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Short Review

A Place to Call Home: Bioengineering Pluripotential Stem Cell Cultures

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Abstract: Pluripotent stem cells (PSCs) have the power to revolutionize the future of cell-based therapies and regenerative medicine. However, stem/progenitor cell use in the clinical arsenal has been hampered by discrepancies resulting from stem cell engineering and expansion, as well as in their (mass) differentiation in culture. Moreover, the manner in which external conditions affect PSC and induced-pluripotent stem cell lineage establishment as well as maturation remains controversial. In this review, we examine novel methods of cell engineering and the role of reprogramming transcription factors in PSC development. In addition, we explore the effect of external environmental signals on PSC cultivation and differentiation by elucidating key components of the primordial stem cell microenvironment, the blastocyst. Furthermore, we assess the effects of hypoxic conditions on DNA editing, gene expression, and protein function in PSC self-renewal and growth. Finally, we speculate on the principal use of gap junction subunit expression as relevant biomarkers of PSC fate. Improving bioreactor design and pertinent cell biomarker classification could vastly enhance manufactured stem cell yield and quality, thereby increasing the potency and safety of therapeutic cells to be used in regenerative medicine.

Keywords: embryonic stem cells; induced-pluripotential stem cells; stem cell niche; stem cell differentiation; cell bioreactors; oxygen metabolism

1. Introduction

Pluripotency is a property of early embryonic cells, defined by their ability to generate all tissue

types of the adult organism [1]. Due to this property, pluripotent stem cell (PSC) populations have the power to revolutionize regenerative medical therapies. One major breakthrough in the field has been the engineering of an induced pluripotent stem (iPS) cell. This achievement vastly expanded the possible avenues of PSC development because it was the first to enable the conversion/reversion of differentiated cell types from multiple sources into stem cells. Two phases of PSC pluripotency have been identified in the early embryo: 1) early uncommitted naive cells at the ground state and 2) more specific developing primed cells [2]. Microenvironmental cues within the blastocyst are thought to trigger the transition of the ground state PSCs, which rely on "minimal" extrinsic stimuli, into the more committed primed cells [3].

1.1. Lessons from the blastocyst: A multifaceted bioreactor

The earliest stage of mammalian development may be characterized by the proliferation and condensation of totipotential blastomeres into a highly compact sphere. During compaction, the sphere's outermost cells that remain in contact with the environment form the trophectoderm (TE). TE cells display epithelial like-properties and give rise to an internal fluid-filled cavity (blastocoel) by exocytosis and fusion of extracellular vesicles, or vacuoles. In this stage of embryonic development, called the blastula, cells of the inner sphere that lack exposure to the external environment aggregate toward the center and form the inner cell mass (ICM) [1,4]. Cells of the ICM reside in a naive pluripotential state. Characterized by a sphere of TE cells which surround an undifferentiated ICM and blastocoel, the blastocyst is completed in humans approximately seven days post-fertilization (Figure 1). The acellular blastocoel structure can act to sequester morphogens and can also play a hydraulic role, distributing or exerting localized pressure on stem cells of the ICM [1,5]. In fact, chemical signals and mechanical cues from the blastocoel on the adjacent ICM may be associated with their arrangement within the blastula, as well as with the expression of the GATA6 protein [5]. GATA6 is primarily involved in the loss of pluripotency and cell differentiation [6,7].

ICM cell cultures give rise to the well characterized naïve embryonic stem cell (ESC) which maintains pluripotency even in the absence of the surrounding blastocyst. Nevertheless, external signals such as leukemia inhibitory factor may be required for long term ESC maintenance. In contrast, internal intervention by genetic "reprogramming" factors, commonly expressed in blastocyst cells, can be used to engineer ESCs. These factors act to revert mature cells to a pluripotent state (as is the case of iPS cells).

1.2. Reprogramming factor activity in the pluripotential cell

Induced pluripotency has been achieved by the insertion and expression of several early transcription factors in differentiated cells. The initial iPS cell experiments included four genes: Sry-related HMG-box (SOX2), Octamer binding transcription factor (OCT4), Kruppel-like factor 4 (KLF4), and cellular MYC (MYC) [10]. Subsequently, other genetic combinations have been reported to reprogram cells. These include NANOG and LIN-28 homolog A (LIN28) proteins [11]. For the purpose of this review we refer to these factors, uniformly listing human and mouse, gene and protein variants in a non-italic uppercase font.

