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PATHOBIOLOGY IN FOCUS

Epigenetic foundations of pluripotent stem cells that recapitulate *in vivo* pluripotency

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In mammalian development, dynamic epigenetic reprogramming occurs in pre-implantation embryos and primordial germ cells and plays a critical role in conferring pluripotency on embryonic cells. Pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells, have been derived and maintained *in vitro* under culture conditions that include stimulators and inhibitors of extrinsic signaling. Recent advances in stem cell cultivation have opened the possibility of capturing naive pluripotency, which is reminiscent of the pluripotency of inner cell mass cells, *in vitro*. However, emerging evidence has revealed complexity of epigenetic regulation in pluripotent stem cells *in vitro* that reflects the developmental stage, gender, and species. In this review, we describe the developmental potential and epigenetic regulation of pluripotent stem cells in rodents and humans *in vitro* and discuss unsolved issues in developing strategies to capture *in vivo* pluripotency *in vitro*.

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In mammals, cell fate specification is governed by highly coordinated and sequential gene regulatory mechanisms. Transcription factors (TFs) and epigenetic modifications, including DNA methylation and histone modifications, play a central role in these programs.[1–3] Once cells differentiate into a particular cell lineage, the committed cell fate is stably maintained by epigenetic control of the gene regulation.[1,4,5] However, during the mammalian life cycle, epigenetic reprogramming occurs in primordial germ cells (PGCs) and pre-implantation embryos, which resets the cellular commitment and confers totipotency on the zygote[3,6–8] (Figure 1a). In PGCs, epigenetic memory, particularly DNA methylation, is erased before establishing novel epigenetic regulation during gametogenesis, which includes genomic imprinting with sperm-/oocyte-specific *de novo* DNA methylation[9,10] (Figures 1a and b). Further epigenetic reorganization occurs after fertilization, which is essential for the acquisition of totipotency and subsequent pluripotency.

Pluripotency is defined as the potential of a cell to give rise to all three germ layers, including germ cells, and is linked with unique epigenetic control of gene regulation. Indeed, after epigenetic reprogramming, inner cell mass (ICM) of blastocysts as well as PGCs display global DNA hypomethylation and characteristic transcriptional profiling[6,7,11,12] (Figure 1a). Pre-implantation embryos undergo dynamic reorganization of the epigenetic regulation. Importantly, however, DNA methylation at imprinting control regions (ICRs) that are inherited from either the oocyte or sperm as 'parental memory' are resistant to demethylation in ICM cells (Figure 1b). The maintenance of genomic imprints in ICM, embryonic, and adult cells is thought to be important, since aberrant imprints are often linked with various diseases including behavior anomalies and tumorigenesis.[13–15] After ICM development, post-implantation epiblasts undergo epigenetic programming including genome-wide *de novo* DNA methylation and differentiate into all three germ layers[5,16] (Figure 1a).

Recapitulation of *in vivo* pluripotency *in vitro* culture has been a major goal of developmental biologists for decades. There exist several types of pluripotent stem cells. In mice, embryonic stem cells (ES cells)[17,18] and epiblast stem cells (EpiS cells)[19,20] are derived from ICM and post-implantation epiblast, respectively, and both are typical pluripotent cell lines isolated from early embryos (Figure 2). Although both ES cells and EpiS cells have pluripotency, they exhibit distinct transcriptional and epigenetic signatures.[21,22] Intriguingly, human ES cells are transcriptionally and epigenetically distinct from mouse ES cells and resemble mouse EpiS cells, despite being derived from developing pre-implantation blastocysts, suggesting different regulatory mechanisms of

