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REVIEW

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Evidence for Functional Roles of MicroRNAs in Lineage Specification During Mouse and Human Preimplantation Development

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Proper formation of the blastocyst, including the specification of the first embryonic cellular lineages, is required to ensure healthy embryo development and can significantly impact the success of assisted reproductive technologies (ARTs). However, the regulatory role of microRNAs in early development, particularly in the context of preimplantation lineage specification, remains largely unknown. Taking a cross-species approach, this review aims to summarize the expression dynamics and functional significance of microRNAs in the differentiation and maintenance of lineage identity in both the mouse and the human. Findings are consolidated from studies conducted using *in vitro* embryonic stem cell models representing the epiblast, trophectoderm, and primitive endoderm lineages (modeled by naïve embryonic stem cells, trophoblast stem cells, and extraembryonic endoderm stem cells, respectively) to provide insight on what may be occurring in the embryo. Additionally, studies directly conducted in both mouse and human embryos are discussed, emphasizing similarities to the stem cell models and the gaps in our understanding, which will hopefully lead to further investigation of these areas. By unraveling the intricate mechanisms by which microRNAs regulate the specification and maintenance of cellular lineages in the blastocyst, we can leverage this knowledge to further optimize stem cell-based models such as the blastoids, enhance embryo competence, and develop methods of non-invasive embryo selection, which can potentially increase the success rates of assisted reproductive technologies and improve the experiences of those receiving fertility treatments.

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Abbreviations: ARTs, Assisted Reproductive Technologies; ICM, Inner cell mass; TE, Trophectoderm; EPI, Epiblast; PrE, Primitive endoderm; IVF, *In vitro* fertilization; sncRNAs, Small non-coding RNAs; miRNAs, MicroRNAs; miRISC, RNA induced silencing complex; mRNA, Messenger RNA; RBP, RNA-binding protein; AGO, Argonaute; ESC, Embryonic stem cell; mESC, Mouse embryonic stem cell; hESC, Human embryonic stem cell; ESCC, Embryonic stem cell cycle; TGFβ, Transforming growth factor beta; BMP, Bone morphogenic protein; KO, Knockout; qPCR, Quantitative polymerase chain reaction; TSC, Trophoblast stem cell; XEN, extraembryonic endoderm; FGF, Fibroblast growth factor; ERK, Extracellular signal-regulated kinase; MAPK, Mitogen-activated protein kinase; ISH, In situ hybridization; PGC, Primordial germ cell.

Keywords: microRNAs, blastocyst, preimplantation development, lineage specification, trophectoderm, inner cell mass, epiblast, primitive endoderm, differentiation, stem cells

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INTRODUCTION

Given the increasing rates of infertility and the global use of assisted reproductive technologies (ARTs), it is essential that we understand the molecular mechanisms underlying early mammalian development. *In vivo*, preimplantation development begins when the sperm fertilizes the oocyte, forming the zygote. During the first few days, the embryo migrates towards the uterus while simultaneously, embryonic cells are dividing and undergoing molecular and morphological changes that culminate in the formation of the blastocyst. At the blastocyst stage, the embryo has completed a process known as lineage specification and consists for the first time of two distinct and spatially segregated cell types; the inner cell mass (ICM) and the trophectoderm (TE; prospective placenta). Subsequently, the pluripotent ICM further specifies into the epiblast (EPI; prospective embryo proper) and the primitive endoderm (PrE; prospective yolk sac and extraembryonic tissue). The blastocyst, with these specified cell types, then implants into the uterine wall to establish a viable pregnancy, making these early events critical for overall development.

Decades of research has provided a great deal of insight into the mechanisms underlying embryonic lineage specification, particularly the spatial/physical cues, cell signaling pathways, and gene networks involved, which have been reviewed extensively [1-3]. However, the role that small non-coding RNAs (sncRNAs), such as microRNAs (miRNAs), may play in the specification of the TE and ICM (and subsequently the EPI and PrE) has not been extensively studied. miRNAs are one of the most widely studied sncRNAs, and they have been functionally implicated in many cellular processes, diseases such as cancer, and development [4-8]. miRNAs are ~18-24 nucleotides in length, and regulate gene expression post-transcriptionally by guiding the RNA induced silencing complex (miRISC) to target messenger RNA (mRNA) through antisense base pairing, leading to inhibition of translation or transcript degradation [9]. In addition, compelling evidence indicates that some miRNAs possess the ability to upregulate the expression of particular transcripts in specific cell types and conditions [10], adding an additional layer of complexity to the post-transcriptional gene regulatory landscape governed by miRNAs.

In general, miRNAs are created either by a canonical (dominant, Figure 1) or non-canonical pathway(s). Canonically, miRNAs are processed from precursor molecules called pri-miRNAs, which are transcribed by RNA polymerase II either from independent genes or introns [5,10,11]. Pri-miRNAs fold into hairpin structures, which serve as substrates for the RNAse III family enzyme Drosha, that cleaves and frees the pri-miRNA [5,10,11]. Drosha works in a complex with Dgcr8, an RNA-binding protein (RBP) that recognizes certain motifs within the pri-miRNA [5,10,11], as well as with other RBPs like DDX17 and DDX5 [12-16], which regulate miRNA biogenesis in part via interactions with cell signaling pathways such as Hippo and transforming growth factor beta (TGFβ) [17,18]. The product of Drosha cleavage, an ~70 nucleotide pre-miRNA, is exported to the cytoplasm by the exportin 5/RanGTP complex where another RNAse III family enzyme, Dicer, removes the terminal loop forming a mature miRNA duplex [5,10,11]. Each strand of this duplex can be a mature miRNA, which are loaded onto the Argonaute (AGO) family of proteins and together form the miRISC mentioned above [5,10,11].

