The Functions of MicroRNAs and Long Non-coding RNAs in Embryonic and Induced Pluripotent Stem Cells

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Abstract

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold immense promise for regenerative medicine due to their abilities to self-renew and to differentiate into all cell types. This unique property is controlled by a complex interplay between transcriptional factors and epigenetic regulators. Recent research indicates that the epigenetic role of non-coding RNAs (ncRNAs) is an integral component of this regulatory network. This report will summarize findings that focus on two classes of regulatory ncRNAs, microRNAs (miRNAs) and long ncRNAs (lncRNAs), in the induction, maintenance and directed differentiation of ESCs and iPSCs. Manipulating these two important types of ncRNAs would be crucial to unlock the therapeutic and research potential of pluripotent stem cells.

Introduction

Embryonic stem cells (ESCs) derived from the inner cell mass, which possess the potential for unlimited proliferation and differentiation into three germ layers, are the ideal cell source for cell therapy [1–3]. The acquisition of human ESCs (hESCs), however, requires the destruction of human embryos. Therefore, possible immunological rejection or religious and ethical concerns greatly hinder the pace of ESCs in basic and clinical applications. In 2006, the Yamanaka group obtained induced pluripotent stem cells (iPSCs) with characteristics similar to those of ESCs by overexpressing four exogenous factors (Oct4, Sox2, c-Myc and Klf4) in fibroblasts [4]. This method of deriving patient-specific iPSCs from donor somatic cells removes many of these medical, ethical and political obstacles, creates disease-specific stem cells and provides a platform to study molecular mechanisms of genetic diseases. Understanding how these regulatory processes function in iPSCs would help to accelerate the basic research and clinical applications of iPSCs [5].

ESCs and iPSCs are characterized by their self-renewal and differentiation into any cell type. Transcription factor networks and epigenetics (including DNA methylation, histone modifications and ncRNAs) undergo a tremendous change during this process [6–8]. ncRNAs can be classified into either housekeeping or regulatory ncRNAs. Housekeeping ncRNAs are most often constitutively expressed, which include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs ( snoRNAs).
Regulatory ncRNAs can be broadly classified by size as lncRNAs (> 200 bp) and small ncRNAs (< 200 bp) such as miRNAs, endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs) [9]. lncRNAs originate from intronic, exonic, intergenic, intragenic, promoter regions, 3' and 5'-untranslated regions (UTR) and enhancer sequences. lncRNAs sometimes are bidirectional transcripts [10]. lncRNAs consists of intergenic ncRNAs, intronic ncRNAs, natural antisense transcripts (NATs), pseudogene transcripts, etc. Long intergenic ncRNAs (lincRNAs) are derived from non-coding DNA sequences between protein-coding genes, whereas intronic lincRNAs are transcribed from within introns of protein-coding genes and NATs are transcribed from the opposite strand of protein-coding sense transcripts [11,12]. Pseudogene transcripts can modulate the expression of their counterpart genes through competing for endogenous RNA (ceRNA) [13]. This review will focus on miRNAs and lncRNAs.

miRNAs are hairpin-derived RNAs that are 20–24 nucleotides (nt) long. They act at the RNA level by destabilizing and repressing target RNAs via binding to the 3' UTRs, 5' UTRs and coding sequences of the transcripts [14–17]. Nonetheless, miRNAs can also enhance mRNA translation by binding to the 5' UTRs [18]. Some miRNA genes are distributed as clusters in the genome and thus these closely distributed miRNAs are termed as the miRNA cluster. miRNA-coding genes are transcribed into long primary miRNAs (pri-miRNAs) by RNA polymerase II in the nucleus, and then the Drosha-DiGeorge critical region-8 (DGCR8) complex processed pri-miRNAs into precursors (pre-miRNAs) of 60–70 nt in length. Drosha is a member of the ribonuclease III family (RNase III) [19]. Drosha and its cofactor, DGCR8, form a multiprotein complex called Microprocessor to mediate the nuclear export. pre-miRNAs possess a short stem plus 2-nt 3' overhang (also known as the nuclear cropping step) [20]. After being exported from the nucleus to the cytoplasm, pre-miRNAs are processed by Dicer (an RNase III enzyme) to produce mature miRNAs, which are incorporated into the RNA-induced silencing complex (RISC) to repress the expression of the target genes or bind directly to DNA preventing transcription [21–24].

