect not only the efficiency of the processing, but also the length of the mature miRNA produced (Lee et al., 2006; Chakravarthy et al., 2010; Fukunaga et al., 2012; Lee and Doudna, 2012; Zhu et al., 2018). Therefore, together with Drosha, Dicer defines not only the abundance but also the ends of the mature miRNAs that will be loaded into Argonaute.

Because of its broader activity, Dicer processes not only pre-miRNAs, but also different endogenous and foreign duplex RNAs to produce other classes of short interfering RNAs (siRNAs). Whereas *Drosophila* has two different Dicer proteins, one specialized for miRNA (*Dcr-1*) and the other for siRNA biogenesis (*Dcr-2*) (Lee Y. S. et al., 2004), many other animals have a single type of Dicer. The involvement of this enzyme in two different pathways creates a bottleneck that in some animals leads to competition between the two different types of small RNA precursors. In *C. elegans* for example, downregulation of the endo-siRNA pathway results in an increase of miRNA-biogenesis, whereas induction of exogenous RNAi competes with both endo siRNA and miRNA production. This suggests that, at least in some contexts, Dicer can be limiting for small RNA production (Zhuang and Hunter, 2011).

Processing by the Microprocessor and by Dicer is subjected to diverse regulatory mechanisms. These can either affect Drosha and DGCR8 or Dicer themselves to broadly impact the biogenesis of multiple miRNAs, or specifically regulate the maturation of individual miRNAs, typically through the action of RNA binding proteins (RBPs) that recognize unique features of primary and precursor miRNAs.

Global and miRNA-Specific Regulation Determine the Rates of miRNA Production

Pri-miRNAs share some broad structural features, but they can differ substantially in the length of their stems, the sequences in their loops, and the sequences flanking the hairpin, posing a challenge for efficient and specific processing by the Microprocessor. Indeed, not all wild-type pri-miRNAs are optimal targets for Drosha or DGCR8 binding, resulting in a broad range of processing efficiencies (Han et al., 2006; Auyeung et al., 2013; Fang and Bartel, 2015; Kim et al., 2017), and different degrees of sensitivity to the presence of co-factors like for example DGCR8's co-factor, the iron-containing porphyrin heme (Partin et al., 2017; Nguyen et al., 2018). A number of sequence motifs that increase the specificity and efficiency of processing by the Microprocessor have been identified; for instance, apical elements in the hairpin, most prominently a UGU motif (Auyeung et al., 2013) and the mGHG-motif on the basal side, which seems to be a key determinant of cleavage site selection in many pri-miRNAs (Kwon et al., 2019). Highlighting the importance of the pri-miRNA sequence and structure for its correct processing, a single nucleotide change in the apical loop of pri-mir-30c-1 found in some patients with breast and gastric cancer results in increased processing and thus higher miR-30c-1 abundance. This was attributed to enhanced binding of SRSF3, a protein of the SR family that promotes Microprocessor cleavage (Fernandez et al., 2017).

The diversity in pri- and pre-miRNA sequences means that different steps might be rate-limiting for individual precursors, and this has enabled the evolution of additional regulatory mechanisms. A number of individual miRNAs are subjected to unique modes of regulation via specific interactions with different RNA-binding proteins (RBPs) (Choudhury et al., 2013; Treiber et al., 2017; Kooshapur et al., 2018; Michlewski and Cáceres, 2018; Downie Ruiz Velasco et al., 2019). A recent mass spectrometry-based screen using various human cell line lysates identified numerous RBPs that impact processing of subsets of miRNA-precursors and ultimately the expression of target mRNAs (Treiber et al., 2017). The binding specificity of many of these seems to be determined by the terminal loop, in which the RNA is single-stranded and typically exposed for contact with other proteins (Choudhury and Michlewski, 2012; Treiber et al., 2017). However, interestingly a few RBPs also bind the stem of specific miRNA hairpins (Treiber et al., 2017). A complementary *in silico* approach using published eCLIP-data has also identified a number of novel RBP:pre-miRNA interactions affecting processing of specific miRNAs (Nussbacher and Yeo, 2018).

Some RBPs have been shown to recruit terminal nucleotidyl transferases, most commonly uridyl transferases or TUTases (Hagan et al., 2009). Post-transcriptional modifications of precursor or mature miRNAs can impact the kinetics of biogenesis or silencing. Uridylation of mature miRNAs by TUTases tends to promote decay. However, uridylation of pre-miRNAs may either promote decay or have stabilizing effects and affect further processing by Dicer, depending on the 3′ end structure of the precursor (Thornton et al., 2014; Bortolamiol-Becet et al., 2015; Kim et al., 2015; Reimão-Pinto et al., 2015). Terminal miRNA modification by TUTases occur in Bilateria as well as in more basal animal clades such as Cnidaria and Porifera. In fact, TUTases acting on small RNAs were already present in the last common ancestor of all animals, underscoring the fundamental involvement of these mechanisms in miRNA biology (Modepalli and Moran, 2017).

The general activities of Dicer and the Microprocessor can also be regulated, with global consequences for miRNA output. A common regulatory mechanism is through post-translational modifications of the enzymes themselves, or their cofactors (Treiber et al., 2019). Regulation of Dicer activity for example is affected by phosphorylation of its binding partner TRBP, resulting in increased stability of the Dicer-TRBP complex and enhanced pre-miRNA processing activity (Paroo et al., 2009; Xu et al., 2015; Warner et al., 2016). Dicer itself can be phosphorylated on two conserved residues, resulting in its nuclear translocation and inhibition in worms, mice and humans (Drake et al., 2014). This inhibitory mechanism has been implicated in the reduction of mature miRNA levels in the *C. elegans* germline, contributing to precise gene expression changes in the oocyte-to-embryo transition (Drake et al., 2014). In general, post-translational modifications may serve to integrate diverse cellular signaling pathways with the production of miRNAs.

Moreover, levels of the processing machinery can vary across different cell types or under different conditions, also impacting the global miRNA output. A survey of Drosha expression levels across different mouse tissues revealed differences in the range of 4–10-fold, with the brain having the highest and the liver the lowest level of Drosha mRNA and protein (Sperber et al., 2014). Lower Drosha expression enhances the inherent differences in processing efficiency of different pri-miRNAs, resulting in the deregulation of subsets of miRNAs with specific properties (Sperber et al., 2014). The contribution of some of these mechanisms to the development and physiology of animals remains to be tested. Nevertheless, the dysregulation of the miRNA biogenesis machinery has been associated with diverse forms of cancer, typically resulting in a global repression of miRNA maturation (Lin and Gregory, 2015).

The final step for producing a functional miRISC is the loading of a miRNA into Argonaute. This seems to be the rate-limiting step in a number of contexts (Diederichs and Haber, 2007; Janas et al., 2012; Reichholf et al., 2019; Bose et al., 2020), likely related to the fact that loading does not simply reflect binding of an RNA to Argonaute, but follows a number of steps that require assistance from other factors, including chaperones and energy from ATP (Kobayashi and Tomari, 2016). As with every other step of miRNA biogenesis, miRNA loading can also be affected by specific features of the miRNA duplex, such as presence of a 5′ phosphate, identity of the 5′ terminal nucleotide and stability of the duplex. These features also determine which of the two duplex strands is preferentially loaded on Argonaute and acts as a mature miRNA; the opposite strand, or miRNA*, is typically rapidly degraded. In addition, specific RNA binding proteins that impact the loading on Argonaute either positively or negatively have been described. For example, TDP43 disrupts loading of miR-1 and miR-206, two muscle-specific miRNAs (King et al., 2014), while hnRNPD0 supports loading of let-7b (Yoon et al., 2015).

The effects of all these regulatory mechanisms at the organismal level can in principle have two different types of consequences. On the one hand, post-transcriptional regulation of miRNA biogenesis may be used to encode temporal or spatial information in a developing animal. Such is the case of LIN28, which acts as a conserved post-transcriptional repressor of let-7-family miRNA biogenesis (Moss et al., 1997; Yang and Moss, 2003; Heo et al., 2008; Newman et al., 2008; Viswanathan et al., 2008). LIN28 recruits TUTases that ultimately prevent Dicer-recognition and promote decay via the exonuclease Dis3L2 (Hagan et al., 2009; Chang et al., 2013). Release of LIN28-mediated repression of let-7 maturation provides a temporal switch in different contexts during progression of animal development (Thornton and Gregory, 2012).