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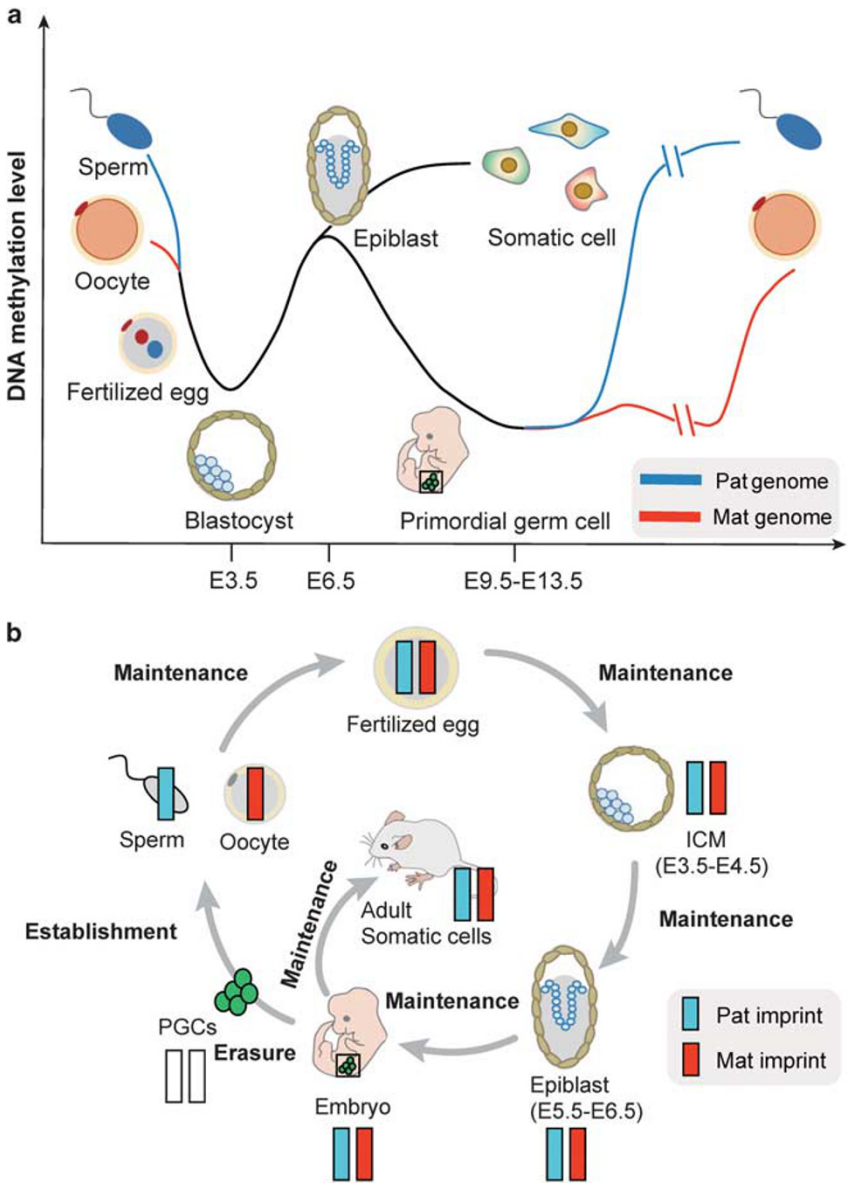

Figure 1 Epigenetic reprogramming and programming in mouse development. (**a**) DNA methylation levels in mammalian development. Global DNA demethylation occurs in pre-implantation embryos and PGCs. DNA methylation in the paternal genome reaches the level in the maternal genome after fertilization, and then both genomes are further demethylated until a zygote develops into blastocyst. After blastocyst development, post-implantation epiblast gains *de novo* global methylation during gastrulation and differentiates into three germ layers. PGCs emerged from the epiblast also undergo global demethylation. Germ cells (sperm or oocyte) gain *de novo* methylation during their maturation in a sex-dependent manner to establish genomic imprints. E indicates the embryonic day. (**b**) Genomic imprints in mammalian development. Imprints are established in germline (oocyte or sperm) in a sex-dependent manner (blue: paternal imprints, red: maternal imprints). The established imprints are strictly maintained in ICM, embryo, and somatic cells throughout life. Parental imprints are erased only in PGCs to re-establishment imprints in the next generation.

pluripotency between rodents and humans.[21] The derivation of induced pluripotent stem cells (iPS cells) has enabled the creation of pluripotent stem cells from somatic cells without the need for embryos. Because ES/iPS cells have the ability of self-renewal and pluripotency, they could serve clinical applications such as regenerative medicine and drug discovery.[23] For these purposes, however, it is important to understand the common and distinctive molecular and functional features of different pluripotent stem cells. In this review, we discuss current understandings and unresolved key issues in the developmental potential and epigenetic regulation of pluripotent states *in vivo* and *in vitro*.