In contrast to miRNAs, some lncRNAs are remarkably similar to messenger RNAs (mRNAs). lncRNAs are transcribed by RNA polymerase II, capped, spliced and get polyadenylated like mRNAs, although they cannot act as templates for protein synthesis [25]. lncRNAs are able to activate or repress gene expression at multiple levels through diverse mechanisms. For example, lncRNAs can recruit repressive (e.g., PRC2) and activating (e.g., the Trithorax group) chromatin modifiers at the DNA level much like molecular scaffolds, leading to regulation of target gene expression [26–29]. At the RNA level, lncRNAs play a role in post-transcriptional events during gene expression and contribute to splicing, mRNA translation and mRNA degradation [30–33]. In addition, certain lncRNAs can inhibit miRNA function, which indirectly enhances protein expression of miRNA targets [34]. Along with the growing understanding of the significance of ncRNAs in mammalian cell differentiation and human diseases [35,36], accumulating examples are being identified that illustrate the specific importance of short and long ncRNAs in PSCs. In this review, we focus on the recent advances in our understanding of miRNAs and lncRNAs, in the induction, maintenance and differentiation of PSCs.

**ESC-specific miRNA family**

Numerous studies identified a set of ESC-specific miRNAs that were preferentially expressed in ESCs and downregulated during differentiation into embryoid bodies [37,38]. The expression signature of ESCs has been characterized (Table 1 and Figure 1). The requirement of miRNAs in ESC self-renewal and differentiation was demonstrated by ESCs lacking the miRNA-processing enzymes, Dicer or DGCR8. Dicer- or Dgcr8-null ESCs exhibited reduced cell proliferation due to G1 cell cycle arrest and resistance to differentiation through embryoid body formation or retinoic acid induction [39,40]. miRNAs, miR-195 and miR-372 participate in hESC cell cycle control by depleting the two main miRNA processing enzymes, Dicer and Drosha [41].

In addition, two clusters of miRNAs—miR-302 cluster and mouse miR-290-295/human miR-371-373 cluster—were

### Table 1  Summary of pluripotency or differentiation associated miRNAs and lncRNAs

| Name      | Validated targets | Cell type      | Function                                      | Ref   |
|-----------|-------------------|----------------|-----------------------------------------------|-------|
| miR-302a  | CyclinD1          | hESCs          | Regulate cell cycle, promote self-renewal      | [42]  |
| miR-302   | NR2F2             | hESCs          | Maintain pluripotency                         | [43]  |
| miR-302b, miR-372 | TGFB1R2, RHOC | hiPSCs         | Accelerate mesenchymal to epithelial transition | [57]  |
| miR-291b-5p, miR-293 | P65         | mESCs          | Maintain pluripotency and self-renewal         | [46]  |
| miR-138   | P53               | miPSCs         | Promote reprogramming                         | [63]  |
| miR-145   | Oct4, Sox2, Klf4   | hESCs          | Induce differentiation                        | [77]  |
| miR-125, miR-181 | Cbx7         | mESCs          | PRC1-mediated differentiation                 | [78]  |
| Let-7b    | TLX, CyclinD1     | Neural stem cells | Reduce proliferation and differentiation     | [79]  |
| miR-9     | Stathmin          | hNPCs          | Coordinate proliferation and migration        | [81]  |
| miR-18, let-7   | Smad2, Acvr1b, Lin28 | mESCs          | Mesoderm differentiation                      | [71,84] |
| miR-27b   | Pax3              | Muscle stem cells | Myogenic differentiation                      | [88]  |
| miR-375   | Hnf1β, Sox9       | hESCs          | Endoderm differentiation                      | [92]  |
| Pcnct1-3  | Oct4              | mESCs          | Maintain pluripotency                         | [97]  |
| lincRNA-RoR | P53            | hiPSCs         | Promote reprogram                            | [99]  |
| Tsix      | PRC2              | mESCs          | X-chromosome inactivation                     | [103] |
| Mistral   | ML1I              | mESC           | Germ layer differentiation                    | [104] |
| lncRNA_N1, lncRNA_N3 | SUZ12, REST | hESCs          | Neurogenesis                                 | [105] |
strongly expressed in ESCs. The miR-302 cluster (containing 8 miRNAs on chromosome 3/4 in human), consists of highly expressed ESC-specific miRNAs including some of the most commonly studied miRNAs [42]. Pluripotency factors Oct4 and Sox2 can bind to a conserved promoter region of the miR-302 to initiate its expression [42]. miR-302a can inhibit the expression of G1 regulator cyclin D1, which contributes to the increased population of hESCs in S phase and regulating the cell cycle of ESCs, thereby promoting self-renewal and pluripotency [42]. A recent study found that in undifferentiated ESCs, Oct4 and miR-302, directly inhibit the expression of nuclear receptor subfamily 2, group F, member 2 (NR2F2; COUP-TFII) at the transcriptional and post-transcriptional levels, respectively. As a positive feedback loop, NR2F2 directly inhibits Oct4 expression [43].

