

REVIEW ARTICLE **OPEN**

New insights into the epitranscriptomic control of pluripotent stem cell fate

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Each cell in the human body has a distinguishable fate. Pluripotent stem cells are challenged with a myriad of lineage differentiation options. Defects are more likely to be fatal to stem cells than to somatic cells due to the broad impact of the former on early development. Hence, a detailed understanding of the mechanisms that determine the fate of stem cells is needed. The mechanisms by which human pluripotent stem cells, although not fully equipped with complex chromatin structures or epigenetic regulatory mechanisms, accurately control gene expression and are important to the stem cell field. In this review, we examine the events driving pluripotent stem cell fate and the underlying changes in gene expression during early development. In addition, we highlight the role played by the epitranscriptome in the regulation of gene expression that is necessary for each fate-related event.

Experimental & Molecular Medicine (2022) 54:1643–1651; <https://doi.org/10.1038/s12276-022-00824-x>

INTRODUCTION

Stem cells exhibit the potential to differentiate into various cell types¹. Therefore, stem cells have been useful in biological and medical research². Stem cells have the advantage of inducibility to obtain and utilize the cell types suitable for a particular research purpose. In particular, stem cell types affect different developmental stages and differentiation lineages^{3,4}. The usefulness of stem cells is based primarily on their ability to undergo various cell development stages, allowing researchers to obtain specific cell types with phenotypes at the appropriate developmental time based on the stage of acquisition⁵. Using lineage differentiated stem cells has enabled research model establishment for the direct investigation of molecular mechanisms, phenotypes, and therapeutic strategies in phenotype-relevant human cells^{6–8}. Stem cells in the early stages of development exhibit a wide range of differentiation properties². For example, zygote cells proliferate to form a morula, a group of stem cells with the same multicellular differentiation potential, including embryonic cells that form an organism and supporting cells needed to maintain proper embryonic development⁹. This broad differentiation potential and totipotency of stem cells gradually decrease as stem cells proliferate and acquire the necessary phenotypes during development, eventually progressing to a state called pluripotency¹⁰. Embryonic stem cells (ESCs) extracted from the inner cell mass (ICM) of a blastocyst are stem cells in the pluripotent state¹¹. Although these cells have lost the ability to differentiate into cells that form extraembryonic tissues, such as the placenta and yolk sac, they retain the ability to differentiate into cells that can form fetal tissues and organs¹². Thus, human ESCs (hESCs) in a pluripotent state are actively employed in developmental and pathological research that integrates aspects of human body pathophysiology^{13,14}. However, both the totipotent and

pluripotent states of cells are dynamic, and the range of differentiation potential changes gradually with the progressive maturation of stem cells¹⁵. Furthermore, whereas totipotent stem cells are characterized by their homogenous proliferation, human pluripotent stem cells (hPSCs) gradually show cellular diversity in the ICM of a blastocyst^{16,17}. The pluripotency of stem cells is thus limited, leading to the generation of three heterogeneous germ populations through pattern and structural formation events and gastrulation¹⁸. Thus, understanding the biological events and molecular mechanisms that control the properties of hPSCs is critical. However, only a subset of the molecular pathways governing stem cell pluripotency is understood.

The state of stem cells is determined by the influence of spatiotemporal location and ambient environmental conditions that change on the basis of developmental processes¹⁹. In a proliferating mass of stem cells, the topological information of each cell leads to diverse cell polarity and plasticity^{20,21}. Both the matrix surrounding stem cells and various environmentally induced signaling pathways trigger alterations in stem cell activity and behavior²². Through the control of gene expression, these factors eventually lead to biological changes, functioning as intrinsic fate regulators operated by the stem cells themselves^{23,24}. Gene expression patterns regulate the biological properties of cells and define cell types²⁵. Similar to those of somatic cells, the functions of stem cells are controlled by the molecular actions of proteins encoded by cell-type-specific genes²⁶. Gene expression control, therefore, is a key factor in determining the state and function of stem cells, regardless of intrinsic or extrinsic triggers^{24,27}. To ensure the timely and accurate regulation of required gene expression, stem cells engage well-established transcription machineries and epigenetic regulatory systems²⁸. For instance, recent studies have suggested that an epitranscriptomic

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Received: 8 April 2022 Revised: 26 May 2022 Accepted: 1 June 2022
Published online: 21 October 2022