EPIGENETIC REPROGRAMMING AND PROGRAMMING IN MOUSE EARLY DEVELOPMENT *IN VIVO*

DNA methylation patterns are dynamically altered during early embryogenesis in mice.[5,7] Paternal and maternal genomes are demethylated asymmetrically after fertilization.[24]

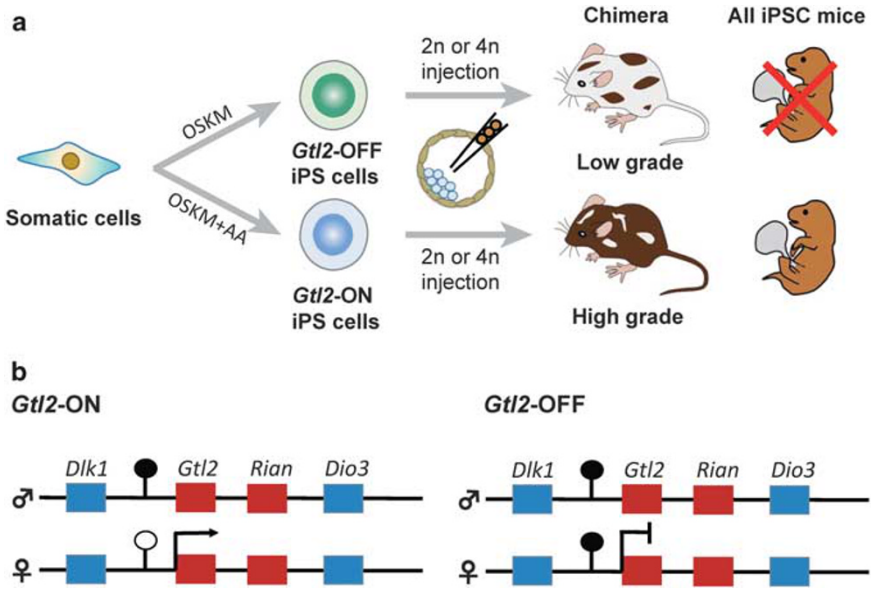

Figure 2 Effects of genomic imprints on stem cell potential. (**a**) The expression of *Gtl2*, an imprinted gene, is frequently silenced in iPS cells (OSKM; *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* and OSKM+AA; *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* with ascorbic acid (AA)). *Gtl2*-OFF iPS cell clones tend to poorly contribute to chimeric mice and fail to produce all-iPS-cell mice by 4n complementation. AA treatment prevents the imprinted region from aberrant methylation and yields iPS cells with *Gtl2* expression. *Gtl2*-ON iPS cell clones are able to efficiently contribute to chimeras and produce all-iPS-cell mice. (**b**) *Gtl2* is expressed only from the maternal allele, which is regulated by paternal allele-specific DNA methylation at the *Dlk1-Dio3* imprinted cluster. The imprinted cluster is often aberrantly hypermethylated in *Gtl2*-OFF iPS cells, whereas it is methylated only at the paternal allele in *Gtl2*-ON iPS cells.

The paternal genome is immediately demethylated in the zygote, while the maternal genome undergoes demethylation during the cleavage stage. Interestingly, the oocyte already exhibits DNA hypomethylation relative to sperm, particularly at transposable elements.[7] However, upon fertilization, sperm methylation rapidly decreases to levels comparable of the oocyte, indicating that the oocyte methylation pattern is a better predictor of epigenetic reprogramming in the zygote. Continuous genome-wide DNA demethylation occurs in pre-implantation embryo until the ICM stage, when the DNA methylation status reaches to a lower level[7,12] (Figure 1a). Mechanistically, both paternal and maternal genomes undergo passive demethylation during the pre-implantation stage. In agreement with this property, DNA methyltransferases are mainly localized outside of the nuclei from the two-cell embryo to blastocyst stage.[25] Despite dynamic epigenetic reprogramming in pre-implantation embryos, unique sequences in the genome are resistant to DNA demethylation. Such representative genetic elements include ICRs and intracisternal A particle transposons.[26] After specification of the ICM, genome-wide remethylation in the epiblast is essential for gastrulation and proper cellular differentiation into the three germ layers including germ line[5,27] (Figure 1a). X chromosome inactivation is one of the essential epigenetic phenomena. In female pre-implantation mouse embryos, paternal X chromosome is reactivated only in ICM and not in extraembryonic cells. Then, one X chromosome is randomly inactivated for dosage compensation after exit from the ICM state.[28–30] This dynamic epigenetic regulation plays an important role in normal mammalian development.