The human homolog of the mouse miR-290-295 cluster, which also forms a tight genomic cluster (miR-371-373 cluster), is specifically expressed in hESCs, which is upregulated in several human tumors [44]. A recent study indicated that several Wnt-signaling pathway genes, including Dickkopf-1 (DKK1), TGF-beta type II receptor (TGFB2), B cell translocation gene 1 (BTG1), and left right determination factor 1 (LEFTY1), were direct targets of miR-372 and -373 [45]. The expression of the miR-371-373 cluster was transactivated via the Wnt/β-catenin pathway by directly binding β-catenin/LEF1 to the miR-371-373 promoter. These findings elucidate a novel beta-catenin/LEF1 - miR-372 and -373-DKK1 regulatory feedback loop, which likely plays a crucial role in ESC maintenance [45]. Luningschror et al. reported that overexpression of the NF-kB subunit p65 results in the loss of pluripotency and differentiation of ESCs, as well as the epithelial to mesenchymal transition [46]. Interestingly, the miR-290 cluster, specifically miR-291b-5p and miR-293, targets the p65 coding sequence to repress its translation, which may also contribute to regulatory networks in pluripotency [46].

The seed sequence of miRNAs is about 6–8 nt in length, which is thought to be the most important feature for miRNA target specificity [47–49]. Interestingly, previous studies showed that several miRNAs from different clusters including miR-106, miR-302-367 and miR-290 have similar seed sequences (AAGUGCU) and are all upregulated in ESCs, suggesting that they may repress similar pools of mRNAs to maintain the stem cell state [50].

miRNAs function during somatic cell reprogramming

Since the Yamanaka’s group claimed that somatic cells can be reprogrammed into iPSCs by expressing four transcription factors Oct4, Sox2, c-Myc and Klf4 (also known as the Yamanaka factors), scientists have tried different methods to obtain the iPSCs [4,51–55]. Several miRNAs have been shown to increase the efficiency of reprogramming when expressed along with a combination of the four or fewer Yamanaka factors Oct4, Sox2, c-Myc and Klf4 (also known as the Yamanaka factors), scientists have tried different methods to obtain the iPSCs [4,51–55].

Several miRNAs have been shown to increase the efficiency of reprogramming when expressed along with a combination of the four or fewer Yamanaka factors [56,57]. For example, MYC can be regulated by the miR-17-92 cluster, and overexpression of MYC leads to increased levels of miR-92 [58]. miR-92 belongs to the miR-17-92 cluster, which is upregulated in cancer [58]. Studies have shown that there are differences in the miRNA patterns between human iPSCs (hiPSCs) and...
miRNAs act as suppressors of the pluripotent state

miRNAs are critical for embryonic development and pluripotency maintenance and are involved in cell fate decisions as well. ESC-specific miRNAs have been described previously [37,38]. Nonetheless, miRNAs can also promote the differentiation of ESCs into the three germ layers—ectoderm, mesoderm and endoderm.