However, most of the RBPs that affect biogenesis and loading are ubiquitously or very broadly expressed, suggesting that they may rather contribute to achieving homeostatic levels of specific miRNAs. This could be necessary to compensate for individual miRNA features that make them better or worse substrates of the biogenesis machinery. It is also possible that some of these RBPs have different expression levels or modifications in different tissues that might directly contribute to the spatio-temporal specificity of miRNA production. Another possibility is that some regulators are used to restrict the production of miRNAs to specific sub-cellular localizations. An extreme example of this is the synapse-specific maturation of miRNAs by Dicer upon neuronal activation (Sambandan et al., 2017).

SILENCING MECHANISM – VARIATIONS ON A THEME

The ancestral mechanism of small RNA guided effector proteins involves irreversible destruction of targeted nucleic acids by cleavage (Park and Shin, 2014; Moran et al., 2017). This miRNA mode of action, which is usually accompanied by near-perfect target complementarity, is prevalent in plants (Voinnet, 2009) and also observed in basal metazoan lineages like Cnidaria (Moran et al., 2014; Mauri et al., 2017). Bilaterian animals on the other hand, predominantly employ a mechanism that relies on partial base pairing between the "seed" region of a miRNA (nucleotides 2–8) and sequences typically in the 3′ UTR of mRNAs. Recruitment of miRISC in bilaterians usually results in the downregulation of protein output through a combination of translation inhibition and target mRNA decay (Jonas and Izaurralde, 2015; Bartel, 2018; **Figure 3**).

Appearance of the slicing-independent, seed-based mechanism laid the foundation for functional diversification of miRNA biology in animals. Compared to a mode of target recognition that involves full sequence complementarity, a mechanism that requires only partial base-pairing has the potential to greatly increase the target repertoire of any miRNA. This likely expanded the overall potential of miRNA-mediated repression in bilaterian animals, enhancing the connectivity of gene regulatory networks, and contributing to cell-type diversification and acquisition of morphological complexity during evolution (Peterson et al., 2009; Moran et al., 2017).

The Metazoan Seed-Based Mechanism

The best understood mechanism of miRNA-mediated repression in animals relies on a protein of the GW182 family, which together with Argonaute forms the core of what is considered

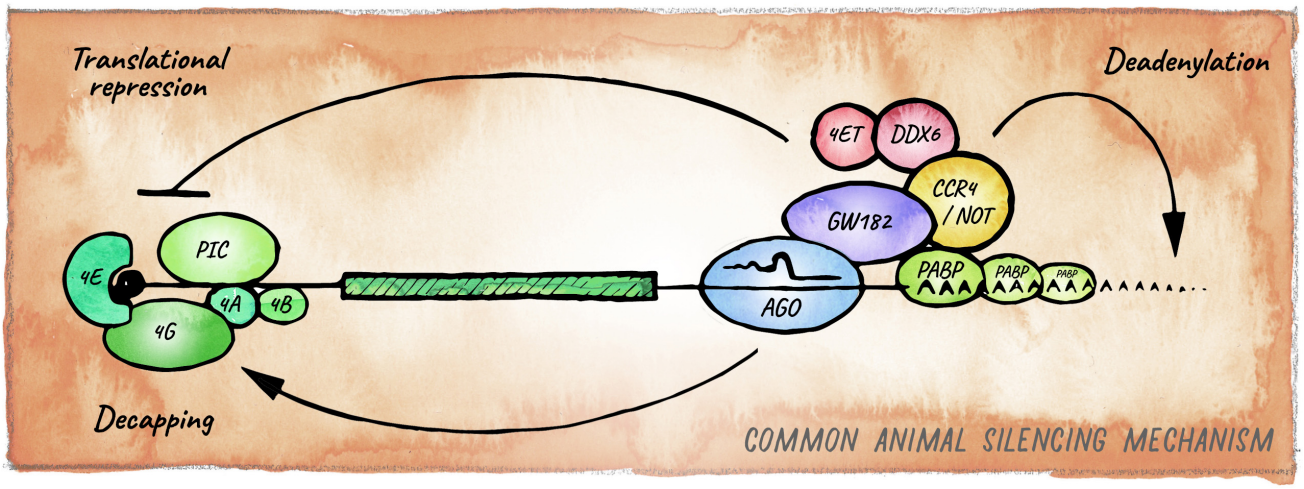

FIGURE 3 | Canonical miRNA-silencing mechanism in animals. miRNAs elicit repression of target genes usually through a combination of translational repression and promotion of mRNA decay. Argonaute is guided by a miRNA to a cognate target mRNA and tethers GW182, forming the core of the most common animal miRISC. GW182 interacts with PABP and recruits the deadenylase complexes CCR4-NOT or Pan2-Pan3 (not shown), leading to deadenylation, decapping and ultimately exonucleolytic decay. Inhibition of translation occurs mainly at the initiation step by interfering with assembly or activity of eIF4F, via eIF4E-T, and DDX6.

the canonical miRISC. Members of the GW182 protein family (e.g., GW182 in *Drosophila*, AIN-1/2 in *C. elegans* and TNRC6 in humans) are rapidly evolving but share Gly-Trp repeats that bind to conserved pockets in Argonaute proteins (El-Shami et al., 2007; Zhang et al., 2007; Eulalio et al., 2008; Pfaff et al., 2013). Through other domains, GW182 proteins recruit RNA-processing factors, repressing translation as well as enhancing mRNA turnover (Chekulaeva et al., 2011; Fabian et al., 2011; Kuzuoğlu-Öztürk et al., 2012; Huntzinger et al., 2013). The precise mechanism for translational repression remains to be resolved; however, the emerging consensus is that it involves inhibition of cap-dependent translation initiation via interaction with eIF4F (Pillai et al., 2004; Mathonnet et al., 2007; Zdanowicz et al., 2009; Ricci et al., 2013; Fukaya et al., 2014). GW182 proteins also recruit the PAN2-PAN3 and CCR4-NOT deadenylase complexes, ultimately triggering mRNA decay (Behm-Ansmant et al., 2006; Guo et al., 2010; Braun et al., 2013; Eichhorn et al., 2014; Jonas and Izaurralde, 2015; Kuzuoğlu Öztürk et al., 2016; Niaz and Hussain, 2018; **Figure 3**). GW182 has been proposed to promote the formation of phase-separated condensates containing miRISC and target mRNAs, increasing the local concentration of deadenylases and other factors for efficient repression (Sheu-Gruttadauria and MacRae, 2018). GW182 is also present in Nematostella, where it is able to interact with Argonaute and the CCR4-NOT complex, suggesting that this mechanism originated in the last common ancestor between Cnidaria and Bilateria (Mauri et al., 2017). Experimental uncoupling of translational inhibition and mRNA decay has proven challenging, as the two processes are intimately linked (Subtelny et al., 2014; Radhakrishnan and Green, 2016). Still, analyses of the dynamics of miRNA-mediated silencing in zebrafish early embryos and in mammalian cells in culture, revealed that miRNAs first repress translation initiation and then induce mRNA decay (Bazzini et al., 2012; Djuranovic et al., 2012). The relative contribution of these two mechanisms to biological function remains a matter of debate (Eichhorn et al., 2014), but as we discuss below multiple pieces of evidence indicate that this may be determined by the cellular context and miRISC composition.

Despite the conserved nature of the canonical miRNA targeting mechanism, there is an increasing number of examples highlighting context-dependent variability in the mode and functional consequence of miRISC targeting (**Figure 4**). This variability can arise from two different mechanistic sources. First, miRISC can have non-canonical composition in different cell types or distinct developmental stages (e.g., differential presence or abundance of GW182 and other proteins that act as facultative Argonaute interactors). In metazoans, such variations are observed most notably between soma and germline, and we expand on this in the next section. Second, as perfect target complementarity is not a prerequisite, there are different possible miRNA:mRNA interaction modes (Lal et al., 2009; Shin et al., 2010; Chi et al., 2012). Whereas the majority of miRNA binding sites are located in the 3′ UTR and involve pairing to the seed region of a miRNA, these features can differ with varying functional consequences; from efficiency of silencing depending on the relative location of miRNA-binding sites within the target mRNAs (Grimson et al., 2007; Forman and Coller, 2010; Zhang K. et al., 2018); to degradation of the miRNA itself, if target pairing extends to the 3′ end of the miRNA (Ameres et al., 2010; Cazalla et al., 2010; De la Mata et al., 2015; Bitetti et al., 2018; Ghini et al., 2018; Sheu-Gruttadauria et al., 2019).