ESTABLISHMENT AND MAINTENANCE OF GENOMIC IMPRINTING IN MICE

Because imprinted genes often directly regulate fetal growth, genomic imprinting is an essential epigenetic phenomenon for mammalian development. Mammalian cells are normally diploid, with the two sets of genes inherited from both parents. Most genes are expressed from both alleles, but a subset is expressed from only one. Monoallelic expression is regulated by ICR methylation that is specifically established during germ cell differentiation (oocyte or sperm) (Figure 1b). Mechanistically, the establishment of imprints in oocyte/sperm is dependent on the *de novo* methyltransferase *Dnmt3a* and stimulatory factor *Dnmt3l*. In fact, genetic deletion of either fails to establish imprints in mice.[31–34]

Parent-specific ICR methylation is maintained in embryos and adults by *Dnmt1* (Figure 1b). The functional significance of the original parental expression for mammalian development can be explained by the lethal phenotype of parthenogenetic and androgenetic mouse embryos as well as uniparental disomies.[35,36] It was previously demonstrated that *Dnmt1* knockout causes embryonic lethal and deletion of *Dnmt1* in mouse ES cells results in global DNA hypomethylation including imprinted loci.[13,37,38] Notably, once imprints are erased in mouse ES cells, they remain unmethylated even after differentiation, indicating that somatic cells lack the ability to re-establish imprints.[13,38] Given that aberrant

imprinting persists in somatic cells throughout the lifetime, the epigenetic stability of imprints should contribute to the developmental potential of pluripotent stem cells.

DERIVATION OF MOUSE PLURIPOTENT STEM CELLS FROM SOMATIC CELLS

Cellular specification and commitment are orchestrated by regulatory mechanisms that mediate spatiotemporal control of gene expression. Crosstalk between TFs and epigenetic regulations governs gene regulatory programs that drive precise control of gene expression patterns during development. Given that the epigenetic control of gene regulatory programs is a largely irreversible process, it was thought that somatic cells are unlikely reprogrammable to the pluripotent state once cells differentiate. However, that idea has since been refuted, as it was shown that somatic differentiated cells are reprogrammable to ES cell-like pluripotent cells by somatic cell nuclear transfer[39,40] and the ectopic expression of four TFs, *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* (hereafter referred to as OSKM).[41] The iPS cells were successfully generated from mouse fibroblasts in 2006[41] and from human fibroblasts in 2007.[42,43] Since then, many laboratories have succeeded in establishing iPS cells with various methods (eg, virus-based, integration-free, and *piggyBac* transposon system, among others).[44–50] Furthermore, a recent study showed that *Sall4*, *Nanog*, *Esrrb*, and *Lin28* could replace OSKM to induce somatic cell reprogramming into iPS cells in mice.[51] Consistent with reprogramming to the pluripotent state, the epigenetic signatures of iPS cells are similar with those of ES cells. Moreover, mouse iPS cells are competent for tetraploid complementation assay,[52] demonstrating that iPS cells are indeed pluripotent and are functionally equivalent to ES cells.[53] Collectively, either *ex vivo* culture of early embryos or somatic cell reprogramming can obtain pluripotent cells *in vitro*.

ABERRANT GENOMIC IMPRINTING IN MOUSE PLURIPOTENT STEM CELLS

Aberrant imprints have been implicated in various diseases. Notably, mouse ES cell lines have been shown to occasionally exhibit aberrant ICR methylation.[54] Previous studies also reported that several imprinted genes within the *Dlk1-Dio3* cluster are aberrantly silenced in most mouse iPS cell lines.[55] The aberrant silencing is accompanied by increased methylation at these ICRs (Figures 2a and b). These epigenetic alterations are not observed in most ES cells but only some iPS cell lines, suggesting that iPS cells exhibit considerable epigenetic variation when compared with ES cells. Together, it appears that genomic imprinting is vulnerable in mouse pluripotent stem cells *in vitro*. Given that genomic imprints are essential epigenetic regulation mechanisms in mammalian development, a key issue is whether the imprint status in stem cells affects stem cell functionality. Notably, mouse iPS cells harboring aberrant imprints at the *Dlk1-Dio3* cluster poorly contribute to chimeras and fail to produce all-iPS-cell mice by tetraploid embryo (4n) complementation, which is one of the most stringent tests for developmental assays (Figure 2a). Of note, histone deacetylase inhibitor and ascorbic acid reactivated the silenced loci in iPS cells and eventually rescued the defect permitting all-iPS-cell mice.[55,56] These findings indicate that the imprint status in pluripotent states significantly affects the developmental potential of iPS cells (Figure 2a). Collectively, the imprint status is key epigenetic regulation for the propagation of ICM-like naive pluripotency *in vitro*.