The let-7 miRNAs are broadly expressed in differentiated tissues and are increased during ES cell differentiation [70,71] (Table 1 and Figure 1). At the early differentiation stage of ESCs, expression of Oct4, Sox2, Nanog and other pluripotency genes are downregulated, which leads to the downregulation of the ES cell-specific cell cycle-regulating (ESCC) miRNAs and Lin28. Lin28, an RNA-binding protein, is a posttranscriptional repressor of let-7 miRNA biogenesis [72]. Therefore, the downregulation of Lin28 dramatically increases the expression of let-7 miRNAs. By targeting the 3' UTR of Lin28, let-7 may inhibit the translational initiation of the genes downstream of Oct4, Sox2, Nanog and transcription factor 3 (Tcf3), thereby accelerating the differentiation of ESCs [73,74]. Further study demonstrated that TUT4 is the uridylyl transferase for the let-7 precursor, which adds an oligouridine tail to downstream targets of the let-7 miRNAs, blocking the biogenesis of let-7 miRNA at the dicing step [75].

Expression of miR-145 is low in self-renewing hESCs, which is highly upregulated during differentiation [76]. A recent study reported that increased miR-145 expression inhibits hESC self-renewal and induces lineage-restricted differentiation [76]. Furthermore, Xu et al. demonstrated that endogenous miR-145 binds to the 3' UTR of the pluripotency genes Oct4, Sox2 and Klf4. Interestingly, as part of a double-negative feedback loop, the miR-145 promoter, is bound and repressed by Oct4 in hESCs [77].

The polycomb group (PcG) contains multiple homologs of the polycomb repressive complex 1 (PRC1) components including five orthologs of the Drosophila polycomb protein (Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8), and is critical for ES pluripotency and differentiation. A recent study demonstrated that Cbx7 is the primary polycomb ortholog of the PRC1 complexes in ESCs and knockdown of Cbx7 expression in ESCs can induce differentiation and increase expression of lineage-specific markers [78]. The miR-125 and miR-181 families are regulators of Cbx7, and overexpression of these miRNAs accelerates ESC differentiation [78].

Studying ESCs can help us understand how miRNAs play a role in suppressing the pluripotent gene expression. However, to clarify the specific role of miRNA in ESC differentiation, further study is needed.

Role of miRNAs in stem cell lineage determination

The iPSC technology provides an unlimited source of stem cells to promote the clinical applications of cell therapy. However, one of the biggest challenges to such clinical application is differentiating these pluripotent cells into the final functional cells of a specific organ. A further understanding of miRNAs demonstrated that the function of different cell types is associated with a unique miRNA expression pattern.

The let-7 family plays an important role in the ectoderm lineage differentiation of ESCs. Further study demonstrated that the let-7b miRNA regulates neural stem cell proliferation and differentiation by targeting the stem cell regulator TLX and the cell cycle regulator cyclin D1 [79]. Expression of musashi 1 (Msi1), an RNA-binding protein, is increased during the
early neural differentiation of ESCs. Ms1 can enhance Lin28 localization to the nucleus and block let-7 family member miR-98 in the nuclear cropping step, thus affecting early neural differentiation of ESCs [80]. miR-9, a brain-specific miRNA, is expressed in human neural progenitor cells (hNPCs) that are derived from hESCs. Further results suggest that miR-9 regulates the proliferation and migration of hNPCs by directly targeting the microtubule-related gene statmin [81]. The highly expressed miR-371-373 cluster in PSCs has also been reported to play a critical role in human PSC neurogenic differentiation behavior [82,83].

Another group found that let-7 and miR-18 downregulated Acrv1b and Smad2, respectively, to increase the mesoderm at the expense of endoderm in mouse ESCs (mESCs) [84]. Expression of miR-125b is upregulated in patients with leukemia and can regulate hematopoiesis by targeting Lin28 in mouse hematopoietic stem cells and progenitor cells [85]. Ivey and colleagues demonstrated that miR-1 and miR-133 can regulate mesoderm formation and cardiac muscle differentiation by suppressing the gene expression in desired lineages [86,87]. PAX3, a regulator of skeletal muscle stem cells, is required for the maintenance of muscle cell differentiation. miR-27b downregulates the PAX3 protein levels by directly targeting the 3’-UTR of PAX3 and accelerates myogenic differentiation in muscle stem cells [88]. However, a recent study demonstrated that miR-489 is a quiescence-specific miRNA in the satellite cell lineage. The highly expressed miR-489 in quiescent satellite cells decreased quickly during satellite cell activation. Further results have shown that miR-489 suppresses the oncogene Dec at the posttranscriptional level, which may be associated with the mechanism for maintaining the quiescent state of a stem cell population [89].

