REVIEW

Maintenance of an undifferentiated state of human induced pluripotent stem cells through migration-dependent regulation of the balance between cell-cell and cell-substrate interactions

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ABSTRACT

We present an outlook on the current strategies for maintaining and culturing human induced pluripotent stem cells (hiPSCs) in an undifferentiated state without affecting their pluripotency. First, cell structures and function are described in relation to interactions between hiPSCs and their surroundings. Second, the phenomenon of spontaneous deviation from undifferentiated hiPSCs in feeder cell-dependent cultures is addressed, with a summary of current topics that are of particular interest to our studies. The key regulatory factors that can contribute to the growth and maintenance of undifferentiated hiPSCs are also discussed, with a summary of recent work toward a culture strategy to control hiPSC fate through balancing cell–cell and cell–substrate interactions. Finally, we discuss culture process design in our previous studies with respect to maintaining and expanding cultures of undifferentiated and pluripotent hiPSCs. We focus on the regulation of migration-dependent balance between cell–cell and cell–substrate interactions. This review offers novel insights into the regulation and processing of stem cells for research in regenerative medicine.

Keywords: Human induced pluripotent stem cells; Undifferentiated state; Cell migration; Cell–cell interaction; Cell–substrate interaction

INTRODUCTION

Induced pluripotent stem cells (iPSCs) are generated from various somatic cells by exogenous expression of defined transcription factors (Oct4, Sox2, Klf4, and c-Myc) that confer properties similar to those of embryonic stem cells (ESCs) (1, 2). Since iPSCs resemble ESCs in their ability to generate cells of all three germ layers, patient-specific iPSCs could resolve the ethical and histo-incompatibility issues surrounding the use of ESCs. iPSCs exhibit ESC-like properties in terms of morphology, proliferation, differentiation capacity, and genomic and epigenomic states (3, 4), suggesting that iPSCs could be regarded as an infinite cell source for many areas of research and medicine.

The expansion of iPSCs remains a challenging process because spontaneous differentiation is accompanied by loss of self-renewal and pluripotent qualities (5, 6). Therefore, iPSCs require a specialized growth environment to retain an undifferentiated phenotype. The components of this optimal growth environment are constantly undergoing revisions and improvements. To facilitate development of *in vitro* culture systems, many researchers have attempted to design microenvironments that mimic the stem cell niche, thereby driving cells down their preferred self-renewal and differentiation pathway (5-10). A typical strategy is to enrich the biochemical environment of the *in vitro* culture media with a combination of soluble growth factors, cytokines, and/or serum protein to achieve successful maintenance of the undifferentiated cell state after several passages (11, 12). In addition to enhancing the soluble biochemical signal, gaining a fundamental understanding of the cell–cell and cell–substrate interactions is required to understand, design, and refine these artificial environments (13, 14). A comprehensive understanding of these interactions will allow for the effective design and development of materials capable of influencing iPSC self-renewal and the lineage into which these cells differentiate. This requires a multidisciplinary approach, integrating various concepts in the stem cell culture process.

In this review, we have focused on recent advances in culture strategies aimed at expanding populations of undifferentiated human iPSCs (hiPSCs), together with approaches to studying the

mechanisms underlying their self-renewal and pluripotency. We discuss morphological and biological features of cellular structures and mechanisms related to cell–cell and cell–substrate interactions. We then introduce principles for creating cell culture bioprocesses by considering the mechanisms of cell–cell and cell–substrate interactions, and delineate the prospects of surface-based culture strategies for clinical application of hiPSCs in the future.