NAIVE AND PRIMED MOUSE PLURIPOTENT STEM CELLS IN *EX VIVO* CULTURE

Since ICM cells can contribute to all cell lineages of the body, they are functionally described as having 'naive pluripotency.'[22] Mouse naive ES cells derived from ICM of blastocysts have historically been maintained in serum and leukemia inhibitory factor (LIF) on feeder cells, and they indefinitely proliferate on culture dish and differentiate into all three germ layers including germ cell line when they are injected into blastocysts[57–59] (Figure 3). After post implantation, mouse epiblast cells form an egg cylinder structure at around E5.5–E6.5. EpiS cells are derived from post-implantation epiblast under media containing basic fibroblast growth factor and activin and defined as having 'primed pluripotency'[19,20,22] (Figure 3). EpiS cells can differentiate into various cell types *in vitro* and form teratomas, but they lack an ability to contribute to chimeras when injected into blastocysts.[19] While the maintenance of mouse ES cells is dependent on the LIF/Stat3 signaling pathway, the maintenance of EpiS cells is dependent on the FGF/ERK pathway.

Consistent with the *in vivo* epigenetic property of female developing embryos, mouse ES cells have two active X chromosomes (XaXa), whereas one copy of X chromosomes is inactive (XaXi) in EpiS cells (Figure 3). Transcriptionally, EpiS cells express core pluripotent marker genes including *Nanog* and *Oct3/4*. However, *Klf4* and *Zfp42* (*Rex1*) are downregulated in primed EpiS cells compared to naive ES cells.[60] Thus, there are marked functional and molecular differences between the naive and primed states.

CAPTURING GROUND STATE *IN VITRO* BY MODULATING EXTRINSIC SIGNALING PATHWAYS IN MICE

Although mouse pluripotent stem cells maintained *in vitro* are able to contribute to all somatic cell lineages, they contain heterogeneous populations in terms of morphology and transcriptional patterns of naive marker genes, suggesting that ES/iPS cells in conventional culture condition fluctuate between the naive ICM-like state and primed epiblast-like state (Figure 3). Moreover, ES/iPS cells exhibit global DNA hypermethylation, whereas ICM cells exhibit global hypomethylation, suggesting that ES/iPS cells *in vitro* do not faithfully capture the naive ICM-like state[22,61] (Figure 3). In 2008, Smith and colleagues discovered key signaling pathways to overcome such metastable characteristics.[62] Based on the