Here, we provide a comprehensive review of the current evidence from embryonic stem cell (ESC) studies that implicate miRNAs in maintenance of pluripotency (modelling ICM/EPI) as well as in differentiation of the TE and PrE (Figure 2). We discuss the few studies which have been performed directly in both mouse and human embryos, highlighting consistencies with the stem cell studies and gaps in our understanding regarding the role(s) of miRNAs during early mammalian preimplantation development, particularly in the context of lineage specification. It is critical that both in this review and going forward a species-specific approach is taken to better translate findings, as molecular differences between not only mouse and human ESCs but also mouse and human preimplantation embryos are being increasingly identified [1,19-21]. Addressing the knowledge gaps identified in this review will deepen our overall understanding of the global gene regulatory landscape in the mammalian preimplantation embryo.

miRNAs IN ESC PLURIPOTENCY

Mouse Model

Mouse embryonic stem cells (mESCs) can be classified into two distinct states, naïve and primed. While both are derived from the EPI of the embryo, naïve stem cells are generally considered to share properties with the EPI of the mouse preimplantation blastocyst, while primed resemble EPI cells of a more advanced, post-implantation stage embryo [22]. Naïve pluripotency has also been defined by the unrestricted developmental potential to give rise to all somatic lineages and the germline [23]. Culture conditions for the generation and maintenance of these stem cells states differ, reflecting the utilization of different molecular mechanisms to maintain pluripotency and self-renewal [22,24,25]. In line with this, deep sequencing of miRNA profiles has uncovered significant differences between naïve and primed mESCs, with approximately one third of miRNAs exhibiting differential expression between the two cell types [25]. Among these

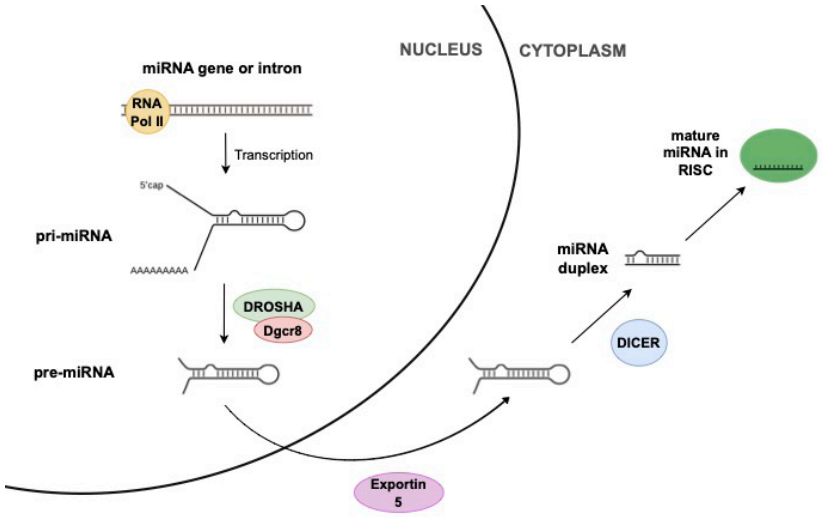

Figure 1. **Simplified overview of the canonical pathway of miRNA biogenesis**. miRNA biogenesis begins with RNA polymerase II transcription in the nucleus, which yields a large capped and polyadenylated transcript called a pri-miRNA. Drosha, along with its cofactor Dgcr8, processes the pri-miRNA into a smaller stem-looped structure called a pre-miRNA. Exportin 5 then transports the pre-miRNA to the cytoplasm, where it undergoes further processing by Dicer into a miRNA duplex. One strand of this duplex becomes the mature miRNA, associating with AGO proteins to form the miRNA-induced silencing complex (miRISC). Mature miRNAs primarily target mRNA 3'-UTRs through Watson-Crick base-pairing, leading most often to post-transcriptional gene silencing.

are pluripotency-associated miRNAs, with naïve mESCs mainly characterized by heightened expression of the miR-290-295 (miR-290, -291a, -291b, -292, -293, -294, -295 [26]) and miR-17-92 clusters (miR-17, -18a, -19a, -20a, -19b-1 -92a-1 [27]), while primed mESCs predominantly express the miR-302/367 cluster (miR-302a, -302b, -302c, -302d, -367) [25]. These clusters are widely recognized as regulators of the embryonic stem cell cycle (ESCC), and share a highly conserved seed sequence, "AAGUGC" [28,29]. Their gene targets and functions in the context of cell cycle regulation of mESCs have been studied and reviewed extensively [28-30].

ESCCs impede differentiation in mESCs when induced by differentiation-promoting miRNAs (discussed in subsequent sections) [31-35], which may be occurring in the ICM/EPI of the blastocyst. Recent findings indicate that other miRNA families share this capability as well, such as the miR-183-182 cluster [35], which also exhibit higher expression in naïve compared to primed mESCs [36]. However, the exact mechanism(s) by which pluripotency-associated miRNAs shape the global gene regulatory network of naïve mESCs is complex and not entirely understood. We speculate that it is likely a combined result of synergistic effects (including those which have been identified in the cell cycle), redundancies, and pathway crosstalk. For example, miR-294, miR-295, and miR-302a (members of the miR-290-295 and miR-302/367 clusters) have been shown to repress multiple genes involved in the epithelial-mesenchymal transition (EMT) such as receptors of the TGFβ pathway and GSK3-beta, as well as regulate the apoptotic pathway, which synergistically contribute to the formation of a barrier preventing the silencing of ESC self-renewal [33]. The miR-290-295 cluster also contributes to the naïve state by regulating DNA methylation of pluripotency factors [37], alternative splicing [38] and bivalent histone modifications [39]. Interestingly, a dichotomy exists whereby the miR-290-295 and miR-302/367 families not only maintain pluripotency, but also allow for exit of pluripotency by promoting the activity of MEK pathway via repression of Akt1 [36]. This functional dichotomy is supported by the finding that when miRNAs are not present, mESCs are unable to silence pluripotency programs when prompted to differentiate [40]. Recently, specific miRNAs have been implicated in the efficient formation of mESC cultures from mouse ICMs, for example, miRNAs from the *Dlk1-Dio3* locus (miR-521-5p, miR-410-3p, and miR-381-3p) in addition to miR-183-5p and miR-302b-3p promote, while miR-212-5p and let7d-3p inhibit ESC formation [41].