Variations in miRISC Composition and Functional Outcome

The mode of miRNA targeting and the effects that different RISC complexes will exert on their targets vary significantly across

different eukaryotic clades. Even within a single animal, the composition and functional consequences of miRISC can differ in specific biological settings. The first evident variation is in the Argonaute component itself, e.g., *C. elegans* evolved more than 25 Argonaute proteins (Youngman and Claycomb, 2014). ALG-1 and ALG-2 are the main miRNA-related Argonautes. They function redundantly to a large degree, and only loss of both simultaneously causes penetrant embryonic lethality (Vasquez-Rifo et al., 2012). However, they do display some functional differences which may stem from differences in expression patterns and levels (ALG-2 appears to be dominantly expressed throughout embryogenesis) as well as in miRNA-binding preference (Vasquez-Rifo et al., 2012; Brown et al., 2017). Interestingly, another Argonaute, ALG-5, has recently been implicated in miRNA-mediated silencing in the worm. ALG-5 is expressed in the germline, where it associates effectively with only a subset of miRNAs and plays a role in gametogenesis (Brown et al., 2017). Insects such as cockroaches, from a basal clade, also have two partially redundant Argonautes for miRNA mediated silencing (Rubio et al., 2018). However, subsequent specialization took place in more derived clades like *Drosophila*, which encodes one Argonaute for siRNA and another one for miRNA-mediated silencing (Förstemann et al., 2007; Iwasaki et al., 2009).

Most of the variation in outcome of miRISC binding to an mRNA stems from differential association of Argonaute with other proteins. This seems to primarily affect the relative contribution of translation inhibition vs. mRNA destabilization. In particular, the larger differences appear to occur between the germline or early embryo versus somatic tissues. In *C. elegans*, the prevalent form of miRISC in the germline has been shown to lack the GW182 proteins AIN-1 and AIN-2 and instead associates with P-granule constituents. The resulting recruitment of target mRNAs to P-granules inhibits protein production but does not promote mRNA decay (Dallaire et al., 2018). This has been proposed to protect maternal mRNAs whose translation products are not beneficial in the germline but are required in the early embryo for robust development. Consistent with these observations, *C. elegans* embryos tolerate mutations that severely impede association of Argonaute with AIN-1/2. Despite Argonautes being essential for embryonic development of *C. elegans* (Vasquez-Rifo et al., 2012), AIN-1/2 play a secondary role at this stage (Jannot et al., 2016). However, the interaction between Argonaute and the GW182 orthologs is necessary for post-embryonic development (Jannot et al., 2016).

GW182-independent function of miRISC has also been observed in other contexts. For instance, in *Drosophila* S2 cells, induction of mitogenic signaling via serum withdrawal results in formation of an ER-associated, GW182-independent miRISC. This complex is a potent inhibitor of translation but has no effect on mRNA deadenylation and decay (Wu et al., 2013). In line with this, depletion of GW182 in *Drosophila* S2 cells abolished miRNA-dependent deadenylation but had practically no effect on translation repression (Fukaya and Tomari, 2012). The diversity in miRNA mediated silencing mechanisms provides organisms with enhanced capacity for gene regulation, while posing additional challenges for a complete understanding of miRNA functions *in vivo*.

Animals like *C. elegans* have adopted variant miRNA activity in the germline, yet other animals seem to have evolved ways to dampen or abolish germline miRNA activity altogether. In *Drosophila* oocytes, Ago-1 is present at very low levels and only increases upon maternal to zygotic transition (Luo et al., 2016), coinciding with the production of a miRNA cluster involved in maternal mRNA decay (Bushati et al., 2008). Zebrafish zygotes also have low miRNA levels, with considerable accumulation starting around the blastula stage (Chen et al., 2005). At that time, miRNAs are also involved in the maternal to zygotic transition. Most notably, miR-430 is expressed at the onset of zygotic genome activation and promotes maternal mRNA clearance (Giraldez et al., 2006). The mouse germline also appears to lack essential miRNA functions, as depletion of DGCR8 results in normal oocytes that give rise to healthy offspring upon fertilization with wild-type sperm (Suh et al., 2010). In the absence of both maternal and zygotic DGCR8, zygotes still undergo normal pre-implantation development but then arrest prior to E6.5 (Suh et al., 2010). Clearly, miRNA-mediated regulation plays important roles in early animal development, yet the contribution and mechanisms of action in the developmental window around fertilization remain an active area of investigation (McJunkin, 2017).

miRNAs provide an outstanding way to confer specificity to a variety of repressor complexes. Different effector mechanisms may operate within one organism, or even within one cell, and may be dynamically regulated under different conditions. It will be interesting to find out how different modes of regulation are used in different cellular contexts within animal development and physiology.

INNOVATION THROUGH EXPANSION OF THE miRNA REPERTOIRE

In the course of animal evolution, the miRNA repertoire expanded drastically in conjunction with complexity (Hertel et al., 2006; Heimberg et al., 2008; Wheeler et al., 2009; Berezikov, 2011; Guerra-Assunção and Enright, 2012; Hertel and Stadler, 2015; **Figure 5**). Often, miRNAs arose in bursts coinciding with major organismal innovations, for example the emergence of vertebrates, or cortical expansion in primates (Peterson et al., 2009; Hertel and Stadler, 2015; Kosik and Nowakowski, 2018; Fromm et al., 2020). While the animal miRNA machinery originated in unicellular holozoans (Bråte et al., 2018), the most conserved animal miRNA, miR-100, first appeared in the last common ancestor of cnidarians and bilateria (Grimson et al., 2008). Organisms diverging early in eukaryotic evolution tend to have few, mostly non-conserved miRNA genes, indicating a high evolutionary flux in basal clades (Grimson et al., 2008; Cock et al., 2017). The availability of high-quality miRNA annotations in diverse genomes has recently upgraded our ability to place miRNAs into an evolutionary context (Berruezo et al., 2017; Cock et al., 2017; Wang et al., 2017; Ylla et al., 2017; Fromm et al., 2019, 2020). This opens up exciting new avenues, enabling connections between miRNA age, expression