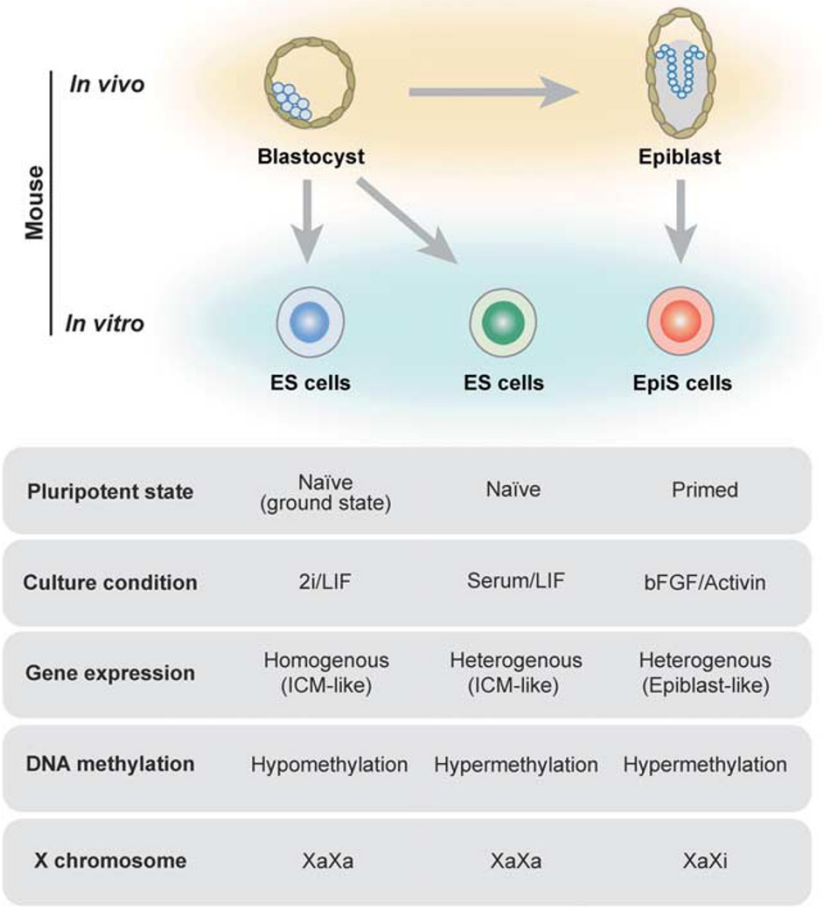

Figure 3 Transcriptional and epigenetic signatures in naive and primed mouse pluripotent stem cells. In mice, ES cells derived from ICM of blastocysts are defined as having naive pluripotency, while EpiS cells derived from post-implantation epiblasts are defined as having primed pluripotency. The X chromosome status is XaXa in naive state and XaXi in primed state (Xa; active X chromosome and Xi; inactive X chromosome). Serum/LIF-cultured ES cells display global DNA hypermethylation and heterogeneous expression patterns of naive pluripotent marker genes. 2i/LIF media enables ES cells to maintain homogenous ground-state pluripotency and global DNA hypomethylation.

fact that *Fgf4*$^{-/-}$ ES cells are compromised in their differentiation to neural and mesodermal lineages,[63] the FGF/ERK signal was identified as a key upstream pathway for cellular differentiation. Another key pathway is Wnt signaling, which enhances the self-renewing activity of ES cells.[64] Notably, dual inhibition of the FGF/ERK and GSK3 (MEK1/2 inhibitor; PD0325901 and Gsk3β inhibitor; CHIR99021), respectively (2i), makes it possible to propagate ground-state mouse ES cells by shielding cells from differentiation and reinforcing the core naive pluripotency circuit[65,66] (Figure 3). Consistent with the naive ICM-like state, exposing ES cells to 2i/LIF induces global DNA hypomethylation to an extent similar to ICM cells *in vivo*[67–70] (Figure 3). Moreover, 2i/LIF culture enhances the derivation of ES cells even from non-permissive mouse strains, which are refractory to ES cell propagation, and other species such as rats.[71–73] Collectively, the 2i/LIF culture system enhances stem cell propagation while reinforcing transcriptional and epigenetic properties of the ICM-like naive state. However, it has not been fully elucidated whether genetic and epigenetic stabilities of 2i-treated ES cells are stable. Recent studies demonstrated that derivation of ES cells directly from blastocysts in 2i/LIF and prolonged culture of ES cells in 2i/LIF result in loss of DNA methylation at imprinted loci[74,75] (Figure 4). Surprisingly, such imprint eroded ES cells compromise autonomous developmental potential by tetraploid embryo complementation and nuclear transfer[74,75] (Figure 4). Mechanistically, the inhibition of MEK1/2 is responsible for these opposing effects. In addition, replacement of the MEK1/2 inhibitor with an Src inhibitor (a2i includes Src inhibitor; CGP77675 and CHIR99021) preserves genetic and epigenetic stability as well as autonomous developmental potential[74,75] (Figure 4). Given that many laboratories have implemented 2i culture condition as standard practice since the discovery of 2i, these findings should be taken into account for future experiments using 2i-treated ES cells.