miR-24 and miR-10a were upregulated to inhibit endodermal differentiation during NaButyrate induction of hESCs [90]. Joglekar et al. showed that the expression of four islet-specific miRNAs including miR-7, miR-9, miR-375 and miR-376 was high during human pancreatic islet development [91]. Overexpression of miR-375 can downregulate the expression of gut-endoderm/pancreatic progenitor-specific markers, hepatocyte nuclear factor 1 beta (Hnf1b) and Sox9, during endodermal differentiation of hESCs. These data indicate that miR-375 may regulate hESC differentiation toward pancreatic islet cells [92].

Taken together, as one miRNA can target more than one gene, the role of miRNAs in cell differentiation is not only related to the level of its own expression but also has a close relationship with factors like the differentiation system, cell type and microenvironment.

**lncRNAs in ESC pluripotency and somatic cell reprogramming**

Recent studies have identified over 900 so-called lincRNAs in mESCs and hESCs, which potentially control the self-renewal and pluripotency of ESCs [93,94]. Intriguingly, more than 100 lincRNAs (with proximal genomic targets located less than 10 kb genomic distance from a gene to the binding site) in mESCs appear to be directly bound by ESC-specific transcription factors, such as Sox2, Oct4 and Nanog [95]. Lipovich’s group observed two lincRNAs that are regulated by Oct4 and Nanog and are essential for maintaining pluripotency. The inhibition or misexpression of these two lincRNAs leads to dramatic changes in the expression of Oct4 and Nanog, indicating the involvement of a feedback loop in the regulatory mechanism [95]. To further examine the role of lincRNAs in pluripotency, Gutman et al. performed loss-of-function studies on 147 lincRNAs using lentiviral-based shRNAs in mESCs [96]. Of these lincRNAs, 26 showed involvement in the maintenance of pluripotency. After deleting these lincRNAs, a reduced Nanog promoter activity was discovered and expression pattern in mESCs was similar to that in the differentiated cell types, suggesting that these lincRNAs repress differentiation programs in mESCs. Another large-scale screen of functional lincRNAs in mESCs was achieved by using RNA interference (RNAi) with transcript localization. Consequently, three non-coding transcripts, Pncat1-3, were identified as modulators of mESC pluripotency based on reduced Oct4 promoter activity [97]. Recent findings showed that lncRNA-RoR (regulator of reprogramming, formerly called lincRNA-ST8SIA3) shares miRNA response elements with Oct4, Sox2 and Nanog, and that lncRNA-RoR prevents these core transcription factors from miRNA-mediated suppression in self-renewing hESCs [98]. Together, these findings connect lincRNAs to the regulatory networks that maintain ESC identity.

In addition to maintaining ESC pluripotency, lincRNAs are involved in the generation of iPSCs (Table 1 and Figure 1). This cellular reprogramming is accompanied by an extensive global remodeling of the epigenome. The research group, led by Loewer et al., found that the expression profiles of lincRNAs in iPSCs were similar to those in ESCs but not to those in the somatic cells of origin, such as fibroblasts and hematopoietic stem cells [99]. Further study showed that expression of 10 lincRNAs was elevated in iPSCs compared with ESCs, suggesting that their increased expression may promote reprogramming [99]. Promoter loci of 3 iPSC-enriched lincRNAs, including lincRNA-SFMBT2, lincRNA-VLDLR and lincRNA-RoR, are bound by Oct4, Sox2 and Nanog. In addition, knockdown of Oct4 led to downregulation of these lincRNAs, suggesting that their expression is directly regulated by the key pluripotency transcription factors. The depletion of lincRNA-RoR resulted in a 2–8-fold decrease in the number of emerging iPSC colonies. Conversely, overexpression of lincRNA-RoR increased the efficiency of iPSC colony formation. Microarray gene expression analysis demonstrated that knockdown of lincRNA-RoR led to p53 upregulation, which induces oxidative stress, DNA damage and cell death, confirming the role of lincRNAs in the induction of pluripotency by promoting the survival of iPSCs [99].