While the miRNAs which are expressed in naïve stem cells provide some insight as to their function in the ICM of the mouse blastocyst, they do not reveal much about how they may contribute to lineage specification, as these cells model an already established ICM. Further, this model does not include the other cell type (the TE), eliminating cell-cell communication between the TE and ICM which may contribute to lineage specification. Only

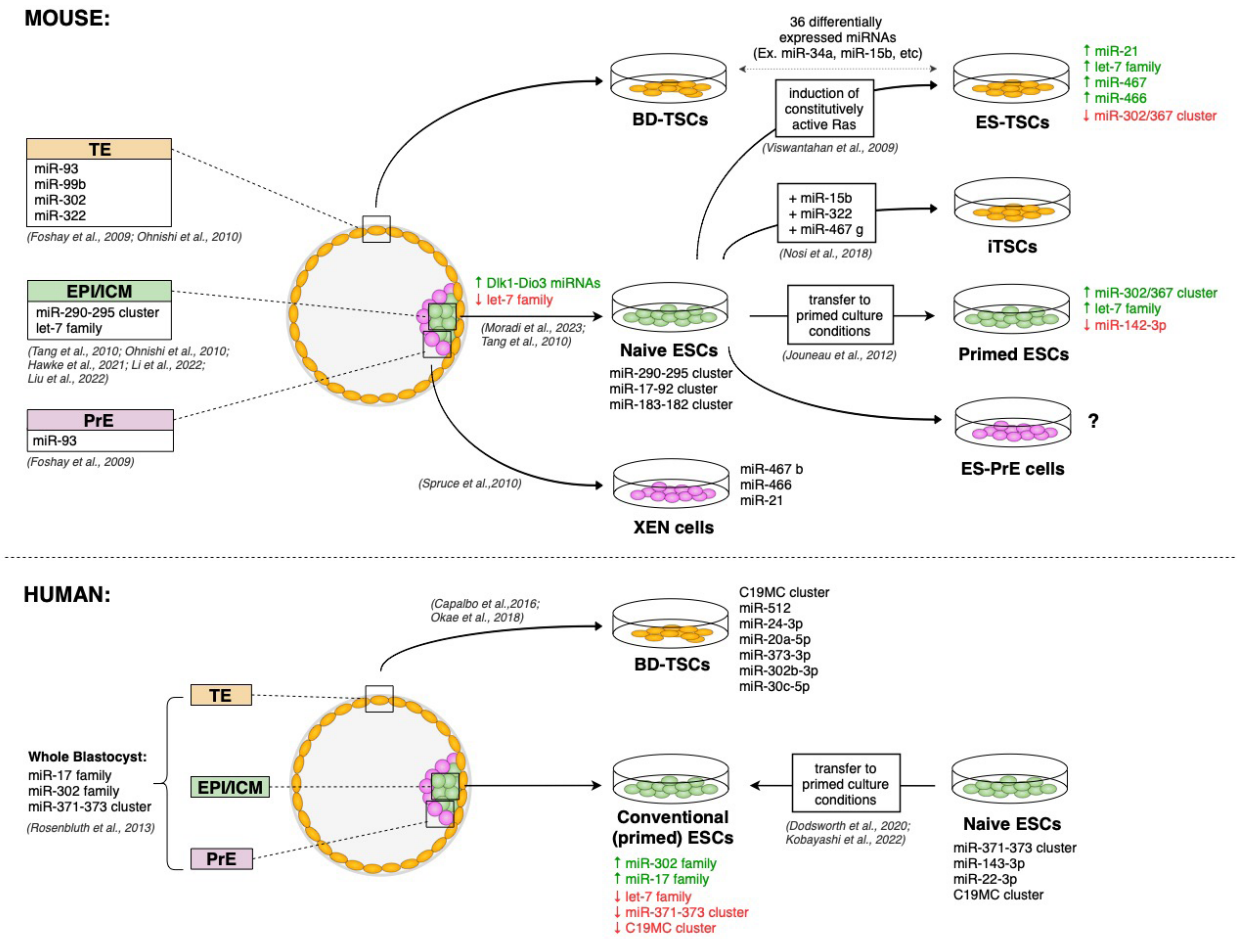

Figure 2. **miRNA expression in the mouse and human blastocyst and stem cells**. TOP: Overview of miRNAs discussed in this review which have been found to be robustly expressed in the mouse blastocyst, in the derivation of mouse *in vitro* stem cell lines, or which undergo dynamic changes during transitions in lineage state. BOTTOM: Overview of miRNAs discussed in this review which have been found to be robustly expressed in the human blastocyst, in the derivation of human *in vitro* stem cell lines, or which undergo dynamic changes during transitions in lineage state. Note, this is a summary of the studies discussed and does not necessarily indicate miRNA markers of lineage, which will require further research to elucidate. ESCs = embryonic stem cells, XEN = extra embryonic endoderm, ES-TSCs = trophoblast stem cells differentiated from stem cells, BD-TSCs = trophoblast stem cells derived from the blastocyst, iTSCs = miRNA-induced trophoblast stem cells, ES-PrE = primitive endoderm resembling cells differentiated from stem cells.