UNDERSTANDING THE CELLULAR STRUCTURES RELATED TO CELL–CELL AND CELL–SUBSTRATE INTERACTIONS

An understanding of the mechanisms that control self-renewal, apoptosis, and commitment to differentiation of hiPSCs is important for developing improved methods of culture and differentiation that could be successfully used in clinical practice. There are three different cell fates that occur within hiPSC colonies during maintenance of cultures (Fig.1). Typically, hiPSC colonies grow as a monolayer and sustain an undifferentiated state. Undifferentiated hiPSCs form compact colonies of tightly associated cells via cadherin-mediated cell–cell interactions (12, 15), exhibiting a small, cobblestone-like morphology. With longer culture, the spontaneous occurrence associated with apoptotic cell death at the central region accelerated the morphological transformation of undifferentiated cells to deviated cells as the culture became prolonged, leading to formation of deviated cells from the undifferentiation at the center of the colony. Some colonies display a double-ring pattern, with large and flattened cells appearing at the center or periphery of the colonies. Once deviation occurs in these colonies, apoptosis triggers rapid invasion of the space between colonies in long-term hiPSC cultures.

Cadherin- and integrin-mediated interactions are known to influence differentiation during maintenance of ESCs and iPSCs (8, 15, 16). E-cadherin has been identified as a key factor in the regulation of pluripotency and self-renewal signaling pathways in human ESCs (hESCs) and hiPSCs. The cytoplasmic domain of E-cadherin contains binding regions for β -catenin, p120-catenin, and various other regulatory elements including phosphorylation sites and regions

1 recognized by the degradation machinery of the cell (17-19). Interaction of E-cadherin with
2 β -catenin and p120-catenin facilitates stabilization of the cytoplasmic cell adhesion complex,
3 protecting it from degradation and maintaining anchorage of E-cadherin to the actin cytoskeleton.
4 E-cadherin expression further maintains the self-renewal capacity of hESCs by influencing the
5 expression of Oct3/4 and Nanog genes (6). Altered levels of E-cadherin expression in monolayer
6 cultures occurs spontaneously, along with apoptosis and differentiation. Down-regulation of
7 E-cadherin stimulates pro-apoptotic Caspase-3 and suppresses the apoptotic inhibitory gene *Bcl-XL*,
8 resulting in increased level of cell death (12, 20, 21). Li et al. (15) observed that >98% of individual
9 hESCs that failed to maintain E-cadherin expression following single cell dissociation exhibited cell
10 death or differentiation within 72 h of seeding. Subsequent up-regulation of E-cadherin expression
11 tended to result in aggregated colonies with an embryoid body-like structure, suggesting that
12 adhesion between cells is preferable to that between cells and the culture substratum. Close contact
13 between cells in these aggregates could lead to paracrine interactions that could generate spatial
14 heterogeneity, and trigger differentiation.

15 Integrin-mediated interactions are also considered essential for maintenance of stem cell
16 survival, pluripotency, and growth. The maintenance of ESCs and iPSCs requires extracellular
17 matrix proteins such as laminin and fibronectin (22). Fate decisions of hiPSCs are reportedly
18 supported by various integrin-mediated signal pathways. Among them, the PI3K-Akt pathway has
19 been shown to inhibit apoptosis and attenuate cell death after hESC dissociation, with constitutive
20 overexpression of Akt partially improving hESC survival (12, 22). Li et al. (16) also demonstrated
21 that functional interaction between the small Rho family GTPase Rac1 and E-cadherin is
22 responsible for regulating hESC self-renewal (20). This ability of the PI3K-Akt pathway to promote
23 cell survival indicates a broader role for Rac1-related GTPases in the regulation of cell survival or
24 death decisions. E-cadherin-mediated interactions are commonly associated with β -catenin
25 signaling, and can also stimulate Rac-PI3K-Akt signaling in hESCs (8, 23). Therefore,
26 integrin-mediated signaling pathways are of interest in the maintenance of hiPSC pluripotency.

