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Running Title: Intra-population heterogeneity in hPSCs

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Abstract

Human pluripotent stem cells (hPSCs) include human embryonic stem cells (hESCs) derived from blastocysts and human induced pluripotent stem cells (hiPSCs) generated from somatic cell reprogramming. Due to their self-renewal ability and pluripotent differentiation potential, hPSCs serve as an excellent experimental platform for human development, disease modeling, drug screening, and cell therapy. Traditionally, hPSCs were considered to form a homogenous population. However, recent advances in single cell technologies revealed a high degree of variability between individual cells within a hPSC population. Different types of heterogeneity can arise by genetic and epigenetic abnormalities associated with long-term *in vitro* culture and somatic cell reprogramming. These variations initially appear in a rare population of cells. However, some cancer-related variations can confer growth advantages to the affected cells and alter cellular phenotypes, which raises significant concerns in hPSC applications. In

contrast, other types of heterogeneity are related to intrinsic features of hPSCs such as asynchronous cell cycle and spatial asymmetry in cell adhesion. A growing body of evidence suggests that hPSCs exploit the intrinsic heterogeneity to produce multiple lineages during differentiation. This idea offers a new concept of pluripotency with single cell heterogeneity as an integral element. Collectively, single cell heterogeneity is Janus-faced in hPSC function and application. Harmful heterogeneity has to be minimized by improving culture conditions and screening methods. However, other heterogeneity that is integral for pluripotency can be utilized to control hPSC proliferation and differentiation.

Introduction

Pluripotent states are recently recognized to be a spectrum of highly metastable cellular states that range from naïve to primed states (1, 2). Pluripotent stem cells (PSCs) in pre-implantation blastocysts represent the naïve pluripotent state, while PSCs in post-implantation embryos are considered to be in the primed pluripotent state. The naïve to primed transition of PSCs occurs in early embryos during the peri-implantation stage and this transition is essential for proper post-implantation development (1, 2). More importantly, the metastable nature of PSCs can be found in single cells within an *in vitro* PSC population. When mouse PSCs (mPSCs) were cultured with serum, individual cells dynamically transit between naïve and primed states, resulting in substantial single cell heterogeneity within a mPSC population (3, 4). Likewise, the coexistence of naïve and primed cells was reported in conventional hPSC culture (5). Given the highly flexible nature of pluripotent states, single cell heterogeneity can be viewed as an intrinsic feature of PSC populations. In contrast, various single cell heterogeneity types can appear in a hPSC population by artificial manipulation such as long-

term *in vitro* culture and somatic cell reprogramming, which poses significant risks on hPSC-based therapy. Inherently, substantial heterogeneity of cells raised in *in vitro* culture is not limited to PSC lines. In fact, studies on HeLa cell lines found that significant genomic and phenotypic variabilities existed in HeLa variants from 13 international laboratories (6). In the field of hPSCs, such artifact-induced heterogeneity is already considered as a serious drawback of the reprogramming method and attempts have been made to develop safer techniques for iPSC derivation. However, technical limitations still exist in hiPSC generation and validation, while rare mutations within a subset of cells can induce genetic and functional mosaicism in hiPSC lines. Thus, the objective of this review is to cover both artifact-induced heterogeneity and naturally existing heterogeneity in hPSCs. Specifically, we focus on four different types of heterogeneity (genetic, epigenetic, cell cycle, and positional heterogeneity) that can potentially affect hPSC biology and applications.

Genetic heterogeneity

Although hPSCs can be derived from a single cell, *de novo* mutations that occur randomly in individual cells create cell-to-cell variation in genetic information within a hPSC population. Majority of *de novo* mutations are considered to be neutral, but some mutations may affect physiological functions of hPSCs or hPSC-derived cells. Particularly, cancer-associated mutations raise safety concerns about hPSC-based regenerative medicine. Besides a few well-characterized mutations such as the ones in TP53, most mutations in hPSCs have poorly understood mechanisms and unspecified effects. Here, we summarize recent understanding of genetic variations that potentially contribute to single cell heterogeneity of hPSCs.

Types of de novo mutations

In hPSCs, karyotypic abnormalities are the most widely observed genetic changes in routine screening by cytogenetic techniques. Abnormal karyotypes include numerical aneuploidies such as gain or loss of whole chromosomes and structural aneuploidies such as translocations, deletions, duplications, or insertions of a chromosome segment (7-9). Interestingly, it was reported that more than 70% of karyotypic abnormalities observed in ESCs are chromosomal gains (10), suggesting that PSCs have more tolerance to gains than losses. In order to figure out genetic changes that arise during hPSC culture, the International Stem Cell Initiative (ISCI) analyzed 125 hESC lines and 11 hiPSC lines from worldwide, representing major ethnic groups (7). In this study, most hPSC lines showed grossly normal karyotypes. However, after prolonged culture, increased propensity of karyotypic abnormalities was observed in some lines. Commonly detected aberrations include gains on chromosomes 1, 12, 17, and 20. Of particular interest was minimal amplicon in chromosome arm 20q that contained three expressed genes in hPSCs, *ID1*, *BCL2L1*, and *HM13*. Strikingly, this structural variant gain appears in more than 20% of the hPSC lines analyzed in the ISCI study. This example suggests that certain types of karyotypic abnormalities could confer enhanced fitness to mutant cells, which potentially drives time-dependent changes of genetic heterogeneity within a hPSC population.