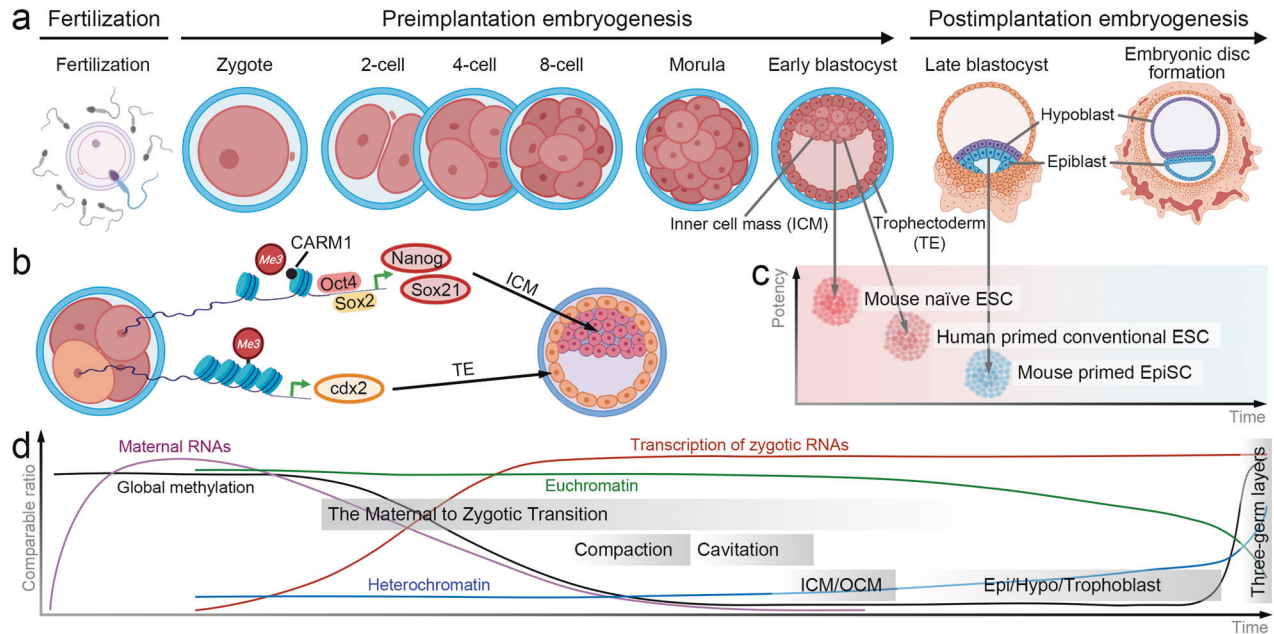


Fig. 1 Dynamics of stem cell development. **a** Morphological changes of stem cells according to the stage of stem cell development. **b** Molecular mechanism in the acquisition of heterogeneity. **c** Pluripotent state of stem cells derived during different developmental stages. **d** Molecular characteristics during stem cell development.

gene regulatory system changes the protein translation efficiency and persistence of messenger RNA (mRNA) through chemical modifications of transcribed mRNAs^{29,30}. Compared with somatic cells, hPSCs have immature genetic and epigenetic regulatory mechanisms^{31,32}, which can affect precise gene expression control, resulting in alterations in protein expression patterns that can be fatal³³. Nonetheless, PSCs in the early stages of development preserve an accurate fate decision process based on tight gene expression control³⁴. In addition to conventional gene regulation, other molecular mechanisms may play a role in the control of gene expression in PSCs.

In this review, we summarize changes in the state of PSCs during the early stages of development and the regulatory mechanisms underlying these changes. Additionally, we discuss recent research on gene regulation at the transcriptome level in relation to the regulation of PSC fate. Although a number of studies have reported distinct properties of hPSCs compared with mouse PSCs due to interspecies differences, the use of mouse embryos can provide sufficient knowledge for understanding human embryonic development. Here, we present studies on both mice and human cells and point out whether the results have been derived from human or animal models.

STATE OF PLURIPOTENT STEM CELLS (PSCS) DURING EARLY DEVELOPMENT

Stem-cell development generates diverse groups of cells with different properties via the generation of identical multiple-twin cells from a starting single cell (Fig. 1a)^{35–37}. Immediately after fertilization, zygote cells continue to divide, forming a group of 16-homogenous cells that comprise the early morula³⁸. As this cell group continues to divide, differences in environmental conditions, including topographical forces applied to each cell in the mass, are created. These differences result in the generation of the first heterogeneous population: the ICM and trophectoderm (TE, Fig. 1b)^{35,39,40}. Each cell group constituting the preimplantation blastocyst exhibits distinct differentiation potential for the production of nonoverlapping progeny. Trophectoderms organize surrounding structures such as the chorion, which supports

embryogenesis, whereas the ICM is critical for the formation of the embryo⁴¹. After implantation of a blastocyst in the maternal endometrial epithelium, the ICM undergoes subsequent morphogenetic changes. The ICM of a postimplantation blastocyst contains epiblasts and hypoblasts (Fig. 1a)⁴². The morphogenetic events include the polarization of the epiblast, which forms the central lumen that develops into the amniotic cavity; creation of the amniotic epithelium, which forms the amniotic sac membrane; and differentiation of primordial germ cells, which are precursors of eggs or sperm^{43–45}. Moreover, extraembryonic mesenchyme cells derived from the hypoblast surround the generated structure to isolate it from the outer cell membrane (OCM) formed by the trophectoderm^{37,43,46}. Thereafter, epiblasts in the ICM form a primitive streak, gastrulate and differentiate into three germ layers: the ectoderm, mesoderm, and endoderm⁴⁷.