one study has demonstrated a functional role of miRNAs (miR-34a) in the early stages of this lineage specification using stem cells [42]. When miR-34a is absent, mESCs injected into recipient morula can yield progenies contributing to both the ICM and TE of blastocysts [42]. This is due to an increase in the expression of *Gata2* (a target of miR-34a), that results in induction of MERVL (a specific and highly expressed marker of totipotent cells of the 2 cell embryo [43]), which enables cells to acquire bipotential cell fate [42]. It can thus be postulated that in the embryo, miR-34a downregulation would be necessary for previously totipotent blastomeres to acquire a bipotential cell fate and specify into the ICM and TE, although this has not yet been shown. miRNAs may also contribute to stem cell fate decisions through regulation of signaling pathways. For example, miR-191 and miR-16-1 are highly expressed in mESCs and repress *Smad2*, an essential mediator of Activin-Nodal signaling, which results in the inhibition of mesendoderm differentiation [44].

The identification of differentially expressed and functionally significant miRNAs in mESCs raises the question of how the mechanisms of miRNA biogenesis undergo alterations that give rise to distinct miRNAs in these cells. As discussed above, there are different levels of miRNA biogenesis which are susceptible to regulation. It has been shown that during the transition of

naïve cells to primed cells, which would recapitulate the peri-implantation period of mouse embryonic development (E4.5-E5.5, when the blastocyst enters the uterus in order to implant) there is a specific phase of "poised" pluripotency characterized by a distinct transcriptome and miRNAs [45]. This poised state is dependent on an alternative mechanism of miRNA biogenesis, whereby an RBP, ISY1, recruits the endonuclease CPSF3 to mediate a pri-miRNA processing into a large intermediate termed a progenitor miRNA (pro-miRNA) [45,46]. This pro-miRNA then serves as a favored substrate for Drosha to generate pre-miRNAs, and this mechanism is necessary for the expression of a large subset of miRNAs in ESCs, including the miR-17-92 cluster [45,46]. While the majority of our knowledge pertaining to miRNAs comes from the study of those produced by the canonical pathway, it is important to note that non-canonical miRNAs may also impact gene expression and they are independent of the Drosha complex and Dicer and therefore not considered when using *Dgcr8* or *Dicer* knockout (KO) ESCs [47,48]. Indeed, two non-canonical miRNAs (miR-320 and miR-702) have been found to contribute to pluripotency in Dgcr8-deficient mESCs by targeting cell cycle inhibitors, similar to the ERCCs discussed above [49].

Human

Differences exist between mESCs and human embryonic stem cells (hESCs) [19,50], in addition to disparities in the expression of pluripotency-associated factors between the human and mouse ICM [21,51]. Moreover, accumulating evidence suggests that although miRNAs typically exhibit substantial sequence conservation across species, there are instances where their expression patterns differ or predicted mRNA binding sites are not conserved [52,53]. As such, it is important to ensure findings in mESCs also apply to hESCs before generalizing results.

In conventional hESCs, little to no expression of the miR-371-373 cluster (homolog of the mouse miR-290-295 cluster) has been found, which has been viewed as a key difference between the two species [54,55]. However, conventional hESCs display a different transcriptome and methylome than that of the ICM from which they are derived [56,57] but do share features with cells derived from the post-implantation mouse EPI, suggesting that they represent a later stage of development [22]. This led to efforts to reset hESCs to an earlier (naïve) developmental state, hallmarks of which include a primate-specific naïve network of transcription factors which are downregulated upon transition to a primed state [23,58]. In naïve hESCs, there is robust expression of the miR-371-373 cluster [59,60], in addition to other identified markers such as miR-143-3p and miR-22-3p [59], suggesting that their absence in conventional hESCs are more likely due to cellular state rather than a species difference. Differentially expressed miRNAs have been identified between the naïve and primed stem cell states [59-61], with miR-363-5p, several members of the miR-17-92 cluster (notably, more highly expressed in mouse naïve ESCs) and the miR-302/367 family being higher in human primed stem cells [59,60]. Further, a primate-specific miRNA cluster on chromosome 19 (C19MC), is highly expressed in naïve hESCs but is epigenetically silenced in primed hESCs, which could be critical for differentiation potential [62] and may play an important role in ICM-TE specification. Meta-analysis of microRNA-seq, RNA-seq, and metabolomics datasets from human and mouse have identified a large set of miRNAs (and importantly their experimentally validated target genes) that show consistent changes in the naïve to primed transitions [63]. Some of these miRNAs which were consistently upregulated in naïve mouse and human cells include those from the Dlk1-Dio3 locus (miR-541-5p, miR-410-3p, miR-381-3p, and miR-495-3p), miR-143-3p, and members of the let-7 family [63]. Interestingly, induced pluripotent stem cells (iPSCs) can be generated from differentiated human and mouse somatic cells using a combination of the same miRNAs; the miR-200c, miR-302, and miR-369 families [64].

In hESCs, limited studies have explored the functional role of miRNAs. The majority of studies have focused on the miR-302/367 cluster and have found that similar to the mouse, this cluster is involved in cell cycle regulation [65], promoting TGFβ signaling by targeting the transcripts of LEFTY1/2 [53,66], and promoting bone morphogenic protein (BMP) signaling by targeting the transcripts of negative regulators TPB2, DAZAP2, and SLAIN1 (enhancing TE fate when differentiation is initiated) [66]. In addition, the miR-302/367 cluster has been found to dually regulate hESC cell cycle and apoptosis in a dose-dependent manner [67]. Interestingly, miR-302a can be upregulated at the transcriptional level by pluripotency factors POU5F1 (otherwise referred to as OCT3/4), SOX2, and NANOG [68], and the miR-200 family is reciprocally regulated by the TGFβ/activin A/nodal-Smad pathways [69]. This could suggest that reciprocal regulation of miRNAs by transcription factors and cell signaling pathways and vice versa may be an important mechanism in establishing the overall transcriptional network necessary to maintain pluripotency and self-renewal. Indeed, a specific miRNA from the miR-200 family, miR-200c, has been implicated in hESC renewal and the differentiation of all three lineages (EPI, PrE, TE), primarily mediated by its targeting of GATA4 [69]. The conserved miRNA families identified thus far may offer valuable insights into miRNA-mediated regulation of pluripotency in the ICM/EPI of mammalian blastocysts. Further focus on the precise functional roles (for example, the gene targets)

of these conserved families, as well as the identification and study of non-conserved families, is of importance in order to provide clearer mechanistic information pertaining to the role of miRNAs in early development between the species.