Copy number variations (CNVs) represent amplification or deletion of small regions that affects the number of copies of particular genes. Analysis of 17 different hESC lines identified 843 CNVs of 50 kb ~ 3 Mb in size (11). Prolonged culture of hESCs resulted in changes in 24% of the loss of heterozygosity sites and in 66% of the CNVs. More importantly,

altered expression was observed in 30% of the genes within the CNV sites, and 44% of them were functionally connected to cancer. These results suggest that hPSCs exhibit a high degree of CNV mosaicism within a population and urge routine screening for CNVs in hPSC culture.

Global analysis of point mutations requires high-throughput sequencing that hampers routine screening during hPSC maintenance. Therefore, the repertoires of point mutations in hPSCs have not been fully investigated. Recently, whole exome sequencing was performed in 140 independent hESC lines that involve 26 lines for potential clinical use (12). To select culture-acquired mutations, they focused on mosaic variants that appear in a subset of cells and identified 263 candidate mosaic mutations. Among these mutations, 28 were predicted to have detrimental effects on gene function. Interestingly, the tumor suppressor gene *TP53* was the only gene with multiple mutations in several independent hESC lines. All identified mutations in *TP53* involved a cytosine of CpG dinucleotide, a highly mutable site, and were related to residues frequently disrupted in human cancer. Additionally, the analysis of published RNA sequencing data from 117 hPSC lines discovered another 9 mutations on *TP53* (12). These results suggest that culture-related acquisition of point mutations is not a rare event in hPSCs and this type of genetic variations could significantly increase single cell heterogeneity in hPSC populations.

Origins of de novo mutations

As in other cultured cells, environmental agents such as carcinogens and oxidative stress likely induce *de novo* mutations during hPSC culture. Whole genome sequencing of hiPSCs exposed to 79 environmental carcinogens revealed that 52% of agents tested in the study generated mutational signatures including base substitution, insertions, and deletions (13).

Distinct from fully differentiated cells, hPSCs exhibit rapid cell cycle, which potentially induces *de novo* mutations by DNA replication stress. Persistent replication stress during the S phase causes significantly higher DNA damage in hPSCs compared to somatic cells. Such replication stress is marked by slower DNA replication speed, collapse of replication forks, and activation of latent replication origins (14-16). Errors incorporated during the DNA repair process can result in genetic abnormalities (17). The DNA replication defects can also lead to the formation of mitotic errors. Through real-time examination of chromosomes during mitosis, abnormal mitosis was observed in over 30% of hPSCs (18). These abnormalities include lagging chromosomes and chromosomal bridges. Furthermore, compared to somatic cells, hPSCs showed more defects in chromosome condensation and segregation (19). Overall, culture-related environmental and replication stresses act as major sources for genetic abnormalities in hPSCs.

For hiPSCs, cellular reprogramming serves as another source of genetic abnormalities. Whole exome sequencing revealed that the rate of coding mutations was significantly elevated during cellular reprogramming of somatic cells into hiPSCs (20). Among point mutations identified from hiPSCs, 7% of them were related to *in vitro* culture and 19% were present as rare mutations in parental somatic cells, suggesting that the remaining 74% of mutations were generated during the cellular reprogramming process. Furthermore, simulation indicates that the mutation rate during reprogramming is nine times higher than of the mutation rate during cell culturing (20). In-depth mutation analysis by whole genome sequencing found that reprogramming-related mutations were underrepresented in protein-coding genes and in open chromatin regions. These mutations were predominantly localized in lamina-associated heterochromatic domains (21). Analysis of mutational signatures of iPSCs implicates oxidative

DNA damage as a dominant source of reprogramming-induced mutations (21, 22). Given the prevalent *de novo* mutations generated by cellular reprogramming, hiPSCs likely exhibit high genetic mosaicism within a population. However, the effect of genetic mosaicism on physiology of hiPSCs and hiPSC-derived cells remains unknown.

Defensive mechanisms for genetic mutations

Using a clonogenic strategy with two clinical grade hESC lines, the mutation rate of hPSCs was estimated as 0.23~0.30 X 10^{-9} single nucleotide variants (SNVs) per cell division (23), which is much lower than the estimated mutation rate of 2.66 X 10^{-9} SNVs per cell division in somatic cells (24). Although no obvious hotspots were observed across all chromosomes, the mutation rate was higher in intergenic regions than in exons and introns (23). The mutation signatures suggested oxidative damage as a major source of mutations, which was supported by reduced mutation rates in hPSCs cultured in low oxygen condition (23). Elevated expression of antioxidant enzymes in hPSCs compared to differentiated cells may serve as an essential factor in reducing the mutation rate (25, 26). Genes related to DNA repair pathways also showed higher expression levels in hPSCs than in somatic cells (26, 27). Consistently, hPSCs were reported to have enhanced DNA repair capacity with faster rate of base and nucleotide excision repair and faster resolution of interstrand crosslinks (27-29). Furthermore, double strand breaks tend to be repaired in hPSCs by homologous recombination that is less prone to errors than non-homologous end joining does (30).