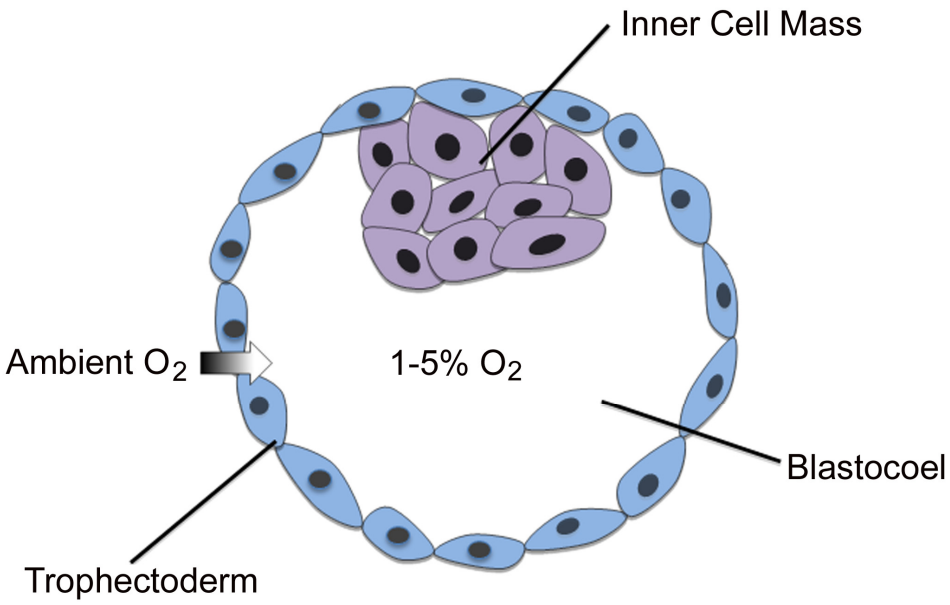

Figure 1. Scheme depicting the oxygen drop in the blastocyst, the primordial pluripotent stem cell niche. While oxygen partial pressures within the ICM and blastocoel were reported to be on the scale of 1–5%, ambient oxygen can be as high as 20% [8,9]. Trophectoderm (TE) cells form an external cage around pluripotent cells of the inner cell mass (ICM) and the blastocoel, a fluid-filled cavity that can serve an important role in hydraulic balance and as a reservoir of morphogenic factors. TE cells develop into structures that nurture the embryo while the pluripotential ICM cells evolve into embryonic germinal and associated layers. Communication between the TE and ICM is considered crucial for proper embryonic development.

OCT4 is a proven transcription factor required for iPS cell engineering. It is also involved in the maintenance of murine and human embryonic stem cells [12,13]. OCT4 is produced early in embryonic development by cells of the ICM and trophoblast. Its expression persists in the ICM during which some of its downstream effectors can be involved in interactions with the TE [14,15]. OCT4 deficient mouse embryos lack an ICM and demonstrate restricted differentiation along the extra-embryonic TE lineage [16]. Importantly, OCT4 expression levels must be maintained within a defined range to establish and/or maintain cell pluripotency [17].

SOX2 is another key transcription factor found to be widely expressed during embryonic development. Predominantly expressed in more specified embryonic cells (epiblast), SOX2 was reported to contribute to cell proliferation and stemness/dedifferentiation through the regulation of genes controlling G1/S transition and epithelial-to-mesenchymal transition [18,19]. It was shown that SOX2 forms a dimer with OCT4 to promote gene transcription. As with OCT4, changes in SOX2 expression levels significantly influence ESC differentiation [19,20].

KLF4 is a factor highly expressed in ESCs. KLF4 and other KLF family members were shown to play a role in the regulation of SOX2, OCT4, and NANOG transcription factors. Moreover, knockdown of KLF4 in ESCs resulted in their differentiation [21,22]. Nevertheless, some studies question the role of KLF4 in promoting self-renewal and pluripotency [22,23].

MYC has been found to regulate self-renewal, growth, differentiation, apoptosis, and metabolic activities in ESCs, hematopoietic stem cells, and more committed progenitor cell types [24–26]. However, MYC was also shown to act as a proto-oncogene in many cancers and to have a mutagenic effect on iPS cells [27]. Although MYC may play a substantial role in cellular reprogramming, these findings have led to a decline in its use in iPS reprogramming [27,28].

LIN28 was first characterized in the nematode *C. elegans* as an important regulator of developmental timing [29]. In mammals, LIN28 was not only found to maintain a pluripotential cell phenotype but also to be down-regulated during ESC differentiation [30]. LIN28 acts to promote the translation of OCT4 mRNA, and like OCT4, tight regulation of LIN-28 expression was required for iPS cell production [30,31].

NANOG is another factor active in embryonic cells whose expression was reported to maintain ESC pluripotency. Regulated by OCT4 and SOX2, NANOG levels decline in the differentiating TE cells of the blastocyst [32,33]. During somatic cell reprogramming, NANOG was found to establish and maintain the pluripotent ground state [34,35]. In mice, NANOG deficiency is embryonic lethal, with development ceasing at the time of ICM formation [34]. However, as studies have shown that NANOG-null ESCs could indefinitely self-renew, and that iPS cells could be converted from NANOG-deficient fibroblasts, NANOG expression may not be beneficial to cellular pluripotency [36–38].