HESC cultures have been established by the in vitro cultivation of cells isolated from a morula, an entire blastocyst, or an ICM^{11,48,49}. Morula-derived hESCs are thought to remain in a naive state, showing unbiased differentiation potential, including the generation of trophoblasts⁵⁰, whereas blastocyst- or ICM-derived cells are considered to exhibit a primed-like state, retaining comparably late-stage stem cell characteristics (Fig. 1c)¹⁵. Despite differences in cell properties, both types of hESCs exhibit the developmental spectrum that enables the generation of all somatic and germline cells^{51,52}. In contrast, murine naive and primed ESCs have shown to exhibit clear differences in the molecular and cellular properties^{53,54}. Previously, murine ESCs were believed to show better differentiation plasticity than human ESCs, but the limited pluripotent state epiblast stem cells (EpiSCs) was revealed. In contrast to mouse ESCs, known to be in the naive state, mouse EpiSCs in the primed state clearly show not only differences in response to activation by external signals and a decrease in self-renewal capacity but also a massive reduction in whole-animal generation efficiency because of a tetraploid composition^{55,56}. Although the definition of the naive and primed state of hPSCs remains ambiguous, evidence of a naive state of mammalian stem cells, including that of primates, has been reported, suggesting the need to understand the conversion of hPSCs to the pluripotent state^{57,58}.

MECHANISMS THAT CONTROL PSC FATE

The state of PSCs, including their development and differentiation, is controlled by both intrinsic and extrinsic conditions. Deciphering the complicated signaling pathways governing the pluripotent state of stem cells is challenging, as multiple biological events, such as maturation, fate determination, and differentiation, concurrently affect stem cell development. In this context, the biological events regulating the pluripotent state of stem cells are categorized into two processes: “acquisition of heterogeneity from a homogeneous population” and “conversion from a naive to a primed state”. Each process is accompanied by an explanation of the mandating molecular pathways.

Acquisition of heterogeneity in a homogeneous population

Among the major questions in stem cell biology, when and how is the control of clonal cells within a homogeneous population changed to produce cells with distinct and orderly fates? To form a highly organized body from an embryo comprised of symmetrical cells, the embryonic symmetry must be disrupted⁵⁹. The importance of acquiring asymmetric fate during gastrulation to enable balanced development of the human body has been investigated in previous studies^{60,61}. Complex signaling at the cellular level, as well as geometrical and topological transformation at the embryonic level, are necessary for asymmetry^{62,63}. Specifically, the process that ultimately disrupts embryonic radial symmetry and triggers anteroposterior axis specification, has been found to be driven by a morphogen signaling gradient⁶⁰. Notably, the first disruption to the symmetric organization of human cells occurs much earlier than previously thought: during the division of stem cells in the totipotent state. The emergence of the TE in blastocyst-stage embryos is the first morphological variation during embryogenesis. TE and primitive endoderm lineages generate the placenta and yolk sac extraembryonic cells, whereas cells inside the blastocyst form the ICM, the cells of which eventually divide to form the primitive endoderm and pluripotent epiblast cells. According to the most recent understanding, each cell in the early embryonic state retains identical developmental potential. Therefore, in the absence of stimulants such as morphogens, cell fate has been assumed to be determined randomly, making the existence of initiating factors driving cellular heterogeneity enigmatic. Based on single-cell transcriptome analyses, recent studies with PSCs and mouse embryos have suggested another possibility^{64–67}. Differences were detected in the totipotency between cells constituting murine or human blastomeres, with each cell in the 16-cell stage exhibiting a transcriptome that differs from that in the 2-cell stage immediately after the first cleavage⁶⁶. The gradual increase in the differential tendency of genes to contribute to lineage specification suggests that the symmetry is disrupted earlier than traditionally thought; that is, it distinguishes trophoblasts from embryoblasts after the morula stage. This finding was consistent with the results observed in several studies suggesting that only one of the cells separated in the 2-cell stage can develop into a mouse^{64,68–71}.