1 The mechanism underlying how crosstalk between cadherin- and integrin-mediated
2 interactions affect cell fate decisions such as cell survival, self-renewal and pluripotency, in hESCs
3 and hiPSCs during maintenance culture have only begun to be understood in recent years (12).
4 Numerous studies have revealed that multiple regulatory mechanisms are involved in the interplay
5 between E-cadherin and integrin in the regulation of hESC fate (8, 15, 16, 24). Several key
6 mechanistic elements participate in a range of regulatory events in hESCs, including cooperation of
7 the actin cytoskeleton with Rho family GTPases, regulation of E-cadherin expression at the hESC
8 surface by turnover and membrane trafficking, and interplay between E-cadherin and integrin (25,
9 26). The inhibition of endogenous Rac1 activity by a specific inhibitor also increased the quantity of
10 E-cadherin/ β -catenin complexes. These findings point to the ability of endogenous Rac1 to
11 modulate E-cadherin and its function. Although the exact mechanisms by which these factors permit
12 the propagation and maintenance of undifferentiated hiPSCs remain unclear, mounting evidence
13 suggests that they play a crucial role in the growth and maintenance of undifferentiated hiPSCs.

14 **WHY DO HIPSCS DEVIATE FROM AN UNDIFFERENTIATED STATE?**

15 We highlight how a stem cell “decides” whether to survive, self-renew or differentiate, and
16 discuss how this information can be used to develop novel strategies for controlling their fate. The
17 distinct qualities that allow assessment of the phenomenon of deviation from undifferentiated
18 hiPSCs in an *in vitro* culture environment deserve particular attention. Undifferentiated hiPSCs are
19 expanded as monolayer colonies in adhesion culture in the presence of feeder cells. Culturing with
20 mouse or human feeder cells is the most widely used method of providing the soluble factors
21 necessary to support the growth and maintenance of undifferentiated hiPSCs (1, 2, 27). We have
22 previously proposed possible mechanisms that control hiPSC self-renewal under *in vitro* culture
23 conditions using SNL and MEF feeder cells (28). For both types of feeder cells, the most important
24 factors influencing the deviation of hiPSC colonies from an undifferentiated state involved cell
25 migration and growth inside a single colony. The process of deviation from undifferentiated hiPSCs

during culture has been outlined (Fig. 2). Colonies of hiPSCs cultured with SNL feeder cells grew outward, dividing while migrating on the surface, and gradually became larger and more tightly packed during culture (Fig. 2A). Motility decreased steadily with increasing population density at the center of colony, and the cells overlapped, indicating that cells at the central region of the colony partially detach from the substrate. These cells in the central region of large growing colonies exhibited morphological changes accompanying apoptosis, and proliferated while undergoing transition from small cells to large and flattened cells. As a result, a large number of hiPSCs within colonies dissociated from one another and spread out as single cells. It appears that the contraction of alone blebbing apoptotic cell could act to drag neighbor cells into the space vacated by the dying cell, thereby spawning deviated cells in the central region. These observations provided important information toward understanding the process of deviation from an undifferentiated state, which occurs at the center of hiPSC colonies. They suggest that a delicate interaction between adjacent cells and their substrate, along with colony growth, is closely related to the occurrence, replication, and transformation of deviated cells during cell expansion. This could explain why the peripheries of undifferentiated colonies displayed enhanced migratory activity, as there would be an extended time-lag before newly-divided daughter cells are ready to divide again.

Conversely, deviation from the undifferentiated state in colonies cultured with MEF feeder cells involved a rim of rapidly proliferating and outwardly migrating cells at the periphery of the colony. These cells underwent rapid division in subsequent cultures (Fig. 2B). It is most likely that the enhanced migration of hiPSCs in response to MEF feeder cells reduce cell–cell interactions at the peripheral region of colonies. It might eventually be possible to facilitate expansion of deviated cells in growing hiPSC colonies. These cells were characterized by a loss of E-cadherin-mediated cell–cell interactions and acquisition of a more motile phenotype. These observations suggest that cells at the periphery of the colonies cultured with MEF feeder cells disrupt cell–cell interactions through accidental events during migration. This migration led to accelerated replication of deviated cells at the periphery, further indicating that appropriate migration is an essential for maintenance of

undifferentiated hiPSCs. Migration should be considered as a key parameter in the determination of hiPSC fate.