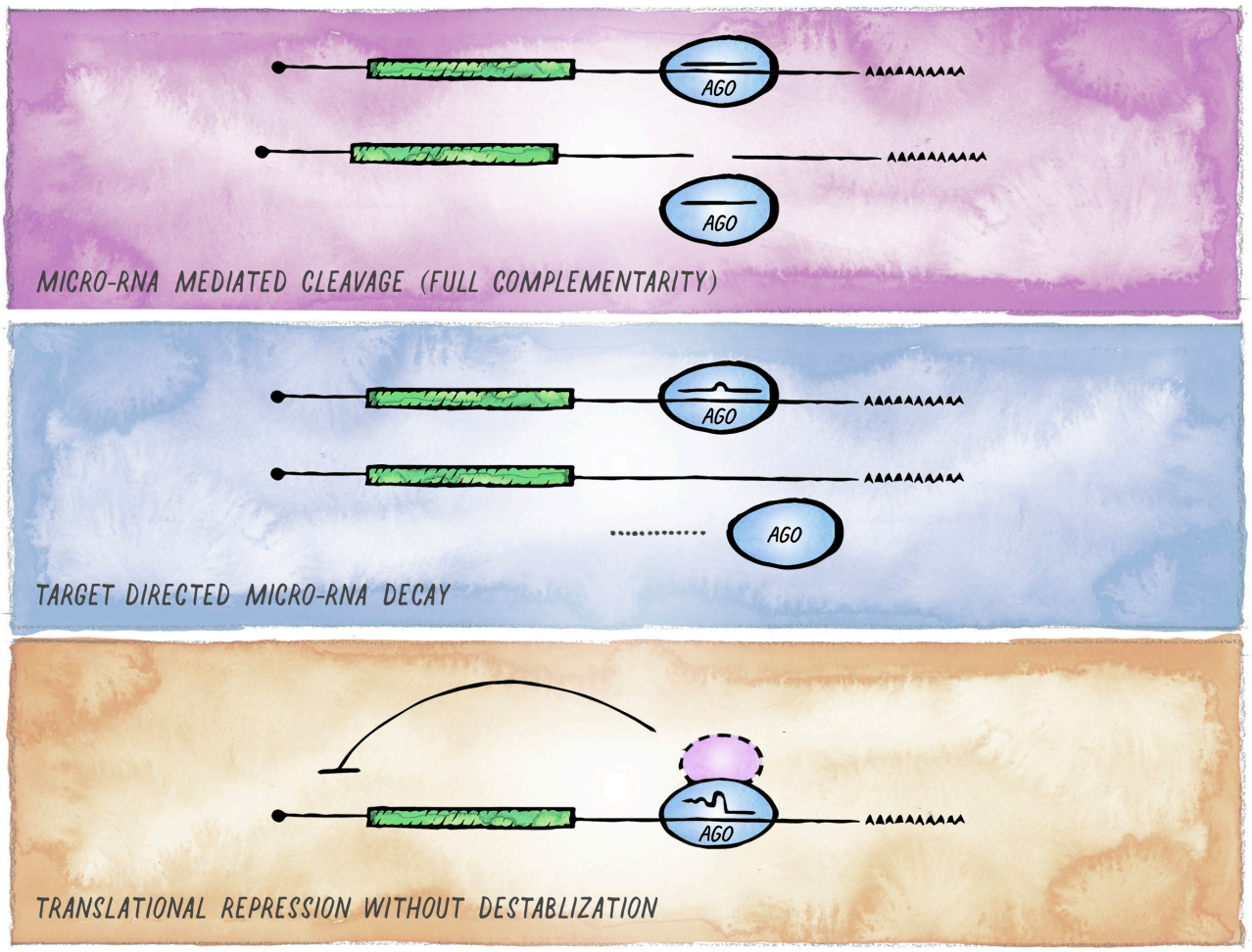

FIGURE 4 | Alternative outcomes of miRNA-mediated targeting. In addition to the common miRISC effects on translation and stability of target mRNAs, other functional outcomes of miRISC binding to an mRNA are possible. **(A)** Full sequence complementarity results in Ago-mediated target cleavage, a mechanism that resembles the mode of action commonly employed in cnidaria and plants. **(B)** Target-mediated miRNA degradation is induced by interaction with targets through extensive pairing, in particular extending to the miRNA 3′ end. **(C)** Recruitment of Argonaute in the absence of GW182 results in inhibition of translation without affecting mRNA stability (likely involving alternative co-factors).

and function during development, as well as the emergence of specific features at the organismal level.

Diversification of the miRNA Repertoire

A widespread view in the field is that miRNAs tend to be rapidly gained and lost in the course of evolution (Nozawa et al., 2010; Fromm et al., 2013; Meunier et al., 2013), although part of this has been recently challenged (Tarver et al., 2018). Nevertheless, a substantial number of miRNAs have acquired important functions and remain conserved. Because miRNA evolution is intimately connected to evolution of target genes (Zhao et al., 2015; Nozawa et al., 2016), conservation of miRNAs is observed primarily over the seed region, which determines targeting specificity. However, features beyond the seed can critically impact miRNA function, underscored by the observation that some mature miRNAs are conserved from the first to the last nucleotide across large evolutionary distances. For example, *let-7* has not accumulated a single mutation between humans and worms (Pasquinelli et al., 2000), or *mir-9a*, has remained identical between Drosophila, mouse, and human (Li et al., 2006).

miRNAs are much younger than protein coding genes (on average 169 Myr vs. 1195 Myr respectively), and many of them arose more recently, after the split of diverse phylogenetic groups. For instance, an estimated 46% of human miRNAs are primate-specific and 14% are human-specific (Patel and Capra, 2017). In general, miRNA genes evolve *de novo* from hairpin-structures located within introns or intergenic regions (Lu et al., 2008; Nozawa et al., 2010), which in turn likely originate through one of three proposed models: (i) inverted gene duplication of a gene that will subsequently become target of the miRNA (Allen et al., 2004); (ii) transposon-insertion followed by derivatization (Li et al., 2011; Qin et al., 2015); and (iii) spontaneous evolution out of random sequences (De Felippes et al., 2008). However, the majority of functionally important miRNAs arose from

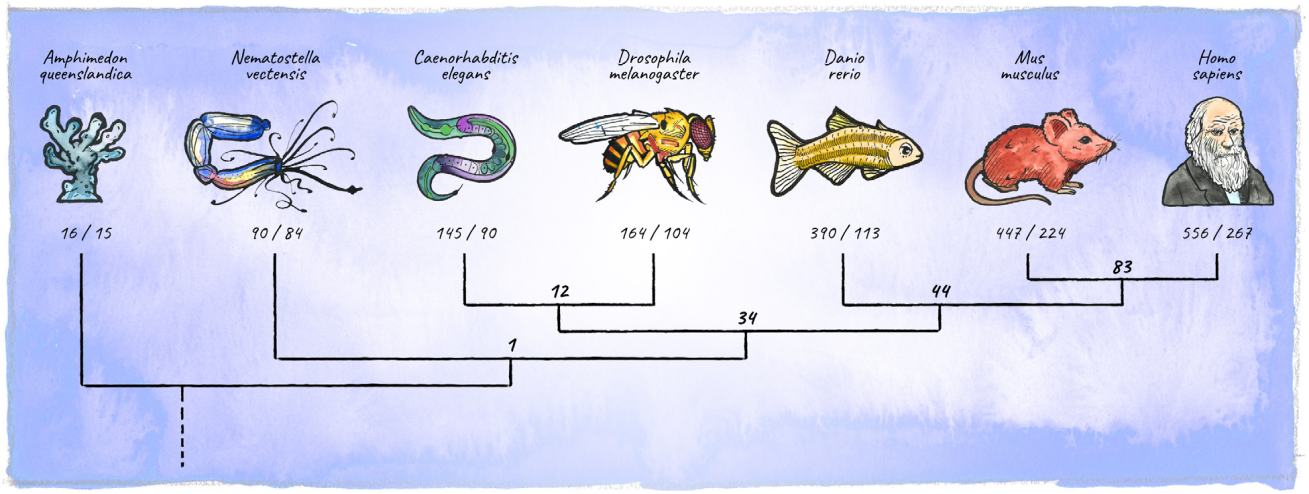

FIGURE 5 | Diversification of the metazoan miRNA repertoire. Shown are numbers of individual miRNAs/miRNA-families (in bold) annotated with high confidence for various clades (https://mirgenedb.org, Fromm et al., 2020). Number of miRNA families present in the last common ancestors of branching clades are noted above the split. Exemplary organisms depicted are *Amphimedon queenslandica*, *Nematostella vectensis*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, *Mus musculus*, and *Homo sapiens*. Data for bilateria is derived from Fromm et al. (2020), for *Amphimedon* and *Nematostella* numbers were retrieved from the work of Grimson et al. (2008); Moran et al. (2014), and Calcino et al. (2018).

duplications of existing miRNAs (Kim and Nam, 2006; Carthew and Sontheimer, 2009; Berezikov, 2011).