The molecular mechanism causing this initial heterogeneity has not yet been clearly elucidated. A recent study reported that individual cells in the 4-cell-stage blastomere exhibit differential expression of epigenetic modifiers, such as that of the histone methyltransferase CARM1 (Fig. 1b)^{72,73}. Differences in the expression of CARM1 regulate the DNA-binding ability of the pluripotency transcription factors SOX2 and OCT4, with activity that is affected by histone H3 arginine 26 dimethylation (H3R26me2)^{74,75}. A differential increase in the expression of genes downstream of SOX2, such as *Sox21*, at the 4-cell stage has been reported to suppress the expression level of *Cdx2*⁷⁶, a lineage specifier for TE differentiation in the 8-cell stage^{77,78}. Subsequently, the reduced expression of *Cdx2* results in the decreased expression of polarity markers that define the apical

domain, in turn leading to TE differentiation. Interestingly, *Cdx2* mRNA has been demonstrated to be asymmetrically localized to the apical pole in the 8-cell stage, when the embryo is undergoing apical-basal polarization^{79,80}. Due to the apical localization of *Cdx2* transcripts, differential inheritance during subsequent division to the 16-cell stage might result in cells on the outside of the apical domain possessing more *Cdx2* transcripts than inner cells. Therefore, the determination of timing of these differences in gene expression between cells is important. The mechanisms underlying the determination of early mammalian stem cell fate remain unclear, largely because whether the first bifurcation in the cell fate sequence is a random event in mammalian embryo during the morula stage or is based on molecular features of differentiation that emerge prior to morphological changes remains unclear^{75,81–84}. Depending on the true pathway, either the role played by an intrinsic gene regulatory mechanism or the cellular environment condition will be predominant. An analysis of the transcriptome in multiple blastomeres revealed that after the first cleavage each cell exhibited different gene expression patterns from the pattern evident in the 2-cell stage⁶⁶. Although evidence supports an autonomous molecular cascade for the regulation of cell fate that directs differences between cells in blastomeres early in embryonic development, the mechanisms that control precise gene expression in the open chromatin structure of early stem cells remain unclear.

Cell conversion from a naive to a primed state

The differentiation capacity of stem cells refers to the ability to generate a range of differentiated cells, which is the basis of classifying cells as totipotent, pluripotent, and multipotent¹⁰. Although pluripotency is a transient property of stem cells in vivo, regulation of signaling pathway activity involved in fate maintenance enables stem cells to remain in a pluripotent state for an extended period in vitro⁸⁵. The pluripotent stage involves multiple stem cells in dynamically changing states, and the differentiation potential of each cell is gradually limited; therefore, the stem cell potential is determined on the basis of the developmental stage of the blastocyst from which the ESCs are derived. Due to the limited accessibility to human embryos⁸⁶, studies using murine cells first elucidated the correlation between the origin, status, and differentiation capacity of PSCs. Two types of PSCs have been established from mouse blastocysts (Fig. 1c): ESCs^{87,88} and EpiSCs^{53,54}. ESCs derived from the ICM of preimplanted mouse blastocysts were in a naive state and were capable of generating all three germ layers and primordial germ cells^{87–89}; in contrast, EpiSCs isolated from postimplanted epiblasts were in the primed state and capable only of generating the three germ layers⁵³. Although both ESCs and EpiSCs show the ability to form the three germ layers, these two types of stem cells are clearly distinguished by their developmental potential, as only ESCs, not EpiSCs, can efficiently generate chimeric progeny from injected blastocysts^{53,54,90}. Differences in the ability of PSCs to generate chimeric animals are representative of stem cell potential in the in vivo counterparts of pre- or postimplantation blastocysts⁹¹. Various efforts have been made to elucidate the mechanism underlying the differences in pluripotency attributed to differences in PSC origin. In contrast to ESCs, which express a variety of pluripotency genes, including *Oct4*, *Nanog*, *Sox2*, *ESRRB*, *Rex1*, *Klf2*, and *Klf4*, EpiSCs exhibit elevated expression levels of differentiation-inducing genes, such as *Otx2* and *Zic2*, and low levels of *Nanog*, *Rex1*, *Klf2*, or *Klf4*^{54,89,92}. Studies designed to optimize in vitro culture conditions to maintain naive or primed PSCs have suggested that the endogenous activity of genes is regulated by signals transduced from outside a cell, such as fibroblast growth factor (FGF)⁹³, bone morphogenic protein (BMP)⁹⁴, transforming growth factor β (TGF β)⁹⁵, and WNT⁹⁶. Therefore, early methods for culturing naive murine ESCs from mitotically inactive mouse