Besides the high expression of anti-oxidant and DNA repair genes, hPSCs are featured by high sensitivity to apoptotic signals. Upon treatment of various DNA damaging agents including ultraviolet C radiation, hPSCs exhibited less damage than somatic cells (29, 31).

Despite the low damage levels, hPSCs responded with strong apoptosis, suggesting a low apoptotic threshold. These results were further confirmed by other apoptosis-inducing agents such as cisplatin, thymidine, and nocodazole (18, 32, 33). Mechanistically, hPSCs exhibited higher expression of proapoptotic proteins such as PUMA, NOXA, BIK, BIM, and BMF, while having lower expression of an antiapoptotic protein, Bcl-2 (31, 34, 35). Efficient eradication of damaged cells by apoptosis establishes a powerful quality control system to safeguard genomic integrity of hPSCs. Overall, hPSCs engage multiple mechanisms including elevated expression of anti-oxidant and DNA repair genes and a low threshold of apoptosis to minimize mutation rates and genetic mosaicism in a population.

Consequences of genetic abnormalities in hPSCs

In hPSC culture, genetic abnormalities occur randomly in low frequency and thus cells with genetic variants initially constitute a rare subpopulation. However, culture-associated population bottleneck that are frequently observed in clonal expansion can select rare mutant cells, resulting in domination of the genetic variants. Nevertheless, recurrence of specific genetic abnormalities in independent hPSC lines implicates that some genetic mutations can confer a selective growth advantage to hPSCs. This idea is supported by the observation in which the proportion of mutant cells gradually increases in culture over time (8, 36, 37). Acquisition of growth advantage is driven predominantly by effects of genetic variants on cellular physiology of hPSCs and hPSC-derived cells, raising significant concerns about hPSC application for human developmental studies, disease modeling, drug screening, and cell replacement therapy.

Trisomy of chromosome 12 is one of the most common karyotypic abnormalities

observed in hPSC culture (7). Trisomy 12 significantly altered the global transcriptional profile of hPSCs similar to that of germ cell tumors (38). As a consequence, trisomy 12 increased the proliferation rate and tumorigenicity in hPSCs (38). *NANOG* is a major candidate for driver gene located on chromosome arm 12p because *NANOG* is essential for pluripotency maintenance (39). Moreover, overexpression of NANOG was sufficient to block stem cell differentiation as well as to enhance colony forming capacity in a clonal density (39, 40). Other genes such as *DPPA3*, *GDF3*, and *KRAS,* located on chromosome arm 12p, can also contribute to the effect of trisomy 12 on the altered behaviors of hPSCs (41, 42).

Amplification of chromosome 20q11.21 that were found in over 20% of hPSC lines (7) increased hPSC growth by reduced sensitivity to apoptosis (43). Of the three genes expressed in hESCs that are located within the minimal amplicon, *BCL2L1* was identified as a candidate driver gene because its splice variant, BCL-XL, acts as a key antiapoptotic protein (43). Overexpression of BCL-XL phenocopied amplification of chromosome 20q11.21 with enhanced hPSC growth, while knockdown of BCL-XL blocked the growth advantage of cells containing the chromosomal abnormality (43). In addition, gain of 20q11.21 influenced hPSC differentiation, specifically impairing neuroectoderm derivation (44, 45). BCL-XL overexpression was sufficient to mirror the differentiation defect of the mutant hPSCs by perturbing SMAD and TGF-β signaling (44). Similarly, another antiapoptotic gene, *BIRC5*, was identified on chromosome arm 17q, whose gain confers a growth advantage to hPSCs by increased resistance to apoptosis (46). Furthermore, gain of chromosome arm 17q altered hPSC differentiation and enhanced mesodiencephalic dopaminergic neuron differentiation (47). In this case, elevated expression of *WNT3* and *WNT9B* was suggested to be involved in the skewed differentiation (47).

As mentioned above, recurring point mutations on the *TP53* gene were reported in multiple unrelated hPSC lines (12), suggesting a selective growth advantage of the mutations. The *TP53* mutations are dominant negative and are frequently observed in human cancers (12). A number of studies revealed that p53 proteins (encoded by the *TP53* gene) play pleotropic roles in PSCs, which include suppression of pluripotency by elevated transcription of non-coding RNAs, differentiation induction by upregulation of developmental genes and lineage transcription factors, and blockade of cellular reprogramming (48). Taken together, some karyotypic abnormalities and point mutations provide selective growth advantages to mutant cells, which enable the genetic mutations to dominate hPSC populations. At the same time, the genetic mutations alter hPSC functions including proliferation, differentiation, and apoptosis. Therefore, genetic mosaicism that arises in hPSC culture is an integral source of functional heterogeneity of single cells within a hPSC population.