Diversification of the miRNA repertoire occurs both through the addition of new miRNAs and the addition or change in targets. An improved understanding of the latter will require better knowledge of what are the biologically meaningful targets of any given miRNA. Whereas for a few miRNAs we know of a number of experimentally well-defined targets, for most miRNAs we rely on computational prediction algorithms. Because such prediction tools yield numerous false positives, substantial experimental validation of targets will be necessary for a deeper understanding of how miRNA-target interactions change over time (Fridrich et al., 2019).

miRNA Families

Duplication followed by sequence diversification of miRNAs can lead to target diversification, changes in expression pattern, and pronounced increases in dose (Luo et al., 2018). In many cases, if the seed sequence is retained, miRNA gene duplication marks the birth of a (homo-seed) miRNA family. Members of a family function largely redundantly on a shared set of target mRNAs. In many cases the full extent of the function of miRNA family becomes apparent only upon removing all members (Miska et al., 2007; Alvarez-Saavedra and Horvitz, 2010; Parchem et al., 2015). Curiously, most animal miRNAs whose loss of function causes severe defects, occur in families with sometimes extreme copy numbers. In *C. elegans*, two miRNA families are required for completion of embryonic development: the MIR-36 family (8 members in *C. elegans*, 29 members in the closely related *C. briggsae*) and MIR-100 family (miR-51-56 in the worm) (Alvarez-Saavedra and Horvitz, 2010). In zebrafish, MIR-430, which plays an outstanding role during embryonic development (Giraldez et al., 2005), has evolved at least 57 family members (according to mirgenedb2.0) located within a 16 kb region on chromosome 4. Mouse embryos lacking the miR-290-295 and miR-302-367 clusters (mammalian miR-430 homologs) arrest early in embryogenesis, with defects that are only partially recapitulated if one or the other family is removed (Medeiros et al., 2011; Parchem et al., 2015).

Underscoring the biological significance of miRNA duplication followed by organization into families, there seems to be an expansive trend among miRNAs with important biological functions. Potential reasons include (i) increasing the dose of mature miRNA to enhance efficient target silencing, or (ii) evolutionary robustness and flexibility, e.g., mutations in one family member are not immediately detrimental, and individual copies can be further diversified in sequence or in expression pattern. Different family members share identical seed sequences, and in some cases, paralogous miRNAs are also conserved around nucleotides 13–17, which underscores the contribution of these positions to efficient targeting (Wee et al., 2012; Schirle et al., 2014; Sheu-Gruttadauria and MacRae, 2017). However, in other cases family members differ in sequences beyond the seed, which can affect targeting properties (Broughton et al., 2016; Brancati and Großhans, 2018; Zhang S. et al., 2018) but also biogenesis or loading efficiency as discussed above. Thus, while miRNA family members appear largely redundant, individual members can acquire specific functions (Abbott et al., 2005; Alvarez-Saavedra and Horvitz, 2010; Brancati and Großhans, 2018; Zhang S. et al., 2018).

A recent analysis found that out of 352 human miRNAs that are conserved among vertebrates, 207 (58.8%) are duplicates and 125 (35.5%) are homo-seed family miRNAs (Luo et al., 2018). miRNAs in families differ from singletons in their evolutionary dynamics and functional roles, with family miRNAs tending to

be: (i) older than singletons, (ii) more conserved at the sequence level than singletons, (iii) enriched for diverse expression in distinct tissues, (iv) broader in target range, and (v) implicated in more diseases. These functional correlations are still significant, albeit less pronounced, when family vs. singleton miRNAs are stratified by age, with older miRNAs tending to contribute more to disease, target more genes, and being expressed in significantly more tissues (Patel and Capra, 2017). In addition, the efficiency of target repression correlates with degree of conservation as well as evolutionary age for many miRNAs (Luo et al., 2018).

The expansion of metazoan miRNAs was likely one of the factors that contributed to the evolution of complexity in present-day animals. In this context, *de novo* evolution but also miRNA duplication followed by sequence diversification played an important role laying the foundation for miRNA families. Among others, family membership and evolutionary age of miRNAs coincides with functional trends, offering a useful context for elucidating the contribution of miRNAs to animal development and homeostasis. Nonetheless, clade-specific singleton miRNAs also provide a source of innovation. For example, miR-791 originated in a class of nematodes called Chromadorea, and at least in *C. elegans* it acquired an important function in its CO_2-sensing neurons (Drexel et al., 2016). Similarly, *lsy-6* originated in the last *Caenorhabditis* common ancestor and plays an essential role in sensory neuron diversification in *C. elegans* (Johnston and Hobert, 2003).

CONCLUDING REMARKS

Since the discovery of miRNAs, the field has made tremendous progress toward understanding the molecular mechanisms of biogenesis and action of this versatile class of repressors. The vast majority of these mechanistic studies have been performed in cell culture models, in which biochemical approaches are feasible. This has given us detailed snapshots of the possible roles of miRNAs at the molecular and cellular levels. At the organismal level however, most of our understanding comes from genetics, either from manipulations of the miRNA biogenesis machinery or individual miRNAs. While it is clear that miRNAs are necessary for the correct development and function of multiple cell types, in most cases we do not understand the functionally relevant relationships of miRNAs and their targets, the consequences on gene regulatory networks, and how the effect on specific cell types impacts the organism. Given the broad implication of miRNAs in physiology and disease, a deeper mechanistic understanding of the roles of miRNAs within complex organisms is highly desirable. We expect that new technologies that enable this depth of analysis in animals will make this possible.

AUTHOR CONTRIBUTIONS

PD and LC wrote the manuscript. PD designed, drew, and painted the figures in ink and watercolor.

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miR-15/107 microRNA Gene Group: Characteristics and Functional Implications in Cancer

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The miR-15/107 group of microRNAs (miRNAs) encloses 10 annotated human members and is defined based on the presence of the sequence AGCAGC near the mature miRNAs' 5′ end. Members of the miR-15/107 group expressed in humans are highly evolutionarily conserved, and seven of these miRNAs are widespread in vertebrate species. Contrary to the majority of miRNAs, which recognize complementary sequences on the 3′UTR region, some members of the miR-15/107 group are peculiarly characterized by the ability to target the coding sequence (CDS) of their target mRNAs, inhibiting translation without strongly affecting their mRNA levels. There is compelling evidence that different members of the miR-15/107 group regulate overlapping lists of mRNA targets but also show target specificity. The ubiquitously expressed miR-15/107 gene group controls several human cellular pathways, such as proliferation, angiogenesis, and lipid metabolism, and might be altered in various diseases, such as neurodegenerative diseases and cancer. Intriguingly, despite sharing the same seed sequence, different members of this family of miRNAs may behave as oncomiRs or as tumor suppressor miRNAs in the context of cancer cells. This review discusses the regulation and functional contribution of the miR-15/107 group to the control of gene expression. Moreover, we particularly focus on the contribution of specific miR-15/107 group members as tumor suppressors in breast cancer, reviewing literature reporting their ability to function as major controllers of a variety of cell pathways and to act as powerful biomarkers in this disease.

Keywords: miR-15/107, miR-195, miR-497, miR-15, miR-107, miR-16, miR-503, Granulin (GRN)

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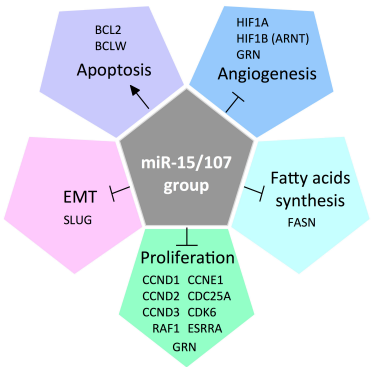
INTRODUCTION

The miR-15/107 gene group contains multiple highly conserved microRNA members, including miR-15a-5p, miR-15b-5p, miR-16-5p, miR-103a-3p, miR-107, miR-195-5p, miR-424-5p, miR-497-5p, miR-503-5p, and miR-646 (Finnerty et al., 2010). The nomenclature referred to these molecules is not uniform in the literature, and six of these miRNAs (miR-15a/b, miR-16, miR-195, miR-424, and miR-497) are frequently referred to as miR-16 family members (Rissland et al., 2011). Inclusion in the miR-15/107 group is based on the presence of "AGCAGC" in the "seed" region starting at either the first nucleotide or the second nucleotide from the 5′ end of the mature (∼22 nt, single stranded) miRNA (**Figure 1A**). Recently, it has been evidenced that also miR-6838-5p contains the AGCAGC sequence in its seed sequence, and this miRNA has then been included as a new member