embryonic fibroblasts and fetal bovine serum⁹⁷ led to modulation of JAK-STAT3 pathway and NODAL signaling via the addition of leukemia inhibitory factor (LIF)⁹⁸ and BMP⁹⁴. Similarly, treatment with MEK/FGF/GSK3 inhibitors led to murine ESC maintenance in the naive state, even without LIF stimulation⁹⁶. When FGF inhibition was released, cell proliferation was promoted while the naive state of the stem cells was maintained by a MEK-ERK-independent mechanism of endogenous FGF^{93,99}. Alternatively, FGF was reported to induce the emergence of ESCs from the naive state. In addition, FGF together with activin A, abrogated the differentiation of murine ESCs into primordial germ cells⁸⁹, whereas it promoted location-specific differentiation in an embryo; that is, it induced the acquisition of the anterior late-gastrula primitive streak cell phenotype by EpiSCs^{100,101}. WNT signaling has also been found to function ambivalently with respect to the naive state of PSCs. For instance, the stabilization of nuclear β -catenin has been shown to enhance naive pluripotency by neutralizing the repressive activity of transcription factor 3 (TCF3) on nuclear target genes such as *Oct4*, *Nanog*, and *Sox2*^{102–106}. The transcriptional activity of β -catenin on differentiation-associated target genes was diminished, thereby comporting with the increased membrane stability of cytoplasmic β -catenin rendered through complex formation with OCT4 and E-cadherin in cells in the naive state¹⁰⁷. In contrast, by promoting mesodermal gene expression under differentiation-optimized conditions, nuclear β -catenin was shown to facilitate differentiation-related priming over naive pluripotency-promoting functions¹⁰⁸.

Although hESCs are derived from blastocysts similar to those of mice, they differ from murine ESCs. For example, in contrast to murine ESCs, which require LIF for survival, hESCs derived from the ICM of blastocysts require FGF2 and TGF- β 1. In addition, hESCs share hallmarks of murine EpiSCs, including partial expression of naive pluripotency markers, deposition of H3K27me3 at genetic loci required for development, lack of global hypomethylation, and an inactive X-chromosome in female ESC lines^{51,109,110}. However, recent evidence has indicated that hESCs in the primed state resemble murine ESCs more closely than murine EpiSCs. Furthermore, hESCs maintain the expression of E-cadherin, which is highly expressed in naive murine ESCs, but not FGF5 or N-cadherin expression, although they are highly expressed in murine EpiSCs⁵¹. The level of expression of naive pluripotency factors such as *NANOG*, *PRDM14*, and *REX1* is relatively stable in hESCs, and ablation of *NANOG* and *PRDM14* induced the differentiation of hESCs¹¹¹. In addition, even when cultured with FGF2/activin A, the DNA methylation pattern of hESCs has been shown to be comparable to that of naive ESCs cultured in LIF/FBS not murine EpiSCs^{112,113}.

To date, the distinction between naive and primed states remains unclear; however, clearly, the stem cell-specific regulation of intrinsic FGF and β -catenin signaling is precisely regulated at the early pluripotent naive stage.

CONTROL OF GENE EXPRESSION IN THE PLURIPOTENT STATE

Cellular identity is driven by epigenomic, transcriptomic, and proteomic heterogeneity. Studies creating pluripotent cells by introducing the exogenous genes *OCT4*, *SOX2*, *KLF4*, and *MYC* have provided evidence for the key role played by the expression of specific genes in the regulation of stem cell fate^{27,114}. More specifically, the aforementioned differences in the expression of symmetry disrupting and pluripotency genes are evident before the important division of PSCs into the three germ layers^{66,87,115,116}. The regulation of autonomous molecular factors participating in the control mechanism of stem cell fate determines the homogeneous and naive states, with the function of these factors leading to cell-specific differences that lead to achieve heterogeneity and a primed cell state^{117–119}.

In general, the regulation of gene expression required by cells is described by the central dogma¹²⁰. Specifically, transcription, translation, posttranslational modifications or subsequent proteolytic processes precisely control the levels of molecular expression. In contrast to other processes that require the systematic organization and regulation of subsequent processes to control the level of a final molecular product, transcriptional regulation is an efficient process for regulating PSC fate because the molecular control mechanisms are immature^{121–123}. Although the epigenome shapes stem cell hierarchies¹²⁴, differential transcriptomes only partially explain protein abundance^{121,122}. As hPSCs are characterized by completely open chromatin conditions and immature epigenetic systems, posttranscriptional control is thought to play a key role in the functional output of genetic programs (Fig. 1d)^{125,126}. A zygote survives and functions through the support of molecular components passed down through gametes, which carried these traits before fertilization¹²⁷. In particular, PSCs in the early stages of development undergo zygotic gene activation (ZGA), which allows precise regulation of selective expression of genes necessary for survival¹²⁸. ZGA of hPSCs begins in the 2-cell stage and proceeds to the morula stage¹²⁹, which is still characterized by immature chromatin reorganization, even in the ICM of a preimplanted blastocyst^{130,131}. Although epigenetic control is thought to occur during this stage, epigenetic modifications are deposited during the heterogeneity acquisition process of the cell transition from a primed state to three germ layer development³¹. This timeline has been supported by studies showing de novo heterochromatin formation and X-chromosome inactivation in hPSCs after ICM formation^{132–134}. In addition, as described above, WNT, FGF, or activin signaling regulates the transcription of molecular factors that affect global methylation through the activity of OCT4 and NANOG in the naive state^{135,136}. This regulatory program requires additional mechanisms to compensate for coarse transcriptome level changes caused by immature transcriptional control prior to the completion of epigenome-driven transcriptional regulatory mechanisms¹²³. Increasing data have suggested a role for posttranscriptional modifications in the early stages of stem cell development that enables the selective regulation of required functional outputs (i.e., proteome content) based on the global transcriptome.