The above studies indicate that transcription factor expression and fine-tuning are involved in the driving of cellular pluripotency. However, their precise role in embryonic and in engineered cells remains ambiguous and may be influenced by environmental cues. By extension, external signals may also impact the establishment of pluripotential cell lines currently produced by several gene targeting techniques.

1.3. Reprogramming factor gene targeting and editing

The emergence of site specific genomic targeting tools have opened the field to rapid and efficient endogenous DNA editing as well as exogenous gene introduction in a wide variety of cell types and organisms. For example, zinc-finger transcription factors were shown to activate OCT4, SOX2, KLF4, and MYC gene expression via their interaction with endogenous enhancer elements [39,40]. Using another tool, the (designer) transcription activator-like effector (TALE) system, in which TALE DNA-binding repeat arrays were linked to transcriptional regulatory domains, up-regulation of endogenous SOX2 and KLF4 but not OCT4 or MYC was achieved [41]. One variation of the TALE methodology is the TALE nuclease (TALEN), which makes use of a TALE DNA binding/FokI nuclease fusion protein. This fusion could be used to edit DNA at predetermined sites on the chromosomes [42,43]. By comparison, the clustered regularly interspaced short palindromic repeat-CRISPR associated-9 (CRISPR-Cas9) technique is simplified making use of site specific DNA binding sequences fused to a Cas9 endonuclease [44,45]. In this effective method, a custom guided RNA delivers the Cas9 protein to genomic sequences deemed for cleavage [46].

DNA editing approaches to target the endogenous Oct4 enhancer resulted in more than a 20-fold increase in Oct4 transcription in mouse NIH3T3 and human HEK293T cells [47,48]. Moreover, using the CRISPR-Cas9 method a super-enhancer region responsible for over 90% of SOX2 expression was identified ~100 kb downstream of the SOX2 gene [49,50]. With an explosion

of numerous (and safe) DNA editing technologies, methods of reprogramming gene expression and their fine-tuning can be adjusted. As the TALE and CRISPR-Cas systems were evolutionary developed for bacterial infection and adaptive immunity, respectively, it is possible that changes in environment may facilitate their DNA editing capability. Thus, adjustment of external stress stimuli including oxygen exposure may significantly influence protein-nucleic acid interactions as well as subsequent reprogramming factor gene expression.

1.4. Oxygen levels and pluripotential cell metabolism

Oxygenation is widely known to impact PSC growth and metabolism [51–54]. Low oxygen tensions are thought to maintain cells in a pluripotental state with increasing oxygen levels leading to differentiation [55–57]. As such, stem cell niches are generally hypoxic (< 5% O_2), with models of stem cell compartments indicating partial pressures as low as 1% [8,9,58,59]. A preference for anaerobic conditions and for glycolysis by PSCs can be partially explained by the fact that normoxic conditions and aerobic respiration elevate reactive oxygen species (ROS) production and oxidative damage [60]. ROS can act on cells to mutate DNA, trigger lipid peroxidation of polyunsaturated lipids, and/or activate cell cycle checkpoints [61]. In fact, to counter ROS, antioxidant enzyme and molecules in the cell are concentrated in regions of high oxidative activity, predominantly in or around the mitochondria [62]. Interestingly, the mitochondria of pluripotential cells were reported to be poorly developed and/or display partial functioning [63,64]. A lack or deficiency in proper mitochondria could explain why pluripotential cells grown under hypoxic conditions (limited ROS production) displayed improved morphology, increased expression of pluripotency markers, reduced chromosomal abnormalities, and a higher rate of proliferation than those exposed to normoxic conditions [65–68].