Based on these findings, we propose that the observed morphological changes occur because altered migration changes the balance between cell–cell and cell–substrate interactions. Ultimately an appropriate balance between interactions of cell–cell and cell–substrate leads to the formation of undifferentiated colonies. When cell–cell interactions overcome cell–substrate interactions, colonies with deviated cells form in the central region (Fig. 2A). Conversely, when cell–substrate interactions overcome cell–cell interactions, colonies form with deviated cells at the periphery (Fig. 2B). A major reason for the differences in migratory capacities could be the response of hiPSCs to two types of feeder cells in culture environments. These differences might be crucial in determining the point of occurrence for deviated cells from the undifferentiated state. Additionally, we investigated how the underlying pattern of colony formation affects the balance between cell–cell and cell–substrate interactions by inhibition of migration in cultures of feeder cells. In culture with MEF feeder cells, blocking Rac1 activation with a specific Rac1 inhibitor NSC23766, revealed that the deviation of undifferentiated hiPSCs resulted in predominantly deviated cells in the central region than at the peripheral region. Deviation of hiPSC colonies that were not treated with NSC23766, remained concentrated at the periphery of colonies (Fig. 2C). These studies suggest that cell–cell and cell–substrate interactions influence migration during maintenance of undifferentiated hiPSCs, and provide a resource for future studies of hiPSCs.

NEW INSIGHTS INTO THE ROLES OF MIGRATION IN MAINTENANCE OF UNDIFFERENTIATED HIPSCS

The interaction of stem cell with specific microenvironmental elements is thought to be a key regulatory mechanism in the maintenance of self-renewal and differentiation capacity (7, 8). The regulation of stem cell fate has been shown to be influenced by cues provided by reciprocal interactions with an artificial extracellular microenvironment, comprising soluble bioactive agents

1 and the synthetic substrates (14). Another aspect of the microenvironment that has been mimicked
2 in the study of stem cell fate is the regulation of behaviors such as morphology and migration.
3 However, there are few reports focusing on culture strategies for developing and maintaining hiPSC
4 cultures that are based on their dynamic behavior.

5 In our recent studies, we addressed possible mechanisms for controlling hiPSC fate decisions
6 by influencing the balance between cell–cell and cell–substrate interactions through altered cell
7 migration (29). We have also discussed recent insights concerning the maintenance of
8 undifferentiated hiPSCs through the regulation of migration (Fig. 3). Deviation from an
9 undifferentiated state by hiPSCs in colonies cultured with SNL feeder cells involve decreased
10 migration during colony formation, and induction of apoptosis at the center of colony. The
11 deviation of undifferentiated hiPSCs cultured with SNL feeder cells occurred spontaneously in cells
12 at the center of the colony, and was dependent on colony size. We have investigated dendrimer
13 surfaces with dendron structure and their regulation of cell morphology by changing the generation
14 number of dendrimers, and attempted to clarify the relationship between cell morphology and
15 function on these designed surfaces. The cell morphological changes on dendrimer surface were
16 supposed, including the ligand display control over cells which is caused by the binding balance
17 between integrin on the cytoplasm and adhesion protein on surface, to topographical control over
18 cells which is nano-topography of dendron structure, and to surface stiffness control over cells (29,
19 30). Colony morphology was either scattered or aggregated, depending on the levels of migration.
20 An appropriate migration pattern leads to formation of an undifferentiated hiPSC colony. When
21 migration level increased, colony morphology appeared to be scattered and was associated with
22 apoptosis. Further migration induces aggregate formation, resulting in differentiation toward cells
23 of the early mesoderm. Modulating survival versus death, and pluripotency versus differentiation,
24 during cell fate decisions-making can therefore be explained in terms of the balance between cell–
25 cell and cell–substrate interactions by altering cell migration.