The epitranscriptome in development and stem cells

In addition to reported posttranscriptional modifications during oocyte development¹³⁷, more than 100 chemical modifications in addition to typical posttranscriptional modifications, such as 5'-capping, polyadenylation, and splicing, have been identified¹³⁸. Methylation is the most common enzyme-catalyzed modification, with N6-methyladenosine (m⁶A)²⁹, N1-methyladenosine (m¹A)¹³⁹, pseudouridine (Ψ)¹⁴⁰, and C5-methylcytosine (m⁵C)¹⁴¹ of RNA being reported. However, the role played by RNA m⁶A in posttranscriptional regulation during stem cell development is unknown.

N6-methyladenosine (m⁶A)

m⁶A is the most prevalent modification of the 3' untranslated regions (UTRs), long internal exons, intergenic regions, and 5' UTRs in human mRNAs^{30,142}. The functional m⁶A network is accurately orchestrated through a series of protein effector molecules, including "writers" for methylation, "erasers" for demethylation, and "readers" for interpretation of methylated mRNAs. A microRNA-guided protein complex consisting of Wilms tumor 1-associating protein (WTAP), methyltransferase-like protein 3 (METTL3), and methyltransferase-like protein 14 (METTL14) targets specific regions on mRNA, depositing a methyl group at the N6-position of adenosine^{143–145}. Subsequent demethylation by eraser proteins, such as fat mass and obesity-associated protein (FTO) or human AlkB homolog H5 (ALKBH5), results in m⁶A being highly