miRNAs IN DIFFERENTIATION AND TROPHOBLAST STEM CELLS (TE MODEL)

Mouse Model

The role of miRNAs in cellular differentiation has been extensively investigated. Dicer is essential to exit pluripotency, and when it is absent from mESCs, they are unable to contribute to embryo development and fail to undergo differentiation when injected directly into mouse blastocysts [70,71]. In mESCs, specific miRNAs (miR-134, miR-296, miR-470, and miR-421) have been found to target the coding regions of the Nanog, Pou5f1, and Sox2 transcripts, leading to the suppression of these stemness factors and resulting in the induction of differentiation [44,72,73]. An additional set of three miRNAs (miR-34a, miR-100, and miR-137) drive the differentiation of mESCs by modulating the expression of epigenetic regulators (*Sirt1, Smarca5,* and *Jarid1b*) which are required for naïve ESCs to undergo differentiation [74]. When silencing of self-renewal must occur, c-MYC releases miR-27a and miR-24, which become free to target the transcripts of pluripotency factors Pou5f1 and Foxo1, as well as TGFβ signal transducers Smad2/3 and Smad4 [32].

The let-7 family of miRNAs are well known to be involved in cellular differentiation. In mESCs, the expression of the let-7 family is low due to the antagonistic effect of Lin28 proteins, which selectively bind to pre- and pri-let-7 and block their processing by Drosha and Dicer [75,76]. When let-7 is overexpressed, it hinders the self-renewal of *Dgcr8* KO mESCs (which lack canonical miRNAs), but this effect is not observed in wildtype or *Dgcr8* KO mESCs that possess miR-290 miRNAs [34], demonstrating that miR-290 is sufficient to counter the differentiation effects of let-7. Further investigation has revealed that the members of the let-7 family downregulate multiple genes within the pluripotency network, including many of those that are positively regulated by miR-290 miRNAs [34]. Overall, evidence from the studies to date suggest that decreased miR-290 and increased let-7 is essential for the exit of the naïve pluripotent state, a mechanism which plausibly could precede ICM-TE specification, as is discussed in subsequent sections.

MiRNAs identified in studies of differentiation have also been specifically implicated in trophoblast stem cell (TSC) differentiation. Upon induced differentiation of mESCs into TE, there are significant dynamic changes in the expression of miRNAs [77,78]. In one study, inducing TSC differentiation resulted in the significant upregulation of 25 miRNAs and significant downregulation of 18 miRNAs relative to controls [77]. The miRNA with the largest increase in expression was miR-21 [77], which is known to have roles in differentiation via targeting the transcripts of Pou5f1, Nanog, Sox2, and c-Myc [79-81]. Other miRNAs that increased in expression include members of the let-7 family, miR-467, and miR-466, while members of the miR-302/367 cluster were decreased (known to maintain hESC stemness [31,82], as discussed earlier) [77]. Although, other pluripotency-associated miRNAs may not be completely negligible in TE identity, as loss of the miR-290 cluster (which remains to be highly expressed in TSCs) in mouse TSCs leads to further differentiation into downstream derivatives [83]. In contrast, gain of the miR-322 cluster in TSCs represses cell cycle activators (Cyclin D1, Cyclin E1, Cdc25b) and Cdx2 (a trophoblast stemness factor) to induce differentiation [83]. In another study, when TSC-enriched miRNAs (miR-15b, miR-322, and miR-467g) were transfected into mESCs, the result was a stable trophoblast phenotype, supported by gene expression changes similar to the preimplantation embryo TE [78]. Remarkably, when these cells were transfected into mouse blastocysts, they were able to incorporate into the TE and further differentiated into downstream cell progenies of the mural TE [78]. Together these data suggest that regulation by only a few miRNAs may be sufficient to drastically change the cell fate trajectory into TE.

Human

Human TSCs have only recently been successfully derived [84], and as such, the roles of miRNAs in their differentiation remain to be thoroughly investigated [85]. While miRNA profiling of derived and primary human TSCs has been performed [84], detailed analysis pertaining to the expression of specific miRNAs in ESCs vs TSCs was not mentioned, and cross-species analysis has not been performed. However, the expression of primate-specific miRNA cluster on chromosome 19 (C19MC) was highlighted and is known to be highly expressed in human trophoblast cells [62,84,86]. Moreover, C19MC has been found to be essential for human TSC maintenance, and forced expression of C19MC in primed hESCs (which typically do not express the miRNA cluster) allows them to give rise to TSCs [62]. Further, C19MC is considered to be a marker necessary for verifying TSC identity [87]. As this is a primate-specific cluster, C19MC likely represents a species-specific mechanism in TE differentiation between the human and mouse. Whether miRNAs such as the let-7 family, which are implicated in mTSCs are also functionally important in hTSCs remains to be determined.

miRNAs IN XEN CELLS (PrE MODEL)