**lincRNAs are implicated in the differentiation of PSCs**

LincRNAs can regulate the differentiation of ESCs as well. For example, X-inactive specific transcript (Xist) plays a role in X-chromosome inactivation (XCI) during female ESC differentiation. In placental mammals, XCI randomly inactivates one of the two female X chromosomes to obtain the proper gene dosage of X-linked genes in females as compared with males [100]. In female ESCs, Oct4, Sox2 and Nanog bind to intron 1 of Xist to suppress its expression, whereas the antagonizing lncRNA Tsix is activated by the pluripotency factors Oct4, Sox2, Rex1, c-Myc and Klf4 [101,102]. Upon differentiation,
downregulation of the pluripotency factors initiates the expression of Xist, which later recruits chromatin regulators such as PRC2 to mediate XCI [103]. In addition to Xist in the early step of differentiation, certain other IncRNAs play important roles in the later lineage commitment. Mixed lineage leukemia 1 (MLL1) is an epigenetic activator involved in embryonic development and hematopoiesis [104]. Bertani et al. found that the lncRNA Mistral is able to recruit MLL1 to chromatin and subsequently induce the expression of the homeotic genes Hoxa6 and Hoxa7 during mESC germ layer differentiation [104]. Another study reported an essential role for IncRNAs in neurogenesis. Cytoplasmic IncRNA_N2 promoted neurogenesis possibly by maintaining the expression of neurogenic miRNAs, miR-125b and let-7a, since both of them are located within the introns of IncRNA_N2. Additionally, nuclear IncRNA_N1 and IncRNA_N3 were identified to physically interact with nuclear factors REST and SUZ12, respectively, suggesting their potential roles in regulating neuronal differentiation [105].

Expression of IncRNAs is correlated with the full development potential of iPSCs. In most of the iPSC clones, the lncRNA Gtl2, which belongs to the Dlk1–Dio3 imprinted locus, is aberrantly silenced by DNA hypermethylation and histone hypoacetylation [106,107]. The Gtl2 gene is maternally expressed and its expression is thought to negatively regulate the expression of paternal Dlk1 gene, which is located within the same gene cluster and gets involved in fetal growth. In addition, a total of 26 miRNAs all localized to the Dlk1–Dio3 cluster are differentially expressed in non-4n complementation-competent and 4n complementation-competent iPSC lines [106]. The silenced status of this cluster in iPSCs is closely correlated with the developmental failure of these iPSCs. In contrast, iPSC clones with normal Dlk1–Dio3 cluster expression contributed to high-grade chimaeras and yielded viable all-iPSC mice. Interestingly, when an iPSC clone with silenced Dlk1–Dio3 was treated with a histone deacetylase inhibitor valproic acid (VPA), the locus that includes Gtl2 got reactivated, thus recovering the capability of this clone to support full-term development of all-iPSC mice.

Future perspectives

In recent years, the miRNAs and lncRNAs have been emerging as important components of gene regulation and have become the new hotspot of current molecular biology. Somatic cell reprogramming technology renders terminally-differentiated cells to revert to a pluripotent state, thus injecting new vitality into the field of stem cell research. Studies have shown that the regulatory interactions between ESC-specific miRNAs and their targets in the cell cycle, DNA methylation, mesenchymal to epithelial transition and apoptosis pathways influence stem cell pluripotency and somatic cell reprogramming and differentiation. A large number of miRNA and IncRNA sequences have been obtained via high-throughput sequencing technologies. However, the underlying molecular mechanism of ESC differentiation and pluripotency maintenance and somatic cell reprogramming still remains elusive. Better understanding of the new functions and mechanisms of miRNAs and IncRNAs in these processes would be conducive to achieving better appreciation of epigenetics and even more extensive impact on life sciences and biomedical research.

Competing interests

The authors have declared that no competing interests exist.

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