A

		Host gene
hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA	*PANK1*
hsa-miR-103	AGCAGCAUUGUACAGGGCUAUGA	*PANK2, PANK3*
hsa-miR-15a	UAGCAGCACAUAAUGGUUUGUG	*DLEU2*
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA	*SMC4*
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	*SMC4, DLEU2*
hsa-miR-195	UAGCAGCACAGAAAUAUUGGCC	*MIR497HG*
hsa-miR-497	CAGCAGCACACUGUGGUUUGU	*MIR497HG*
hsa-miR-503	UAGCAGCGGGAACAGUUCUGCAG	*MIR503HG*
hsa-miR-424	CAGCAGCAAUUCAUGUUUUGAA	*MIR503HG*
hsa-miR-646	AAGCAGCUGCCUCUGAGGC	*MIR646HG*
hsa-miR-6838	AAGCAGCAGUGGCAAGACUCCU	*POLM*

B



C

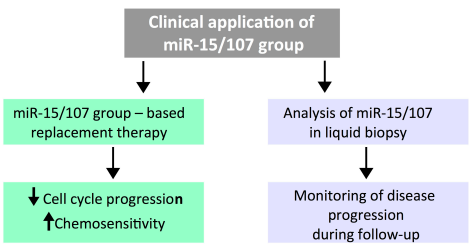


FIGURE 1 | (A) Members of the miR-15/107 gene group are highly conserved and share an AGCAGC hexamer in their "seed" region. All members of the miR-15/107 group are enclosed in host genes, indicated on the right. **(B)** Diagram describing the main tumor suppressive functions exerted by members of the miR-15/107 group in breast cancer. Experimentally demonstrated targets relevant to the various functions are indicated. **(C)** Diagram describing putative fields of application of the miR-15/107 group members in the clinical practice in breast cancer. Delivery of modified oligonucleotides mimicking members of this microRNA (miRNA) group could strongly impair the proliferative potential of breast cancer cells and increase the response to therapies. In addition, evaluation of the levels of various miR-15/107 group members in liquid biopsy is a promising approach for the diagnosis and the monitoring of disease progression, especially for triple-negative breast cancer.

of the miR-15/107 group (Wang F. et al., 2019). miR-15/107 family members are only expressed in chordates, with several being mammal specific (miR-195,-497, -503, -424, and -646) and miR-646 appearing only in humans and chimpanzees (Finnerty et al., 2010; Kozomara and Griffiths-Jones, 2014; Wang S. et al., 2019).

All members of this group are interestingly located in host genes (**Figure 1A**), including: (a) coding genes, as *PANK1* (containing miR-107), *PANK2* and *PANK3* (containing miR-103a-3p), *SMC4* (containing miR-15b-5p and miR-16-5p), *DLEU2* (containing hsa-miR-15a-5pand miR-16-5p), *POLM* (containing miR-6838-5p); (b) non-coding genes, as *MIR497HG* (containing miR-497-5p and miR-195-5p), *MIR503HG* (containing miR-503-5p and miR-424-5p and contained in the H19X locus), and *MIR646HG* (containing miR-646) (Finnerty et al., 2010; Necsulea et al., 2014). The conserved genomic organization of this group in four pairs of neighboring miRNA clusters, including miR-15a-5p/miR-16-5p, miR-15b-5p/miR-16-5p, miR-195-5p/miR-497-5p, and miR-424-5p/miR-503-5p within the common host genes, suggests that the miR-15/107 group members might be transcribed in pairs, except for the miR-646 and miR-6838-5p. Moreover, as both miR-103a-3p and miR-107 are hosted into the pantothenate kinase family (PANK) genes, even though located at entirely different chromosomes, they might be possibly subjected to common regulatory mechanisms. It has been reported that intronic miRNAs tend to be coexpressed with their protein-coding "mother" genes, although this is not always the case (Baskerville and Bartel, 2005; Monteys et al., 2010). Supporting a common phylogenetic origin, miR-15/107 family members have similar expression patterns and functions. The 5′ end sequence homology confers similar specificity in terms of targeting mRNAs for posttranscriptional decay and/or translational inhibition (Finnerty et al., 2010). miRNAs from this group are expressed in a wide variety of tissues, and given that many of their validated targets are involved in cell cycle, metabolism, and angiogenesis, it follows that dysregulation of these miRNAs is a hallmark of many disease states (Aqeilan et al., 2010; Li et al., 2011; Furuta et al., 2013).

miR-15/107 GROUP EXPRESSION PATTERNS AND TARGET SPECIFICITY

While all vertebrates examined to date express miR-15a, miR-15b, miR-16, miR-103, and miR-107, only mammals are known to express miR-195, miR-424, miR-497, miR-503, whereas miR-646 appears to be human specific (Finnerty et al., 2010). Overall, it can be considered that the miR-15/107 gene group of miRNAs is ubiquitous in that, as far as we know, no human cell type lacking the expression of at least one miR-15/107 gene group member has been described. Expression of mature miRNA forms of miR-15/107 family has been recently analyzed on 11 human tissues. Overall, these miRNAs were found highly expressed in a variety of these tissues including the brain, heart, lung, liver, kidney, spleen, stomach, and skeletal muscle (Wang et al., 2014). From RNA isolated from human brain samples, miR-16, miR-103, miR-107, and miR-497 are the most highly expressed miRNAs among the family members. Moreover, analysis of human tissues interestingly showed that the miRNAs apparently expressed at the highest levels include the 7-nt common sequence AGCAGCA (Finnerty et al., 2010; Wang et al., 2014). Analysis of tissue samples evidenced that individual members of the miR-15/107 group are expressed at medium-to-high levels across many tissue types, but there is some tropism in terms of both tissue- and cell type-specific expression. However, these data reveal that there is

not, among these miRNAs, a sharply tissue- or cell-specific miR-15/107 gene. Interestingly, analysis of miRNA precursors in the same tissues showed that correlations between the expression of mature miRNA and that of the precursor transcript (pri-miRNA) were generally not strong for most miRNAs of the miR-15/107 group.

With regard to the ability of miRNAs belonging to this group to target mRNAs, a number of studies focused on the dissection of their shared functions, attributable to the presence of the common AGCAGC hexamer in the seed sequences, as well as of their specific functions, conferred by the rest of each miRNA sequence besides the AGCAGC. The group of Peter T. Nelson (University of Kentucky) contributed extensively to this topic leading to the publication of seminal papers on the characterization of the miR-15/107 group function. In the attempt to better understand the implications of both 5′ and 3′ portions of miRNA in terms of mRNA targeting, Nelson and colleagues considered the target mRNAs that are associated with AGO protein in RIP-Chip experiments, and also those mRNAs downregulated following transfections of miR-103, miR-107, miR-16, and miR-195 in cultured H4 glioneuronal cancer cells (Nelson et al., 2011). Analysis of recruited mRNAs showed that, as expected, miRNA 5′ seeds appear to be a critical targeting determinant; however, in contrast to the majority of previously reported miRNAs that predominantly target the 3′-UTR, miR-103 and miR-107 preferentially bind to the CDS (coding sequence) of the target transcript. This property depends on the 3′ end of the miRNA, which pairs with target mRNA. Indeed, mutation of the 3′ portion of the miRNA impairs its preference for CDS vs. 3′-UTR. Interestingly, authors identified, in the 3′ portion of miR-107, a sequence motif AGCCCUGU that was significantly enriched in a group of 110 genes targeted by miR-107. Within this motif, three adjacent pentamer words were significantly enriched, with anti-sense sequences found in 33 of the 110 target genes.

Concerning the identification of common regulatory programs driven by different members of this miRNA group, the comparison of transcriptome modulations caused by various members of miR-15/107 group with relative AGO-interacting mRNAs, indicated that miR-16, miR-103, miR-107, and miR-497 share high degrees of overlap in targets, with 20% of mRNA targets shared by all four miRNAs in H4 glioneuronal cell line (Nelson et al., 2011). On the same line, Wang and colleagues recently analyzed putative and validated target mRNAs of miR-15/107 group to dissect collective and specific functions of these miRNAs through bioinformatic and experimental approaches (Wang S. et al., 2019). In agreement with results previously obtained by Nelson's group, their study showed the existence of a massive overlapping of target mRNAs among the miR-15/107 family; indeed, dozens of target genes could be affected by this family collectively, being subjected to regulation from more than 5 members of this family. Among the target mRNAs of the miRNA group, the most significantly regulated pathways included fatty acid metabolism/biosynthesis/degradation/elongation,various signaling during carcinogenesis, and some crucial pathways for cell survival such as cell cycle. The same study highlighted that, considering the validated targets, there are three pairs of miRNAs, including miR-15a-5p/miR-15b-5p, miR-103a-3p/miR-107, and miR-424/miR-497, closely resembling each other, while three of them, including miR-6838-5p, miR-503-5p, and miR-646, vary independently.