Mouse extraembryonic endoderm stem (XEN) cells, derived from the blastocyst, are a useful model of PrE cells. With their *in vivo* counterparts, XEN cells share the expression of lineage-specific transcription factors (eg, *Gata4* and *Gata6*), differentiation potential and the requirement of exogenous signaling pathway components (fibroblast growth factor (FGF) signaling) to maintain cell identity [88,89]. qPCR measurement of 312 miRNAs in mouse XEN cells showed that the profile of miRNA expression was similar to TSCs, with the top 22 expressed miRNA families in XEN cells (94% of the total quantity of miRNAs expressed) also being highly expressed in TSCs [90]. Some of these common highly expressed miRNAs include miR-467b and miR-466a,b,c,e-3p [90], which were among those identified as highly expressed in the TSC study mentioned previously [77]. Interestingly, miR-34a, discussed above for its role in mESC differentiation and inhibition of bipotential cell fate, is highly expressed in TSCs and not XEN cells or ESCs [90], suggesting it may also be important for TE identity. When Dicer is deleted from XEN cells, proliferation is blocked resulting in an up-regulation of downstream lineage markers, such as those of the extraembryonic visceral endoderm (*Gata4, Ttrm, Alk2,* and *Bmp2*) and parietal endoderm (*Pdgfrα* and *Follistatin*) [90]. The prevention of differentiation by miRNAs in XEN cells is achieved, at least in part, by maintaining extracellular signal-regulated kinase (ERK)1/2 phosphorylation, through blocking the expression of mitogen-activated protein kinase (MAPK) inhibitors (*Sulf2, Rasa2* and *Dusp1*) [90]. The miRNA dynamics during the transition from naïve mESCs to PrE, similar to studies described above examining the ESC to TSC transition, have not yet been performed. However, it is possible that miRNAs are involved in this differentiation process as Ago2 is required for Gata6 expression during mESC conversion to extra embryonic endoderm [91], which is a critical transcription factor for PrE identity.

miRNAs IN LINEAGE SPECIFICATION OF THE PREIMPLANTATION EMBRYO

While stem cell studies have provided valuable insight into the potential importance of specific miRNAs in pluripotency and differentiation, there are many genetic and epigenetic changes that manifest during the transition from the embryo to stem cell culture [20,92,93]. Among these changes is a differential expression of miRNAs, for example, a large number of miRNAs are differentially expressed upon removal of the ICM from the embryo to form ESC outgrowths, including downregulation of differentiation associated miRNAs such as the let-7 family [41,92]. In addition, differentially expressed miRNAs have been identified between blastocyst-derived TSCs and TSCs differentiated from ESCs [77]. This highlights the need for embryo-specific studies regarding miRNAs and their function to confirm that stem cell findings hold true in the highly complex and interconnected embryo. To date, very few studies have directly investigated the functional roles of miRNAs in mouse and human preimplantation embryos and even fewer in the context of lineage specification. This is largely related to limited access of human embryos and the difficulty of interpreting results from KO models or activation/inhibition of different miRNAs, as many exhibit high sequence homology and likely have multiple overlapping or redundant targets/functions [94].

Mouse Model

In the mouse preimplantation embryo, canonically-produced miRNAs have been thought to have a minor role in lineage specification, likely due in part to the decreased mRNA expression of genes encoding proteins involved in miRNA biogenesis and function from the zygote to blastocyst stage [95,96]. In addition, zygotic *Dgcr8* KO embryos are able to reach the blastocyst stage at the same rates as controls with similar morphologies, number of cells per embryo, and distributions of cells between the ICM and TE [97]. The same results were observed in maternal-zygotic KO, eliminating the possibility that lingering maternally-loaded transcripts from the oocyte rescued any effects [97]. However, the loss of zygotic *Dgcr8* does lead to embryonic arrest prior to embryonic day (E)6.5 [40], suggesting that the molecular circuitries of the lineages and embryo competence may still be altered. Silencing of *Dicer1* by RNA interference at the zygote stage did not inhibit development to the blastocyst stage, but there was decreased expression of *Pou5f1*, *Sox2*, and *Nanog* in these embryos [96], suggesting that mature miRNAs may be involved in proper establishment of the pluripotency network. Similarly, *Dicer1* zygotic KO post-implantation embryos possess defects in TE derivatives, with decreased expression of trophoblast stem cell markers *Eomes, Cdx2,* and *Esrrb* at E6.5 [90]. However, this study was unable to determine if miRNAs have a role in the first lineage specification of the blastocyst due to persisting maternal Dicer and/or processed miRNAs at the blastocyst stage [90]. Despite the decreased expression of the miRNA biogenesis machinery, the overall quantity of mature miRNAs themselves steadily increases during preimplantation development, with dynamic changes at each of the developmental stages [95,98] suggesting they may contribute to the different processes throughout mouse development. In addition, differentially expressed miRNAs have been identified between mouse ICM and TE using bulk small

RNA sequencing data [99], suggesting their regulation of gene expression may contribute to acquisition of lineage identity.

Some of the most well-studied miRNAs are those of the miR-17 family, which consists of the miR-17-92 cluster as well as two mammalian paralog clusters, miR-106a-363 and miR-106b-25 [27,100,101]. In particular, the miR-17-92 cluster has been implicated numerous times in different aspects of development and disease and, as discussed above, it is highly expressed in naïve mESCs. The expression of this cluster is variable throughout the 2-8 cell stages of mouse development, but there is a modest increase between the morula and blastocyst stage [77]. In E4.0 mouse blastocysts, in situ hybridization (ISH) has found that miR-17-5p and miR-20a (of the miR-17-92 cluster) are slightly increased in within cells of the TE[102], while miR-106a is slightly increased in the ICM [102]. In addition, miR-93 (from the miR-106b-25 cluster) is almost entirely restricted to the TE and the future primitive endoderm, but minimally expressed in the ICM, suggesting it may have an important function in differentiation [102]. While it is expected from stem cell studies that miR-93 allows for differentiation in TE cells by targeting the transcript of *Stat3*, a known murine ES cell regulator [102], this has not been definitively shown in the embryo itself. While zygotic KO of the miR-17-92 cluster has no obvious phenotypic consequences, mutant mouse embryos lacking both this and the miR-106b-25 cluster die before E15 [100], suggesting there may be functional redundancy. Simultaneous KO of all three clusters and culture to the blastocyst stage, while technically complicated, could shed more light on the role of the miR-17 family in specification of the ICM and TE.