Biological roles of epitranscriptomic modifications

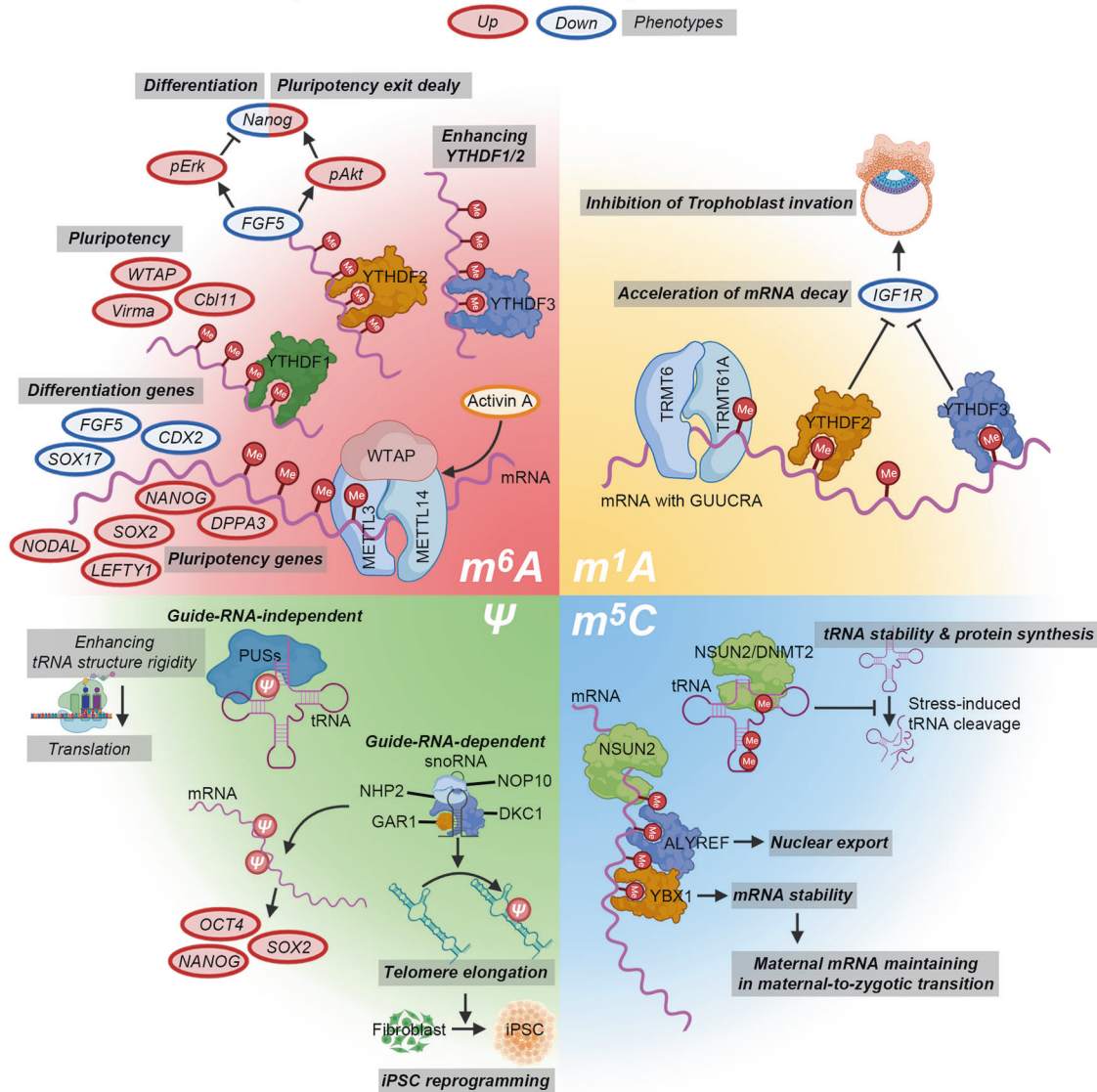


Fig. 2 Various features of pluripotent stem cells are regulated by epitranscriptomic modifications. Molecular regulatory functions of epitranscriptomic modification m⁶A (red), m¹A (yellow), pseudouridine (green), and m⁵C (blue) are associated with each biological phenotype in pluripotent stem cells.

enriched in specific transcriptome sites^{146,147}. The presence of eraser proteins that correct a methylation pattern suggests that m⁶A-associated posttranscriptional control might be a dynamic process that can quickly reflect environmental changes such as developmental progression and cellular stress. An m⁶A-loaded mRNA is recognized by RNA-binding protein YTH domain-containing families 1 (YTHDF1), 2 (YTHDF2), or 3 (YTHDF3) after being delivered to the cytoplasm through 5' capping and polyadenylation^{30,148,149}. Emerging evidence has suggested that YTHDF1, YTHDF2, and YTHDF3 target distinct mRNAs for cap-dependent translation, acceleration of mRNA decay, or promotion of translation and decay, respectively. In contrast to the prevailing model, recent studies have reported that all 3 YTHDFs act redundantly to mediate biological functions through specific m⁶A sites and shared target mRNAs¹⁵⁰.

Recent findings have also demonstrated the role played by the m⁶A modification in various molecular processes, such as translation efficiency, stability, localization, and splicing, which are involved in stem cell development and fate control

(Fig. 2)^{151–153}. Deficient METTL3 and METTL14 have been reported to inhibit the expression of pluripotent genes such as *SOX2*, *NANOG*, and *DPPA3* but to promote the expression of developmental regulators such as *FGF5*, *CDX2*, and *SOX17*¹⁵¹. Furthermore, deletion of *Mettl3* and *Mettl14* increased mRNA stability in a HuR- and miRNA-dependent manner¹⁵², a finding was further supported by a subsequent study showing that *ZC3H13* forms biochemical complexes with WTAP, VIRMA, and CBLL1¹⁵⁴. This complex formation led to a decrease in global levels of the m⁶A modification and self-renewal ability, triggering the differentiation of murine ESCs. These results differed from those of a study reporting that *Mettl3*-knockout mice presented with embryonic lethality between embryonic days 3.5 and 6.5 when PSCs lost lineage differentiation potential¹⁵³. In *Mettl3*-knockout murine ESCs, stabilized *FGF5* mRNA activated pErk and downregulated Nanog expression, FGF5-mediated concurrently coactivated pAkt to reestablish the expression of *Nanog*. The induction of differentiation and a concurrent delay in pluripotency loss might explain the discrepancy in these studies¹⁵⁵. Ultimately, m⁶A loss

causes confusion in determining whether cells maintain and advance in their pluripotent state and or undergo differentiation. Another study investigating the interactome of SMAD2/3 revealed that SMAD2/3 promoted m⁶A deposition on nuclear RNA by interacting with the METTL3-METTL14-WTAP complex in response to the activation of activin A/TGFβ signaling in primed hESCs¹⁵⁶. Accumulation of the mRNA of NANOG, NODAL, and LEFTY1, which are activin A signaling targets, likely affects processes such as the transition from homogeneity to heterogeneity or from the naive to the primed state by dynamically reflecting extracellular signaling in hPSCs.

Pseudouridine (ψ)

Pseudouridine is the most abundant modification on transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs); it has also been found on mRNAs, long noncoding RNAs (lncRNAs), and small nuclear RNAs (snRNAs)^{157,158}. RNA is modified by pseudouridine synthases (PUSs) in a guide-RNA-independent manner¹⁵⁹, affecting RNA structures by promoting base stacking interactions via a hydrogen bond donor to increase RNA backbone rigidity, thereby regulating the interaction of other biomolecules with RNAs¹⁶⁰. Ultimately, pseudouridylation plays a role in increasing tRNAs stability and simultaneously alters translation termination, thereby regulating protein synthesis^{161,162}. Interestingly, guide-RNA-dependent pseudouridylation is regulated by dyskerin (DKC1)¹⁶³, which promotes the elongation of telomeres and increases the expression of OCT4 and SOX2¹⁶⁴. However, mutations in DKC1 result in failed iPSC reprogramming¹⁶⁵. In line with this outcome, DKC1 mutations have been found to be involved in the pathogenesis of human diseases characterized by short telomeres¹⁶³. Moreover, deletion of *Dkc1* led to early embryonic lethality in a mouse model¹⁶⁶. Although its association with the state of PSCs has not yet been clarified, pseudouridine might be related to stem cell development, maturation, and aging (Fig. 2).