FUNCTIONAL RELEVANCE OF miR-15/107 GROUP IN CANCER

Tumor Suppressor Activity of the miR-15/107 Group and the Control of the Cell Cycle

The deletion or downregulation of both miR-15a and miR-16 in cases of B cell chronic lymphocytic leukemias (B-CLL) was the first evidence that suggested that these members of the miR-15/107 group could act as tumor suppressors (Calin et al., 2005; Cimmino et al., 2005). The formal demonstration came from *in vivo* experiments showing that, in mouse models, deletion of the genomic 13q14 region, which encodes the mir-15a/16-1 locus, recapitulates B-CLL phenotypes observed in humans (Klein et al., 2010). Starting from those initial observations, numerous additional evidences concerning the downregulation of members of this group of microRNAs in cancer reinforced the hypothesis of their role as tumor suppressors. Indeed, they have been found often downregulated in a variety of cancer types, such as colorectal cancer, prostate cancer, mantle cell, and other non-Hodgkin B-cell lymphomas, lung adenomacarcinomas, and breast cancer (Calin et al., 2005; Bonci et al., 2008; Bandi et al., 2009; Klein et al., 2010; Liu et al., 2010; Zhang et al., 2012; Bertoli et al., 2015). Interestingly, miR-15a/miR-16-1 downregulation was shown to rely on the repressive activity of transcription factor Myc in B-cell lymphomas (Zhang et al., 2012).

Restoration of the expression of some of these downregulated miRNAs in cancer cells represses the tumorigenicity of cells, impinging on cell cycle and apoptosis (Liu et al., 2010). However, important differences in these effects were observed depending on the cell types analyzed. For example, Cimmino et al. showed that miR-15a and miR-16-1 induce apoptosis through the downregulation of the antiapoptotic gene BCL2 in leukemic cells (Cimmino et al., 2005). On the contrary, miR-16 expression caused G0/G1 accumulation without evidence of apoptosis in colon, lung, breast, and ovarian cancer cells (Linsley et al., 2007). A huge number of reports evidenced the strong impact of miR-15/107 group on cell cycle regulatory genes, especially genes controlling the G1/S transition, as Cyclin D1, D2, D3, Cyclin E1, CDC25A, and CDK6 (Cimmino et al., 2005; Linsley et al., 2007; Bonci et al., 2008; Liu et al., 2008; Marasa et al., 2009; **Figure 1B**).

In addition to the control exerted by these miRNAs on cell cycle progression, it has been reported that the expression of this miRNA group is, in turn, regulated in response to cell cycle changes (Rissland et al., 2011). Specifically, miR-15a, -15b, -16, -424, and -503 are dynamically upregulated during serum starvation and contact inhibition, with miR-503 showing the highest fold change, thus reinforcing the cell

cycle arrest through the targeting of cell cycle-promoting genes, such as cyclins and CDKs. Conversely, as cells are released from G0 arrest, levels of some miR-16 family members rapidly decrease. Interestingly, while miRNAs are generally considered quite stable molecules, authors here demonstrated the intrinsic instability of some members of the miR-15/107 group, with miR-503 showing the highest instability. Specifically, the seed region and 3′ end of miR-503 were coordinately required for its instability. Identification of the nucleases responsible for miR-503 degradation remains an important question. The possible contribution of nucleases involved in cell cycle control, as for example the members of MCPIP family of endonucleases (Miekus et al., 2019), to the downregulation of miR-15/107 group during serum starvation is certainly a fascinating field that merits to be explored.

According to the tumor suppressor activity that might be exerted by these miRNAs, various studies highlighted that miR-107 expression may be induced by p53. Initially, miR-107 has been identified as a transcriptional target of p53 by Yamakuchi et al. (2010), who showed in colon cancer cells that p53, by inducing miR-107 expression, negatively impacts on angiogenesis, thanks to the ability of miR-107 to target HIF-1beta (also known as ARNT). Subsequently, miR-107 has been found upregulated by p53 also in glioma cells, where this miRNA is responsible for the inhibition of proliferation through the targeting of CDK6 and Notch-2 (Chen et al., 2013). Moreover, it has been shown that lethal doses of stress induce p53-dependent upregulation of both miR-103 and miR-107, which, in turn, inhibit LRP1 translation thus promoting cancer cell death (Leslie et al., 2018). Interestingly, a very recent report shows that, in the liver, p53 is responsible for the transcriptional induction of miR-107 and of its host gene *PANK1*, which contribute, respectively, to high−fat diet-induced insulin resistance and metabolic reprogramming (Yang et al., 2020).

Contrary to the positive transcriptional regulation of miR-107, a growing number of reports are currently showing that miR-107 activity is tightly controlled by sponging molecules, such as, for example, circular RNAs, that inhibit miR-107 tumor suppressor activity in cancer cells. Specifically, two circRNAs, namely, cTFRC and circTCF25, were reported to sequester miR-107 in bladder cancer cells, thus favoring cell cycle progression (Zhong et al., 2016; Su et al., 2019), while in gastric cancer, circHIPK3 was shown to sponge miR-107, enabling the release of brain-derived neurotrophic factor (BDNF) expression (Wei et al., 2020).

miR-15/107 Expression and Function Are Altered in Breast Cancer

The miR-15/107 group members have been also reported to be modulated and functionally relevant in breast cancer (BC). Various studies and a meta-analysis recently reported miR-195 and miR-497 as consistently downregulated in BC tissues compared to normal tissues in almost all subtypes of BC (Li et al., 2011; Ouyang et al., 2014; Tahiri et al., 2014; Tashkandi et al., 2015; Cecene et al., 2016; Thakur et al., 2016; Adhami et al., 2018). The methylation state of CpG islands upstream of the miR-195/497 gene was found to be responsible for the downregulation of both miRNAs (Li et al., 2011). However, levels and activity of miR-195 were recently shown to be also dependent on a sponging circular RNA, circAGFG1, in triple-negative breast cancer; of note, circAGFG1 is able to cause Cyclin E1 (CCNE1) upregulation as a consequence of miR-195 sequestering (Yang et al., 2019). miR-503 has been also reported as downregulated in breast cancer and to confer sensibility to chemotherapy treatment (Gong et al., 2014; Long et al., 2015).

From a functional point of view, it has been reported that forced expression of miR-195 or miR-497 suppressed breast cancer cell proliferation and invasion. Raf-1 and CCND1 were identified as direct targets of miR-195 and miR-497 in BC cells (Li et al., 2011; Yang et al., 2013). Importantly, in the same study, miR-195/497 expression levels in clinical specimens were inversely correlated with malignancy of breast cancer (Li et al., 2011). Additional studies reported the ability of miR-195 and miR-497 to downregulate CCNE1 and CCND1 in BC cells (Hannafon et al., 2011; Luo et al., 2013, 2014). Interestingly, miR-497 has been also reported to target estrogen-related receptor alpha (ERRα), a nuclear receptor overexpressed in ERα negative breast cancer; downregulation of miR-497 is then responsible for ERRα induction and increased proliferation of triple-negative breast cancer cells (Han et al., 2016). Enforced miR-497 expression is also able to cause reduction of SMAD7, suppressing MDA-MB-231 and MCF-7 breast cancer cell growth (Liu et al., 2016). According to what was reported by Li et al. (2011); Liu et al. (2016) showed that high expression of miR-497 confers a better prognosis, indicated by the Kaplan–Meier test, especially in HER2 overexpressing and triple-negative breast cancer (TNBC).