Epigenetic heterogeneity

Unlike genetic heterogeneity, epigenetic variations do not change the genomic sequence. Whereas, these variations are inherited to daughter cells and possess the potential to alter gene expressions and cellular phenotypes. In hPSCs, epigenetic alterations often result from cell culture passaging or somatic cell reprogramming and major alterations occur in DNA methylation, imprinted epigenetic marks, and X chromosome inactivation. The focus of this section will be on the three different types of epigenetic variations, their mechanisms, and their consequences within a hPSC population.

DNA methylation

DNA methylation is an epigenetic process where the methyl groups are added to the fifth carbon of cytosine residue to form 5-methylcytosine (5-mC). In mammals, DNA methylation usually occurs at the CpG dinucleotides in various genomic regions including transposable elements, imprinted regions, gene bodies, and some inactive regulatory elements (49). Considered as a stable epigenetic modification, DNA methylation may induce heterochromatin and gene repression. However, dynamic regulation of this modification was observed during embryonic development (50, 51). After fertilization, the demethylation process is rapidly activated, resulting in global hypomethylation in the blastocyst stage. Then, DNA methylation is re-established during post-implantation development. Although hPSCs are derived from inner cell mass of the human blastocysts, they resemble post-implantation epiblasts. Consistently, hPSCs exhibit high levels of global DNA methylation, which is critical for hPSC maintenance. For instance, DNMT1 is an enzyme that catalyzes addition of 5-mC to the newly synthesized DNA strand during S-phase and knockout of this enzyme causes robust cell deaths in hPSCs (52).

During long-term *in vitro* culture, various aberrations of DNA methylation accumulate in individual hPSCs (53, 54). Interestingly, the characteristic feature of these aberrations is gene silencing by hypermethylation, which is frequently found in tumors (55). Specifically, Weissbein et al, utilized genome-wide DNA methylation profiles and identified a gene named *TSPYL5* that recurrently acquires hypermethylation upon prolonged culture (56). Silencing of *TSPYL5* by methylation upregulated growth promoting genes while suppressing differentiation-related genes and tumor-suppressor genes. Another study in hPSCs found recurrent hypermethylation of an anti-oxidant gene, *CAT* (53). Interestingly, methylation-

driven downregulation of anti-oxidant genes could make hPSCs vulnerable to oxidative stress-induced genetic aberrations, linking the epigenetic changes to genetic abnormalities.

Given that massive epigenetic remodeling drives cellular reprogramming, single cell variability in DNA methylation could arise during the reprogramming process and could be maintained within an iPSC population. Indeed, many reports showed that cellular reprogramming was insufficient to completely remove the identity of the donor cell and thus the reprogrammed cells possess residual epigenetic patterns similar to its origin. Such somatic epigenetic memory influences differentiation propensity of iPSCs towards a similar lineage of the origin cells. For example, iPSCs derived from fetal brain retain some DNA methylation patterns of brain tissues and have a higher tendency to differentiate into neural lineage cells (57). Similarly, hiPSCs derived from beta cells maintained epigenetic memory in DNA methylation and open chromatin structure at beta cell genes and consequently showed increased ability to generate insulin-producing cells both *in vitro* and *in vivo* (58). Taken together, long-term culture and cellular reprogramming induce variation in DNA methylation across individual hPSCs and some of the variations affect proliferation and differentiation of hPSCs.

Parental imprinting

Parental imprinting is an epigenetic process that induces the parental specific monoallelic expression in selected gene groups. In this process, differentially methylated regions (DMRs) are established at different loci of the oocyte and sperm genomes, which discriminates maternal and paternal alleles. At the imprinted loci, DMRs remain stable and induce silencing of nearby genes, resulting in monoallelic expression of about 100 imprinted

genes (59). In order to achieve proper embryonic development, imprinting is demanded and acts as a barrier for uniparental reproduction as normal development does not occur in the same-sex genome oocyte experiment (60, 61). Imprinting is highly stable across different tissues and loss of imprinting (LOI) is frequently associated with human developmental disorders, such as Prader-Willi, Angelman syndromes, and cancers (62-64). LOI is defined as loss of monoallelic gene expression that can lead to either complete silencing or biallelic transcription of affected genes.

In hESCs, low incidence of LOI was observed, suggesting a high degree of imprinting stability during hESC derivation and maintenance (65, 66). However, examination of hiPSCs and somatic cell nuclear transfer (SCNT)-derived hESC revealed significantly high LOI incidence (67-69). Recently, a large-scale analysis of LOI with more than 270 hPSC lines confirmed that hiPSCs acquire higher frequency of LOI than hESCs. These results suggest that global epigenetic changes that occur during somatic cell reprogramming are the major source of LOI in hPSCs. Although hiPSCs are initially derived from a single parental cell, global epigenetic changes can induce LOI at different loci across individual reprogramming cells, which likely leads to increased epigenetic heterogeneity within a hiPSC population.