Another miRNA cluster of interest is the miR-290-295 cluster, as multiple members have been found to be highly expressed in isolated mouse ICMs [99], consistent with their roles identified in naïve mESCs as is described above. MiR-290 is abundantly expressed at the 2 cell stage and continues to dramatically increase to the blastocyst stage [77,92,103-105], and the precursor miRNA for this family, *pri-miR-290*, is primarily expressed in the ICM compared to the TE [105]. In addition, ISH has shown that family members miR-294 and miR-295 are localized exclusively in the ICM of mouse blastocysts [103]. Microinjection of a small interfering RNAs (siRNA) against *pri-miR-290* in mouse zygotes downregulated the expression of the core pluripotency genes *Pou5f1* and *Sox2* at the blastocyst stage, however no changes in the number of ICM cells were observed. In addition, mESC colonies derived from these *pri-miR-290* inhibited blastocysts had decreased proliferation and pluripotency, as well as upregulated differentiation-related genes (including *Gata4, Gata6, Dppa4,* and *Cdx2*) [105]. One of the mechanisms underlying the changes in *pri-miR-290* inhibited colonies is thought to be miR-294-3p targeting the P21 transcript (a CDK inhibitor which can promote cell cycle arrest), which is expressed more highly in the TE than the ICM [105]. The majority of miR-290-295 family zygotic KO mouse embryos are lost between E11.5 and E18.5, and among the ~25% of embryos that do survive postnatally, the females have a greater than 80% loss in ovary volume, no follicular structures, and a significantly reduced number of oocytes due to a defect in the migration of primordial germ cells (PGCs) [106]. While similar phenotypic consequences arising from the defective PGC migration during development are also observed in the KO male animals, they remain fertile likely due to the extended proliferative lifespan of male germ cells [106]. This suggests that miRNA regulated changes of gene expression during early embryogenesis could result in major post-natal repercussions. Interestingly, the miR-302/367 cluster, mentioned above for its role(s) in maintaining pluripotency, was not detectable at appreciable levels throughout preimplantation development [77], possibly related to the fact that they are more characteristic of a primed rather than a naïve mESC state, as mentioned in previous sections.

The let-7 family, mentioned above for its role in ESC differentiation, is implicated in cell fate determination and formation of mouse blastocysts [107]. Although, contrary to what would be expected given the importance of let-7 family in differentiation, the expression of let-7 is higher in the ICM when compared to the TE [107]. Forced expression of let-7a at the 1 and 4 cell stages inhibited blastocyst formation (most arrested at the morula stage), increased the number of Pou5f1+ (ICM) cells, and downregulated expression of *Cdx2* in the TE of mouse blastocysts [107]. This finding is consistent with the fact that let-7 targets *Tead4* [107], a transcription factor known for its role in facilitating Hippo-induced expression of TE genes (such as *Cdx2*) in outer embryonic cells [1-3]. While the action of let-7 on ICM fate seems surprising, it has been shown that the expression of let-7a, let-7e, let-7f, and let-7g is reduced between 4- and 12-fold in ESCs compared to ICM [92]. The mechanism(s) that alter the action of the let-7 family in the embryo compared to ESCs, as well as how Lin28 fits into this process in the early embryo, warrants further investigation.

Additional miRNAs outside of the major pluripotency families have also been implicated during early mouse embryogenesis. For example, miR-199a-5p is down-regulated in *in vitro* fertilization (IVF)-produced mouse blastocysts compared to those conceived *in vivo* [108]. Inhibiting miR-199a-5p in *in vivo*-produced embryos leads to the increased expression of *Hk2* (an enzyme that catalyzes glycolysis), resulting in a higher glycolytic rate [108]. Interestingly, downregulation of miR-199a-5p also

leads to a decreased ICM to TE ratio [108], suggesting it may have impacts on lineage specification/allocation, although the gene(s) this miRNA may target in order to elicit this effect have not yet been investigated. Another example is the large microRNA cluster within the mouse *Sfmbt2* gene. KO of the *Sfmbt2* miRNA cluster results in severely impaired development of the mouse placenta, with phenotypes in trophectoderm derived cells apparent as early as E8.5 [109]. While believed to target tumor suppressors and various differentiation/pattern-regulating genes [109], further experiments are necessary to identify the exact mechanism which manifest in the placental phenotype, and whether these effects are evident as early as during specification of the preimplantation TE. Interestingly, this is a rodent-specific cluster (not found in the human, bovine, or pig) [109], but points to a potentially convergent role of miRNAs in trophoblast formation. More detailed studies profiling the miRNAs of the different cell types in the blastocyst has the potential to provide more insight into the functions of these miRNAs in the context of lineage specification and divulge more miRNAs outside of the major families which may have important roles.

Human

Given that access to human embryos is extremely limited and there are greater ethical and legal legislation governing their use, minimal experiments have been conducted to determine if results pertaining to miRNAs from hESC and mouse embryo studies are conserved in the human embryo. However, it has been shown that target selection of the same miRNAs can be different from species to species [52,53], and therefore the extrapolation of results from mouse studies to the human should be performed with caution.