EPIGENETIC AND GENETIC INSTABILITY IN FEMALE MOUSE ES CELLS

Gender differences are thought to be another important aspect of mouse naive ES/iPS cells *in vitro*. Female ICM cells retain XaXa only for a transient period *in vivo*, while female ES/iPS cells sustain XaXa during cultivation *in vitro*. Recent accumulating studies showed that female mouse ES cells unexpectedly display global DNA hypomethylation including reduced methylation at imprinted loci.[76] This property is attributable to XaXa, since XO cells and XY cells exhibit similarly higher levels of DNA methylation.[76,77] Mechanistically, the expression levels of *Dnmt3a* and *Dnmt3b*, but not *Dnmt1*, are markedly lower in XX cells than in XO and XY cells,[76,77] suggesting that lower activity of *de novo* methyltransferases due to XaXa is responsible for the global hypomethylation. Indeed, ectopic expression of *Dnmt3a* or *Dnm3b* can rescue the reduced methylation levels at particular regions in female ES cells.[76] More recent studies showed that female mouse ES cells retaining XaXa displayed lower protein level of Uhrf1 than male ES cells, although mRNA level of Uhrf1 is comparable between male and female.[74,78] Moreover, reduced Uhrf1 protein level is linked with XaXa state since XO ES cells and XX MEFs exhibiting XaXi maintained Uhrf1 protein level.[74,78] These results suggest that female-specific hypomethylation is in part caused by impaired maintenance of DNA methylation due to two active X chromosomes. It is also interesting that female ES cells tend to lose all or part of one X chromosome during their propagation, while male ES cells occasionally lose Y chromosome, indicating that female ES cells are genetically unstable.[76] Collectively, female mouse ES cells often display epigenetic and genetic variations that are associated with XaXa. Considering persistent XaXa *in vitro*,

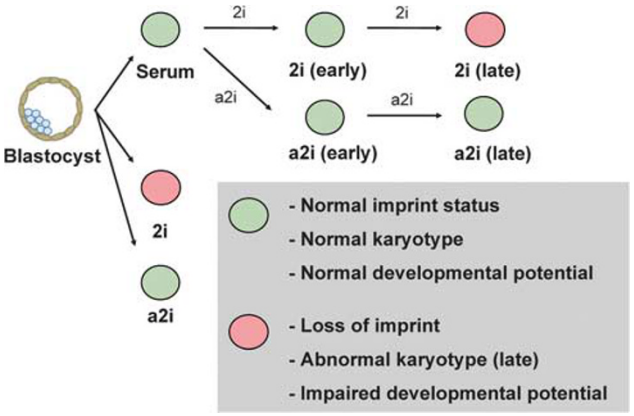

Figure 4 2i affects genetic and epigenetic stability. Mouse ES cells directly derived from blastocyst in 2i and exposed for a prolonged period in 2i display genetic and epigenetic instability (eg, loss of imprints and karyotypic abnormality), which affects full-term autonomous developmental potential by tetraploid embryo complementation. a2i condition (including Src inhibitor and Gsk3β inhibitor) preserves genomic imprints, chromosomal stability, and developmental potential.

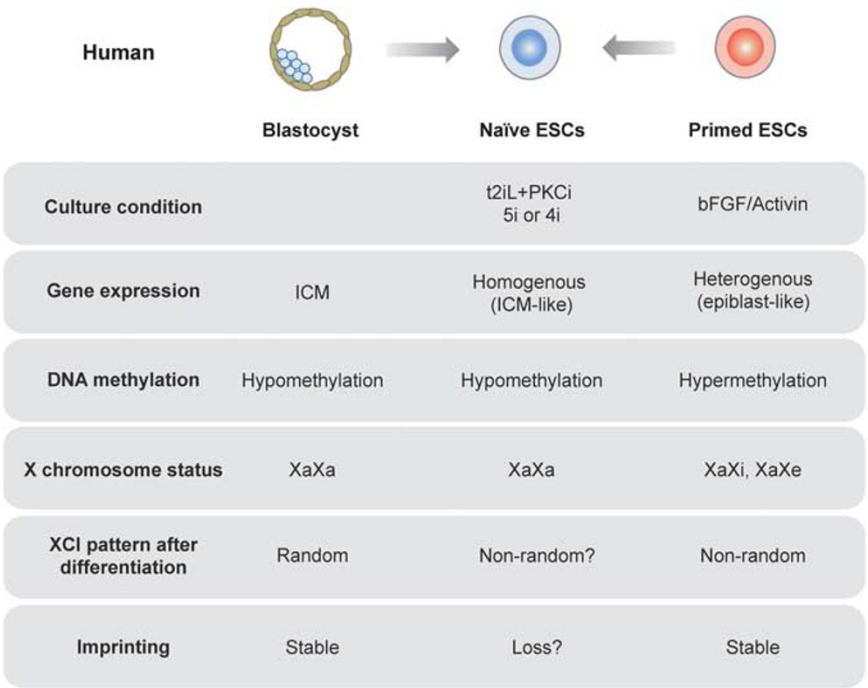

Figure 5 Molecular dynamics during naive conversion of human pluripotent stem cells *in vitro*. Recent studies revealed that human naive-like ES cells can be generated from primed ES cells or directly from blastocysts. Human naive-like ES cells acquire transcriptional profiling reminiscent of ICM cells and display global DNA hypomethylation. Notably, erosion of X chromosome (Xe)[89,90] in primed cells is canceled by naive conversion. However, genomic imprints are lost in human naive-like cells and non-random X chromosome inactivation occurs after differentiation, both of which are not observed in normal early development in humans. Collectively, current human naive-like cells do not fully recapitulate human naive pluripotency *in vivo*.

such variations should be considered for capturing naive ICM cells *in vitro*.