Conventional hPSCs are considered to be in a primed pluripotent state and they resemble post-implantation epiblasts. Recently, various medium conditions were developed to culture hPSCs in a naïve pluripotent state that represent the pre-implantation stage. Naïve hPSCs are featured by global reduction of DNA methylation, which is similar to pre-implantation epiblasts. Despite the low DNA methylation, pre-implantation epiblasts in embryos exhibit high imprinting stability. In contrast, naïve hPSCs in culture showed LOI at significant number of loci (70, 71). These results suggest that current medium conditions for

naïve pluripotency do not fully capture the *in vivo* state of pre-implantation epiblasts. Given the importance of imprinting in embryonic development and cancer, further improvements are required in order to use *in vitro* naïve hPSCs for developmental biology and cell therapy.

Difference in LOI abundance was also detected across genes. Different genes showed dynamic levels of resistance to imprinting aberrations. For example, genes like *KCNQ1OT1*, *SNRPN*, and *PEG3* showed high resistance to aberrations while other genes such as *RHOBTB3*, *TFPI2*, *H19*, *IGF2*, and *ZDBF2* were susceptible to aberrations (72). The genes that were prone to LOI have been consistent in hPSCs and cancer cells, meaning that LOI on these genes could provide selective advantages such as self-renewal promotion and apoptosis resistance to cells (73, 74). Gene-specific difference in sensitivity of aberration is related to the parent-of-origin of methylation because LOI appears more abundantly in paternally silenced genes (66, 72). Additionally, LOI at specific loci is related to human embryonic development. In Prader-Willi syndrome patients, epigenetic silencing of the paternally expressed gene, *NDN*, is considered to dysregulate GABA signaling because *NDN* is required for GABAergic neuron differentiation in mouse brain culture. Silencing of the maternally expressed gene, *UBE3A*, in Angelman syndrome is involved in cortical atrophy and Purkinje cell loss (75). These results highlight the significant effects of imprinting aberrations on stem cell differentiation. Overall, LOI emerges in hPSCs mainly by dramatic epigenetic changes that occur during somatic cell reprogramming or prolonged culture in naïve medium conditions. Furthermore, LOI at specific loci can not only confer selective growth advantages to individual cells, but also influence differentiation potential of stem cells, thereby contributing to functional heterogeneity in a hPSC pool.

X chromosome inactivation

In mammals, X chromosome inactivation (XCI) is a dosage compensation mechanism that transcriptionally silences one of the two X chromosomes in female cells (76). Unlike genomic imprinting, where the process occurs at specific loci, XCI takes place over the whole X chromosome. In mouse embryos, both X chromosomes are active in pre-implantation epiblasts. Random XCI appears in the peri-implantation stage with monoallelic expression of the non-coding RNA *Xist*. *Xist* plays a key role in XCI by coating on the inactive X chromosome (Xi). In contrast, recent studies suggest compelling difference in timing and mechanisms of XCI between mouse and human. Single cell transcriptomic analysis on human embryos found that dosage compensation of X-lined genes occurs while the genes maintain biallelic expression, which is called X dampening (77). The results suggest that different, yet unknown mechanisms are involved in X chromosome dosage compensation in early human embryos. Nevertheless, the ongoing debates are about whether dosage compensation is achieved by XCI or X dampening during early human development (77, 78).

Cumulative evidence suggests that female hPSC lines have different X chromosome states, not only between different lines, but also between different passages of the same line (65, 79-84). Three major types of XCI states can be identified in hPSCs. These are no XCI (XaXa), full XCI (XaXi), and partial XCI due to an erosion (XaXe). Unlike primed hESCs that have inactive X chromosome (XaXi), naïve hESCs derived either from primed hESCs or from blastocysts contain two active X chromosomes (XaXa) (82, 85). Upon differentiation, naïve hESCs induce XCI. In contrast to the random XCI *in vivo*, differentiating naïve hESCs exhibit non-random XCI with skewed silencing of the same X chromosome (82), suggesting that the current naïve culture conditions do not fully capture the epigenetic state of pre-implantation epiblasts. Surprisingly, derivation or maintenance of primed hESCs under physiological

oxygen (5% O_2) exhibits two active X chromosomes and random XCI upon differentiation (86). Given the dynamic nature of X chromosome states, metastable stem cell states and local fluctuation of oxygen concentrations in hPSC culture could induce single cell heterogeneity in XCI and the expression of X-linked genes.