DROSHA and DICER1 are expressed in human embryos at the mRNA and protein levels as early as the 4 and 8 cell stage [56,110]. Similarly, mRNA expression of AGO class genes (AGO1-4) are detected throughout preimplantation development, with *AGO2* specifically showing significant up-regulation at 4 cell stage [110]. Recently, the sncRNAs of human oocytes and early embryos (zygotes, 4 cell and 8 cell embryos) have been profiled using an RNA-sequencing method, which showed that the majority (70%) of miRNAs were expressed across all these developmental stages [110]. Not surprisingly, similar to both human and mouse stem cell studies, miRNAs that were consistently abundant in these early human embryos include miR-371a-3p, miR-371a-5p, miR-372-3p, and miR-373-3p, members of the pluripotency-associated miR-371-373 cluster (homolog of the miR-290-295 mouse cluster) [110].

While limited studies have looked at miRNAs in the human TE and ICM independently, miRNAs in the human blastocyst have been profiled using bulk methods, although the source cell type (ICM, TE, or both) cannot be deduced from this method [111]. Of the 754 miRNAs assayed by Taqman low density array (TLDA), among those that were highly expressed in human blastocysts were members of the miR-17 family (miR-106a, miR-17, miR-19b, miR-20a, and miR92a), the miR-302 family (mIR-302b, miR-302c, miR-302d, miR-302a) and the miR-371-373 cluster (miR-372) [111], which as discussed above, are all associated with pluripotency in stem cells. When TEs were mechanically isolated from human blastocysts and the miRNAs were screened, miR-512-5p, miR-522-3p, miR-24-3p, miR-20a-5p, miR-373-3p, miR-302b-3p, miR-146a, miR-512-3p, and miR-30c-5p were all abundantly expressed [112]. Interestingly, 96.6% of miRNAs detected in human blastocyst spent culture media are also expressed in TE cells, suggesting TE cells are the main secretors of miRNAs by human embryos [112]. It should be noted that while not thoroughly discussed in this review, miRNAs secreted by the embryo and the maternal tract are known to be involved in the process of blastocyst implantation [113,114]. As such, it is not inconceivable that erroneous establishment of the miRNA network in the TE would have downstream impacts on implantation. Indeed, miRNAs assessed from spent culture media of euploid blastocysts which implanted versus euploid blastocysts which did not implant highlighted two miRNAs (miR-20a and miR-30c) that showed increased concentrations in the former, and were predicted to be involved in multiple implantation-related pathways [112]. Further research is necessary to validate the targets of the aforementioned miRNAs in the embryo, and whether these targets are conserved between the mouse and the human.

CONCLUSION

To fully comprehend the intricate and interconnected functions of miRNAs during mouse and human preimplantation development and lineage specification, further research is required. The stem cell studies discussed in this review have generated valuable hypotheses regarding the miRNA families associated with pluripotency, self-renewal, and differentiation which have guided some successful embryo studies. However, disparities between stem cell models and their embryo counterparts, some of which have been discussed in this review, emphasize the need for more thorough embryo-specific investigations. A fundamental distinction between *in vitro* cultures and the embryo lies in the unique environment cells of the embryo exist in, whereby various cellular lineages coexist, interact, and organize into complex structures. To this end, investigating miRNA dynamics in the recently developed mouse and human blastoid models [115],

whereby the different stem cell lines are cultured together aggregate to form a blastocyst-like structure, holds promise for elucidating the potential variations in miRNA behavior between this model and the conventional cultures.

There is also a need for more human-specific studies, and cross-species analysis to compare findings between the mouse and the human. As discrepancies in the molecular mechanisms governing lineage specification between the species are being identified [1], it is not farfetched to presume that the molecular landscape is also differentially regulated, including the regulatory role of miRNAs. Indeed, the differences in miRNA expression, some of which are discussed in this review, between mouse and human stem cell lines emphasize the importance for cross-species studies. For example, the significance of the primate-specific C19MC cluster in human TSCs suggests miRNAs may regulate TE specification differently in each species. Furthermore, it is not guaranteed that mRNA binding sites of miRNAs are always conserved across species [52,53]. A more detailed analysis of miRNA expression at the various stages of mouse and human preimplantation development is needed to provide a comprehensive understanding of the precise changes taking place within each cell type at the different developmental stages, perhaps leading to the identification of some candidate miRNAs which may be functionally important in driving lineage specification and governing embryo competence in each species.

Finally, while not discussed in this review, it should be noted that a great deal of work has been done investigating miRNAs in the bovine embryo, where miRNAs are considered essential for preimplantation development [116]. Future cross-species analysis incorporating the bovine model may provide valuable insights into the regulatory role of miRNAs. Additionally, examination of miRNAs in other species including the non-human primate, rabbit, guinea pig and rat would be of interest and may provide important evolutionarily conserved mechanism(s) for embryo formation and pluripotency.

An intriguing research direction which remains to be studied is the activity of canonical versus non-canonical miRNA biogenesis mechanisms during preimplantation development. Identifying the miRNAs which remain expressed in *Dgcr8* or *Dicer* KO embryos could assist in determining whether these have redundant functions with those whose expression is abolished in the KOs, which may help explain the lack of phenotype in KO blastocysts. In addition, the mechanisms through which miRNA biogenesis is altered leading to differentially expressed miRNAs between pluripotent or differentiating cells have yet to be elucidated.

Addressing the knowledge gaps identified in this review will enhance our understanding of the global gene regulatory landscape in the preimplantation embryo. This could ultimately pave the way for the development of non-invasive embryo selection methods such as miRNA-profiling of spent culture media, and overall enhance the efficacy of assisted reproductive technologies. In addition, it is possible that an increased understanding of miRNA regulation of gene expression during lineage specification can be leveraged to further improved stem cell models, including the aforementioned blastoid.

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