EPIGENETIC PROPERTIES IN HUMAN NAIVE PLURIPOTENT STEM CELLS

Human ES/iPS cells display primed pluripotency features, which correspond to an advanced stage of pluripotent cells that resembles post-implantation epiblast. A number of studies have sought to acquire naive pluripotency in human cells *in vitro*.[79–83] Based on recent transcriptome and epigenetic analyses, it seems that two methods, (i) transgene (*NANOG* and *KLF2*)-dependent system in conjunction with 2i/LIF (t2i/L+PKCi) media[82,83] and (ii) chemical conditions with 5i/L/A or 4i/L/A,[80] succeeded the transition of primed pluripotent stem cells to the naive state[84,85] (Figure 5). Naive human ES cells were similarly established directly from human blastocysts.[83] Consistent with ICM-like pluripotency, human ES cells in these conditions display global DNA hypomethylation and the gene expression patterns of naive markers and transposons, which are reminiscent of pre-implantation human embryos[84–86] (Figure 5). Of particularly note, inactive X chromosome becomes active in naive-like female cells *in vitro*, suggesting that reactivation of X chromosome occurs during naive conversion[84,87,88] (Figure 5). Therefore, it is expected that human naive cells serve as a powerful tool for studying human early embryogenesis and human diseases.

However, more recent studies demonstrated that naive human ES cells tend to exhibit non-random X chromosome inactivation upon their differentiation[87] (Figure 5). This phenomenon does not mimic human development *in vivo*. Similarly, several studies reported that ICR methylation in human naive cells was markedly decreased although the original primed pluripotent stem cells retained the methylation[84,86] (Figure 5). Such aberrant imprinting in naive state was inherited even after re-priming, which is consistent with the fact that cells outside of germline lack the ability to re-establish imprints in mice. Taken together, current human naive cells fail to faithfully recapitulate ICM cells *in vivo* in terms of epigenetic aspects. Considering the critical role of genomic imprints in developmental potential, there remain notable differences between *in vitro* naive cells and *in vivo* ICM cells in humans.

PERSPECTIVE

Pre-implantation embryos undergo dynamic epigenetic reprogramming, which is essential for the mammalian life cycle. Proper reprogramming confers pluripotency on early embryos. Pluripotent stem cells *in vitro* capture many aspects of pluripotency *in vivo* and therefore provide a powerful experimental platform to study early embryogenesis. Accumulating evidence has suggested that 2i/LIF culture system maintains transcriptional and epigenetic signatures reminiscent of naive ICM-like cells in mice.[65] Indeed, 2i/LIF-cultured mouse ES cells display a number of shared characteristics with ICM cells.[66–68] Moreover, previous studies have provided insightful clues for naive transition of primed human pluripotent stem cells using culture conditions containing 2i. However, recent studies indicated that MEK1/2 inhibitor may cause genetic and epigenetic instability of mouse ES cells, which is associated with impaired developmental potential. Indeed, current human naive-like cells harbor karyotypic abnormalities and epigenetic abnormalities, such as a loss of imprints and distinct X chromosome regulation. Notably, a2i culture condition, in which a MEK1/2 inhibitor is replaced with an Src inhibitor, can be used for mouse ES cell maintenance. Moreover, a2i-cultured ES cells maintain genetic and epigenetic stability as well as autonomous developmental potential. These findings may help for generating human naive pluripotent state, which retains genetic and epigenetic stability. Given that pluripotent stem cells offer hope not only for understanding of human embryogenesis, but also for cell transplantation therapy as well as drug discovery, it will be necessary to integrate the complexity of epigenetic regulation in pluripotent stem cells *in vitro* into standard approaches that faithfully capture *in vivo* ICM cells on culture dish.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest. S.Y. is a scientific advisor of iPS Academia Japan without salary.

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