XCI erosion is widespread in hPSC lines and is associated with *XIST* loss and upregulated biallelic expression of some X-lined genes. Large-scale analysis with 23 hPSC lines revealed that XCI erosion occurs during the early stages of *in vitro* culture (87). Therefore, it is plausible that the culture-associated XCI erosion at different loci of X chromosome induces epigenetic and gene expression variations across individual cells. More importantly, this abnormal epigenetic alteration present in hPSCs is maintained in differentiated cells, which results in elevated X-linked gene expression and consequential dysregulation in cellular phenotypes (81, 87). Taken together, XCI is dynamically regulated in hPSCs depending on the developmental stages and culture environments, whereby the XCI states would significantly contribute to single cell heterogeneity in X-linked gene expression within a hPSC population. Therefore, it is crucial to develop hPSC culture conditions that ensure homogenous XCI states and minimize X chromosome erosion.

Cell cycle heterogeneity

During cell cycle, most cell lines and differentiated cells spend more time in G1 phase than in other phases. However, hPSCs are featured by a unique cell cycle pattern with relatively short G1 phase and long S/G2/M phase (88, 89). G1 lengthening is associated with hPSC differentiation, suggesting that short G1 phase of hPSCs plays an important role in stem cell self-renewal (90). Previously, G1 lengthening was considered to be a general phenotype of

stem cell differentiation. However, recent reports revealed that G1 lengthening was regulated in a lineage-specific manner. Significant G1 lengthening was observed during the early stage of neuroectoderm differentiation (25), whereas short G1 length was maintained during mesendoderm differentiation (25). Elongated G1 phase induces neuroectoderm fate specification by activating primary cilia and the downstream autophagy-Nrf2 pathway (25). These results suggest that the cell cycle is tightly connected to differentiation machinery of stem cells.

The development of Fluorescent Ubiquitin Cell Cycle Indicators (FUCCI) system revolutionized the cell cycle research by enabling single cell analysis of the cell cycle in live cells (91). Based on cell cycle phase-dependent degradation of CDT1 and Geminin proteins, the FUCCI system allows visualization of cell cycle states in different fluorescent colors. For example, G1 phase cells display red fluorescence and cells in S/G2/M phase show green fluorescence. Asynchronous cell cycle progression was clearly visualized in a hPSC population with individual cells being in different cell cycle states (92). Given the dramatic changes in gene expression and cellular phenotypes during the cell cycle, this asynchronous cell cycle represents a major source of single cell heterogeneity in a hPSC population. To test if cell cycle states influence differentiation potential of hPSCs, FUCCI-hPSCs were sorted based on cell cycle states, followed by differentiation (92). Cells in S/G2/M phase were refractory to differentiation and maintained undifferentiated states (92, 93). However, cells in G1 phase readily underwent differentiation, suggesting that G1 phase establishes a critical widow during which stem cells can execute fate transition in response to differentiation cues. More importantly, cells in early G1 phase showed biased differentiation toward the mesendoderm lineage, while cells in late G1 phase predominantly differentiated into the neuroectoderm

lineage (92). These results propose an interesting idea that a hPSC population utilizes asynchronous cell cycle to produce multiple lineages upon differentiation. Mechanistically, the cyclin D-CDK4/6 complex regulates nuclear shuttling of SMAD2/3, thereby modulating the developmentally important Activin/Nodal signaling pathway (92). Moreover, cyclin D can directly binds to lineage genes and regulates the transcription (94). Cell cycle-dependent epigenetic changes link asynchronous cell cycle in hPSCs to gene expression heterogeneity. 5-hydroxymethylcytosine (5-hmC) is generated from 5-mehtylcytosine (5-mC) by TET enzymes (95). FUCCI-based cell cycle fractionation revealed that the global 5-hmC level increases during G1 phase, which is associated with elevated expression of lineage genes in late G1 phase (96). Taken together, asynchronous cell cycle creates single cell heterogeneity in gene expression and signaling pathways. Thus, the cell cycle heterogeneity is exploited by hPSCs to produce multiple germ layers.

A recent study further extended the role of cell cycle heterogeneity in pluripotent differentiation by combining the FUCCI system with time-lapse imaging (97). Live cell imaging of FUCCI-hESCs enabled the measurement of absolute time of each cell cycle state in a single cell level. This analysis revealed that individual hPSCs showed high variation in the length of G1 phase ranging from 4h to over 10h. Furthermore, the absolute G1 length of single hPSCs is related to the differentiation potential. Cells with short G1 length showed biased differentiation potential towards the mesendoderm lineage. However, cells with long G1 length acquired neuroectoderm differentiation potential. Consequently, the single cell distribution of G1 length determines differentiation propensity of hPSC populations, linking the high G1 length variation to multi-lineage differentiation potential. These results further consolidate the idea that cell cycle heterogeneity contributes to the pluripotency of hPSCs.

Positional heterogeneity

Inside blastocysts, epiblasts form a cellular aggregate called ICM. Upon implantation, a major morphogenetic change occurs to transform ICM into a single layered epithelium of epiblast (98). This transition is well conserved in amniotes and plays a key role for three germ layer derivation. *In vitro* culture of hPSCs nicely captures the morphological and functional features of post-implantation epiblasts as they grow in an epithelialized colony and are capable of producing three germ layers upon differentiation (99). Recently, micropatterning technology was employed to finely control the size and shape of hPSC colonies (100). Strikingly, cells in a hPSC colony showed different differentiation fates depending on their spatial locations. Upon BMP4-triggered differentiation, an epithelial hPSC colony formed an ordered structure of germ layers along the radial axis with neuroectoderm lineage cells in the center, endoderm cells in the periphery, and mesoderm cells in between the regions. These results suggest that positional heterogeneity in individual cells could play a key role in multi-lineage differentiation potential of hPSCs.