With regard to additional functions, besides cell cycle control exerted in BC by miR-497, this miRNA has been shown to target HIF1A and SLUG, leading to the negative regulation of, respectively, angiogenesis and epithelial-to-mesenchymal transition (Wu et al., 2016a,b). miR-497 downregulation in BC was also shown to release BCL2 and BCL-W expression, thus inhibiting apoptosis (Shen et al., 2012; Wei et al., 2015). Expression of BCL2 in BC is also controlled by miR-195, whose overexpression enhances the sensibility of BC cells to both chemo- and radio-therapy (Singh and Saini, 2012; Zhu et al., 2015). miR-195 as well as miR-15a/miR-16 also impinges on the expression of fatty acid synthase (FASN), leading to the impairment in fatty acid synthesis pathway (Singh et al., 2015; Wang et al., 2016). Involvement of other members of the family, namely, miR-107 and miR-103, in the control of lipid metabolism had been previously shown by Wilfred et al. (2007), while experimental evidence demonstrating the ability of miR-107 to target FASN was also provided in normal and transformed hepatocytes (Bhatia et al., 2014).

Unexpectedly, and contrarily to miR-497, miR-195, and miR-503 that have been found consistently downregulated in BC, an increasing number of conflicting results is emerging with regard to the expression and function of other members of this group, for example, miR-103 and miR-107. These miRNAs, indeed, have been shown to exert oncogenic functions in the context of BC. Specifically, Martello et al. (2010) reported that miR-103 and miR-107 inhibit the expression of a key component

of the miRNA processing machinery, Dicer, finally favoring the metastatic capacity of BC cells; this effect is obtained also as a result of the miR-200 family dowregulation caused by decreased Dicer activity. Coherently with this molecular network, authors show that high levels of miR-103/107 are associated with metastasis and poor outcome in BC. In agreement with the oncogenic model proposed by Martello et al., miR-103 and miR-107 were also shown to favor genomic instability through their ability to downregulate key players of DNA repair, as BRCA1 and RAD51 (Huang et al., 2013; Quann et al., 2015). An oncogenic network has been also evidenced, whereby miR-107 promotes tumor progression by targeting the tumor suppressor miRNA let-7 (Chen et al., 2011).

Despite these results, there is also literature highlighting the tumor suppressive function of miR-107 in BC. An interesting study from Polytarchou et al. (2012), for example, identifies miR-103/107 and miR-15/16 as microRNAs that are downregulated in cancer stem cells (CSCs) and that inhibit the growth of CSCs. These miRNAs were identified among other miRNAs previously reported as tumor suppressors.Authors show that miRNAs downregulated in CSCs affect common target genes that encode the Bmi1 and Suz12 components of the polycomb repressor complexes (PRCs) as well as the DNA-binding transcription factors Zeb1, Zeb2, and Klf4. Of note, they also show that an inverse relationship is present between the levels of CSC-regulating miRNAs and their respective targets in samples from triple-negative breast cancer patients, providing evidence for the relevance of these interactions in human cancer.

We found evidence in a recent study that the downregulation of miR-107 and of other members of the miR-15/107 group, namely, miR-15b and miR-195, in tumor-associated macrophages (TAMs), participate in the reprogramming of these cells in a pro-angiogenic sense. Specifically, miR-107, miR-15b, and miR-195 are decreased after culturing of macrophages with conditioned media from BC cells. This downregulation depends on the presence of ID4 protein in breast cancer cells as well as of VEGF in the conditioned medium. Functionally, the downregulation of these miRNAs enables released expression of proangiogenic factors, being Granulin (GRN) the most markedly affected (Donzelli et al., 2018). GRN had been previously demonstrated to be targeted by the miR-15/107 group in human cancer (Wang et al., 2010a,b). This soluble factor is attracting increasing interest due to its involvement in the regulation of tumor stroma function. GRN is associated with poor prognosis in BC (Elkabets et al., 2011; Yeh et al., 2015), and specifically in triple-negative breast cancer, a population of bone marrow cells secretes GRN to support stromal activation and robust tumor growth in young mice (Marsh et al., 2016). Interestingly, GRN has been recently reported as a key player also in pancreatic cancer metastasis, where macrophage-derived GRN induces liver fibrosis and contributes to cytotoxic CD8+ T-cell exclusion in metastatic livers (Nielsen et al., 2016; Quaranta et al., 2018).

The above described results suggest that members of miR-15/107 that exert an oncogenic function in BC cells, such as miR-107, might, on the contrary, act as tumor suppressors when expressed in cells of the tumor stroma, such as macrophages, or in the cancer stem cell compartment, highlighting the importance of the cell context for miRNA-associated functions.

miR-15/107 as Biomarkers for Breast Cancer in Liquid Biopsy

Using circulating miRNAs as biomarkers has gained tremendous research interests. They are usually incorporated into exosomes or extracellular vesicles (EVs), secreted by cells, transferred to body fluids, and delivered into recipient cells, profoundly impacting the expression profile of these last. While extremely relevant for the understanding of cancer biology, these circulating miRNAs constitute, at the same time, a new category of potential non-invasive disease markers. With regard to miR-15/107 group, a growing body of evidence shows the potential of miR-195 as non-invasive biomarker for diagnosis and prognosis definition in breast cancer (**Figure 1C**). miR-195 has been found upregulated in the majority of studies analyzing liquid biopsies from BC patients. A meta-analysis by Liu et al. (2018), which analyzed six studies with a total of 464 patients and 287 healthy controls highlighted that miR-195 is suitable as a potential biomarker for early diagnosis of breast cancer with high sensitivity and specificity. A summary of the studies reported in literature is included in **Table 1**.

As already mentioned in the previous section, miR-195 expression is downregulated in BC tissues, compared to

TABLE 1 | Studies reporting the analysis of circulating miR-15/107 group members in breast cancer patients.

miRNA	Disease	Sample	Expression change (notes)	References
miR-195-5p	BC	Serum	up (vs. healthy donors)	Peña-Cano et al., 2019
miR-195-5p and miR-15a-5p	TNBC	Plasma	up (vs. healthy donors)	Qattan et al., 2017
miR-195-5p	BC	Serum	up (vs. healthy donors)	Fan et al., 2018
miR-195-5p	BC	Whole blood	up (vs. healthy donors and other cancers)	Heneghan et al., 2010
miR-195-5p	BC	Whole blood	up (high in postoperative patients with relapse)	Igglezou et al., 2014
miR-195-5p	BC	Plasma	down (vs. healthy donors)	Nadeem et al., 2017
miR-195-5p	BC	Serum	down (vs. healthy donors) up (after neoadj. chemo)	Zhao et al., 2014
miR-195-5p	TNBC	Serum	down (vs. triple positive BC)	Thakur et al., 2016
miR-103 and miR-107	TNBC	Serum	up (relapse vs. no relapse)	Kleivi Sahlberg et al., 2015
miR-424	BC	Serum	up (vs. healthy donors)	Zhang et al., 2015
miR-15a and miR-107	ER + BC	Serum	up (vs. healthy donors)	Kodahl et al., 2014

their normal tissue counterparts. On this basis, Qattan and colleagues hypothesized that cancerous cells may selectively export tumor suppressor miR-195 in order to maintain their oncogenic features (Qattan et al., 2017). This is an extremely interesting possibility that merits further investigation to be definitely proven. However, despite this, a couple of studies identified decreased miR-195 serum levels in BC patients compared to healthy controls (Zhao et al., 2014; Nadeem et al., 2017).

CONCLUSION

microRNAs belonging to the miR-15/107 group are characterized by the presence of the "AGCAGC" hexamer in the "seed" region starting at either the first nucleotide or the second nucleotide from the 5′ end of the mature miRNA. This is an important determinant for target recognition and enables these miRNAs to recognize a huge number of common targets, mainly involved in the promotion of cell cycle progression, negatively regulating proliferation. Despite this, different members of this group may exert opposite functions in cancer cells, behaving as tumor suppressors or oncogenes, suggesting that the transformed context dictates their ability to recognize different panels of target mRNAs. The strong functional impact of the members of the miR-15/107 group on the proliferative potential of cancer cells makes them ideal candidates for the development of novel miRNA-based replacement strategies for the treatment of cancer. In addition, their presence in liquid biopsy samples from cancer patients prompts further investigations aimed at evaluating their usefulness for the monitoring of disease progression during follow-up.

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All authors have contributed to the manuscript writing.

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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.

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