N1-methyladenosine (m¹A)

In human cells, m¹A is widely distributed on tRNAs¹⁶⁷. The m¹A modification is mediated by TRMT10C, TRMT61B, and TRMT6/61A methyltransferases and is associated with the maintenance of the structure, stability, and function of tRNAs in mitochondria or cytoplasm^{162,168,169}. Less than 0.1% of mRNAs contain an m¹A modification, which is generally located near the 5' UTR close to a translation initiation site¹³⁹. Although the role played by TRMT6/61A in depositing m¹A modifications in cytoplasmic mRNAs carrying the GUUCRA tRNA-like motif is known, its contribution is minor¹⁶⁷. Interestingly, similar to the m⁶A modification, the m¹A modification involves a dynamic and reversible process. m¹A demethylation is mediated by ALKBH1 and ALKBH3, and m⁶A and m¹A share a reader protein^{170,171}. YTHDF2 and YTHDF3 are known to contribute to the interpretation of m¹A, causing the rapid decay of modified mRNAs^{172,173}. Recent studies have suggested that YTHDF3 promotes the degradation of *IGF1R* mRNA, leading to the inhibition of trophoblast invasion in trophoblast-associated pregnancy disorders¹⁷³. Although neither the biological role nor the molecular mechanism of m¹A has been established to date, it is thought to be involved in the pathophysiology of stem cells because of the similarity in the dynamics and shared molecular machineries of the m¹A and m⁶A modifications (Fig. 2).

C5-methylcytosine (m⁵C)

Similar to m⁶A, the m⁵C modification is reversible and controlled by methyltransferases (writers: NSUNs, TRDMT1, and DNMT2)¹⁷⁴ and demethylases (erasers: TETs and ALKBH1)^{175–177}. The molecular function of m⁵C is mediated by reader proteins (ALYREF and YBX1)¹⁷⁸. Although the m⁵C modification is highly enriched on tRNAs and rRNAs, it reportedly plays a role in mRNA export¹⁷⁸, RNA stability maintenance¹⁷⁸, and translation¹⁷⁹. In zebrafish, deletion of maternal Ybx1 enhanced translation and triggered the

unfolded protein response, resulting in oogenesis and embryogenesis defects¹⁸⁰. In addition, Ybx1 has been shown to play a role in maintaining maternal m⁵C-containing mRNAs during the maternal-to-zygotic transition (MZT) during initial embryogenesis¹⁸¹. These mRNAs have been reported to facilitate the MZT in early zebrafish development. A pathological association between m⁵C and the activation of JAK-STAT signaling and dysregulation of tRNAs has been reported in various cancers and neurodegenerative disorders in humans^{178,182}. Although the role played by the m⁵C modification in the regulation of early human stem cell properties has not been elucidated, further research into the function of m⁵C in health and disease is warranted because of its effect on early zebrafish embryogenesis and importance of TET proteins in early stem cell development (Fig. 2).

CONCLUSIONS AND PERSPECTIVES

Although somatic cells control chromatin accessibility to transcription machineries through their well-organized heterochromatin structure and epigenetic control, hPSCs accommodate the uncontrolled accessibility of transcriptional machineries to euchromatin throughout the nucleoplasm. However, PSCs strictly control the levels of expression of certain proteins, which are the functional end products of strictly controlled gene expression. More specifically, the molecules that determine the differentiation potential and developmental stage of PSCs are controlled at the epitranscriptomic level. Epitranscriptomic regulation, which controls the stability and translational efficiency of transcribed mRNAs, overcomes unfavorable conditions faced by PSCs. In particular, m⁶A is the most prevalent epitranscriptomic modification of mRNAs and is involved in the regulation of major signaling pathways in hPSCs. However, a number of studies have shown that mRNA modifications in addition to m⁶A were correlated with early developmental changes in animal models or modulated by molecular mechanisms during early developmental changes in PSCs. Therefore, future studies should be directed to investigating the complex epitranscriptomic gene regulatory system, which involves the mutual interaction of multiple types of RNA modifications, as well as the molecular mechanism and biological implications of certain types of RNA modifications.

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ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (Grant No. NRF-2021M3E5E5096744 and NRF-2022M3A9H1016308). All illustrations in this article were created with BioRender.com.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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