Traditional studies in developmental biology focused on the role of diffusible factors in multi-lineage derivation. Consistently, recent studies suggested that spatial restriction and gradients of signaling molecules contribute to geometrical derivation of three germ layers in a hPSC colony (101). Upon BMP4 treatment, secreted inhibitor NOGGIN restricts BMP4 responses to the colony edge (100, 102). At the same time, a gradient of Activin-Nodal signaling is established along the radial axis of hPSC colonies (100). On top of the morphogen gradients, receptor relocation was proposed as a cell-autonomous mechanism for positional heterogeneity (102). In the central region of hPSC colonies, TGF-β receptors were

predominantly localized at the lateral side of cells, which impedes cellular responses to apically applied TGF-β ligands. In contrast, cells in the colony edge showed apical localization of TGF-β receptors. These results suggest that the gradients of signaling molecules and differential receptor localization collectively contribute to positional heterogeneity in differentiation potential within a hPSC colony.

Cell-cell and cell-ECM interactions play a key role in embryonic morphogenesis. In pre-implantation ICM, the epiblast aggregate is formed and maintained mainly by cell-cell interaction (103). During implantation, increased cell-ECM interaction drives the morphogenetic transition towards epithelized epiblasts (104). Consistently, hPSCs rely on cell-ECM interaction for survival and proliferation (105, 106). Within a hPSC colony, mechanical asymmetry arises from spatial polarization of cell-cell and cell-ECM interactions (107). Cells in the periphery of hPSC colonies exhibit higher cell-ECM interaction with well-established integrin-based focal adhesions, while cells in the central region rely more on the E-cadherin-mediated cell-cell interaction. More importantly, this mechanical asymmetry plays a key role in spatial derivation of mesendoderm lineage cells (107). These results suggest that heterogeneity in cell adhesion leads to spatially heterogeneous hPSC colonies.

Single cell gene expression analysis revealed that hPSC subsets defined by surface antigen expression show different self-renewal abilities and lineage priming (108). A subpopulation of hPSCs with high expression of GCTM2, CD9, and EPCAM exists at the top of stem cell hierarchy and possesses the highest capacity for self-renewal (108). Recently, another study screened 12 different cell surface markers and identified N-cadherin with heterogeneous expression (109). N-cadherin was exclusively expressed in a subset of hPSCs that localizes in the periphery of colonies. N-cadherin+ cells served as founder cells of hPSCs

with elevated self-renewal ability. Single cell RNA-seq analysis revealed that N-cadherin+ founder cells share transcriptomic profiles with primitive endoderm cells, suggesting dynamic cell state transition in a hPSC population (109). Taken together, individual cells within a hPSC colony display functional heterogeneity depending on their spatial positions of either the center or the periphery of colonies. Furthermore, this positional heterogeneity plays a crucial role in multi-lineage derivation from hPSCs as well as in pluripotency maintenance.

Conclusions

Although single cell sequencing provides a powerful tool to identify and characterize various cell types that exist in a complex tissue and organisms (110), the high dropout rate hampers application of this tool to dissect single cell heterogeneity in a seemingly homogenous cell population. Nevertheless, recent advances in reporter systems and single cell imaging techniques have made significant contributions to expanding our understanding in cell-to-cell variation within a PSC population. Long-term *in vitro* culture of hPSCs, particularly the naïve culture conditions, induces genetic and epigenetic abnormalities in a subset of cells, resulting in heterogeneous hPSC populations. Furthermore, global epigenetic changes that occur during somatic cell reprogramming significantly increase genetic and epigenetic heterogeneity in hiPSC populations. Because some abnormalities related to cancer may affect cellular phenotypes of hPSCs, it is crucial to monitor these types of heterogeneity in hPSCs.

Asynchronous cell cycle in hPSCs establishes another type of heterogeneity where individual cells are present in different stages of the cell cycle. Moreover, absolute lengths of G1 phase cause single cells to exhibit extreme variability, adding additional complexity to the cell cycle heterogeneity. Because hPSCs grow as an epithelialized colony, spatial positions of

individual cells also contribute to the heterogeneity in part through differential cell adhesion. More importantly, cell cycle and positional heterogeneity play a key role in multi-lineage derivation from a hPSC population because the variability primes cells to make different responses to differentiation cues. Interestingly, such heterogeneity mentioned above may occur from alterations in the genomic instability, and vice versa. Although the direct correlation between various heterogeneity is not well known, it is probable to consider that variations in hPSCs are intricately linked to each other.