26 In a recent study, we examined the morphological and functional responses of hiPSCs to a

dendrimer surface (30). We investigated the expression of genes associated with the undifferentiated state of hiPSCs (Oct3/4, Sox2, and Nanog), and the genes associated with lineage commitment in hiPSCs cultured on the first-generation dendrimer surface or on a gelatin-coated surface with feeder cells. We successfully maintained two hiPSC lines on dendrimer surfaces over 10 passage (Fig. 4). Semi-quantitative reverse-transcriptase polymerase chain reaction data demonstrated that there were no significant differences between the hiPSCs cultured on the dendrimer surface compared with those cultured on the gelatin-coated surface. Cell commitment to differentiation was evident in the hiPSCs on the dendrimer surface, as genetic markers indicative of the three germ layers were not activated in hiPSCs on either the dendrimer surface or the gelatin-coated surface. Pluripotent signaling networks in hiPSCs are tightly controlled *via* the coordinated regulation between cadherin- and integrin-mediated interactions. Small perturbations in these pathways are sufficient to regulate maintenance of the undifferentiated state. Application of this culture surface is a useful alternative for obtaining cell preparation enriched with hiPSCs in an undifferentiated state.

Fig. 4

One important factor to consider is that hiPSC culture occurs in a transient environment with bidirectional signaling between cells and substrate. However, many culture surfaces developed to date might not be conducive to incorporating this dynamic behavior and therefore might not truly mimic the extracellular environment. Cell behavior is constantly governed by instructive biochemical and biophysical cues from the environment. Likewise, cells are continuously remodeling their surrounding environment. The required design of synthetic substrates varies across different cell types, and synthetic environments should be individually generated with distinct cell lineages in mind. Our studies can serve as a culture model to maintain an undifferentiated state of hiPSCs through balance between cell–cell and cell–substrate interactions by altering cell migration, and we believe they also pave the way in the design of suitable culture mediums and substrates while preserving undifferentiated hiPSCs.

CONCLUSIONS

We outlined strategies for the expansion of undifferentiated hiPSCs that are well-suited for understanding and regulating the balance between cell–cell and cell–substrate interactions in feeder cell-dependent cultures. We suggest that changes in migrations lead to guidance of hiPSC fate decisions in cultures with two types of feeder cells. Moreover, we found that the coordinated regulation of balance between cell–cell and cell–substrate interactions was important for controlling cell fate decisions such as survival, self-renewal, and pluripotency. Endogenous Rac1 and E-cadherin signaling pathways form an intrinsic key for the regulation of hiPSC fate decisions. Our results from various studies provide insight into the design of culture media and substrates, based

on interactions between the microenvironment and cells. These could serve as useful alternatives for obtaining cell preparations enriched with cells in an undifferentiated or desired differentiation state. Our proposed method offers new strategies of culture process design for the expansion of undifferentiated hiPSCs.

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Figure legends

FIG. 1. Schematics depicting the potential involvement of multiple pathways during hiPSC fate determination in feeder cell-dependent cultures. (A) Self-renewal. (B) Apoptosis. (C) Deviation from the undifferentiated state.

FIG. 2. Schematic outlining our hypothesis that two types of hiPSC colonies with deviated cells form in cultures with feeder cells. Colonies with deviated cells in cultures grown on SNL feeder cells (A) and MEF feeder cells (B) formed in the central and peripheral regions through slow and fast migration, respectively. A1-A4 and B1-B4 show the deviation of hiPSC colonies in cultures with SNL and MEF feeder cells, respectively. (C) The Rac1 inhibitor, NSC23766 (100 μ M), was added to cultures grown on MEF feeder cells. Deviated cells occurred predominantly in central region of colonies through reduced migration.

FIG. 3. Schematic showing regulation of hiPSC fate decision between the undifferentiated state and deviated state with relation to the balance between cell–cell and cell–substrate interactions in cultures grown on feeder cells (Ref. 29; modified).

FIG. 4. Long-term maintenance of undifferentiated hiPSCs (Tic and 201B7 hiPSC lines) cultured on a first-generation dendrimer surface or a gelatin-coated surface with SNL feeder cells. We conducted conventional RT-PCR analysis for undifferentiated stem cell markers and early commitment to differentiation markers after passage 10.

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