In conclusion, single cell heterogeneity is Janus-faced in hPSC function and application. In certain cases, intrinsic variability may underlie pluripotent differentiation potential, whereas harmful heterogeneity induced by culture and cellular reprogramming can jeopardize therapeutic application of hPSCs. Therefore, technical improvements in single cell sequencing are needed to provide a comprehensive view of single cell heterogeneity that exists in a hPSC population.

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Conflicts of interest

The authors declare no conflict of interest.

Figure legends

Figure 1. Single cell heterogeneity that exists in a hPSC population. (A) Genetic heterogeneity can be caused by karyotypic abnormality, copy number variation, and point mutations within a hPSC population. (B) Major epigenetic heterogeneity in hPSCs occurs in DNA methylation, parental imprinting, and X chromosome inactivation. (C) Asynchronous cell cycle stages across individual cells and G1 length variation establish a major single cell heterogeneity in a hPSC population. (D) Positional heterogeneity distinguishes cells in the center of hPSC colonies from ones in the periphery. Differences in cell adhesion, receptor localization, and N-cadherin expression contribute to functional heterogeneity in a hPSC colony.

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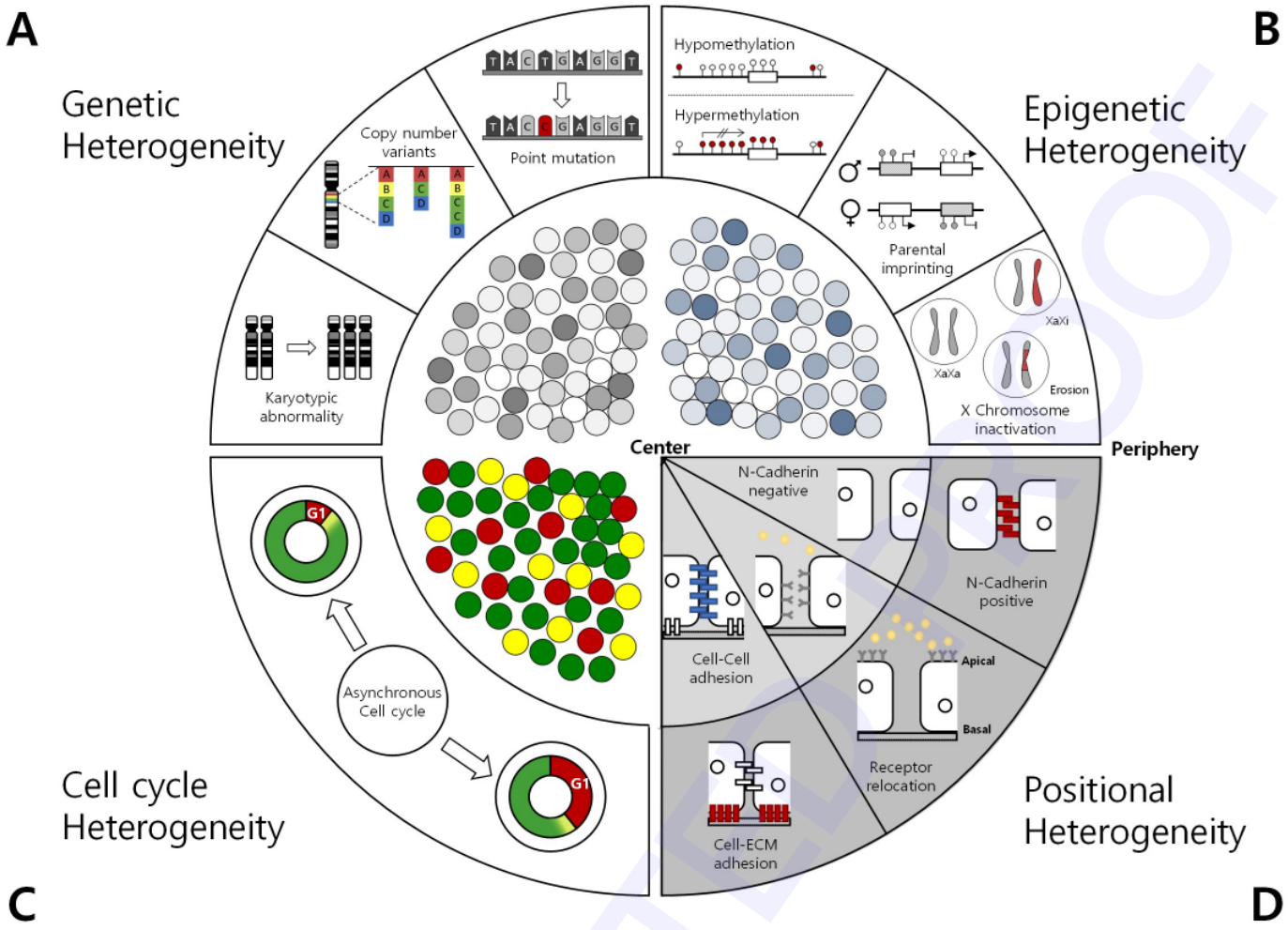

Fig. 1.