At the molecular level, hypoxic conditions were shown to stimulate OCT4, SOX2, and NANOG expression in human embryonic stem cells, while MYC and LIN28 proteins were found to promote a glycolytic shift during human and mouse iPS cell derivation [55,64,69,70]. The mechanism of OCT4 and MYC function involves their interaction with a family of hypoxia-inducible factor (HIF) proteins and their downstream enzymes [69]. HIF protein involvement in iPS cell development is supported by the discovery that ablation of HIF-1α function disrupts reprogramming efficiency and that activation of HIF-1α significantly improves cell fate conversion [71]. In fact, HIF-1α was sufficient to drive ground state/naïve ESCs that utilize both glycolytic and oxidative phosphorylation to primed cells which are more exclusively dependent on glycolysis [72]. Therefore, it is feasible that a strong association between hypoxia and OCT4, SOX2, and HIF-1α proteins exist and that these factors may act in conjunction with additional external cues to regulate PSC growth. An important signaling cascade that may be involved in PSC development includes those of the receptor tyrosine kinase (RTK) family [73]. RTK activity is known to regulate cellular growth and intercellular interactions, often by means of the MYC proto-oncogene [74,75]. As under some circumstances hypoxic conditions were shown to differentiate ESCs, we conceive that environmental and genetic signals cooperatively act to convert or maintain a cell in the pluripotental state [76,77]. Understanding the relationship between oxygen tension, gene expression and consequent stem cell development is crucial and particularly relevant to disease, where elevated oxygen tension may stimulate PSC differentiation (Figure 2) [78]. This association would also be important when outlining the design of new and innovative methods meant to manufacture PSCs on a mass scale [79,80]. Particularly, when

devising stem cell culture workstations which encompass automation, improved sterility and reproducibility. A detailed understanding of the hypoxic variable within the PSC niche may dramatically advance stem cell production processes and lead to the discovery of biomarkers that better reflect cellular fates.

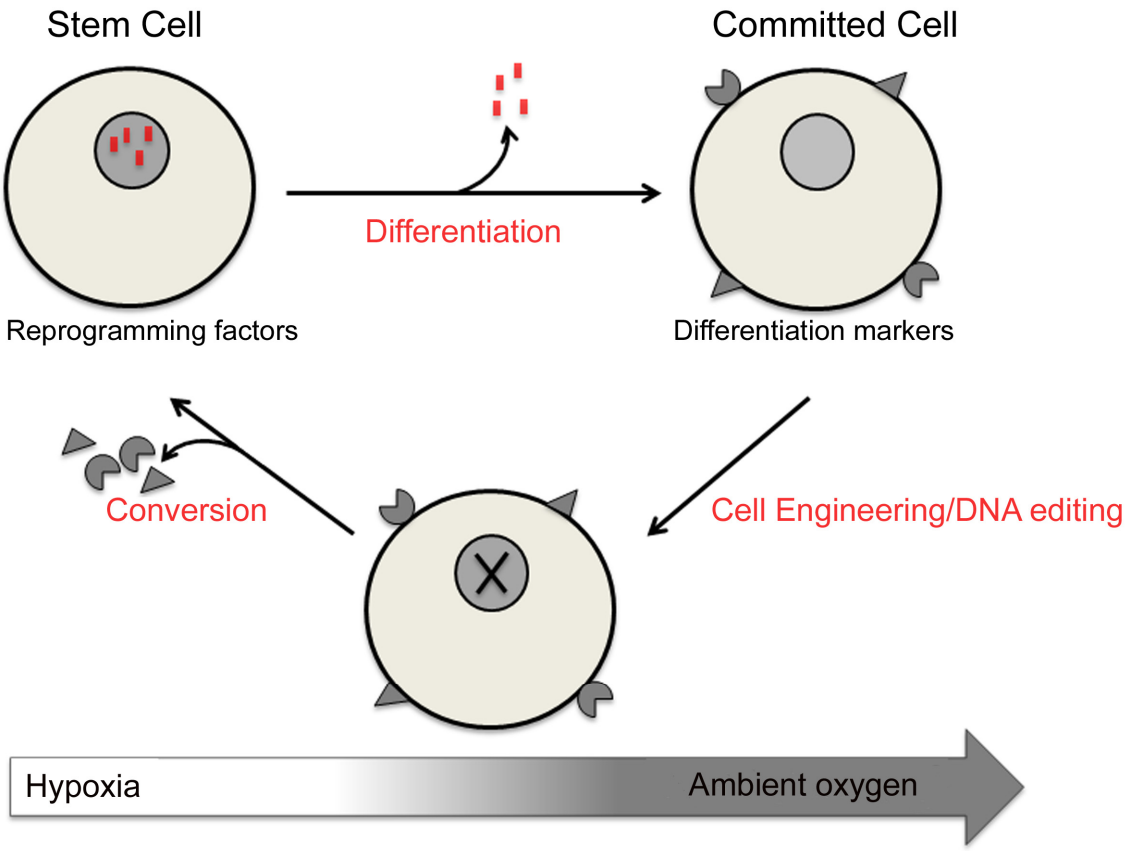

Figure 2. Potential relationship between oxygen tension and PSC differentiation. *Top left*. Stem cells residing in a hypoxic microenvironment express commonly known reprogramming factors (rectangles). *Top right.* Upon release from a hypoxic niche and/or exposure to higher oxygen tensions [during tissue injury], cell commitment would activate new genetic programs of aerobic metabolism (HIF [72]; not shown) leading to the ensuing expression of lineage-specific differentiation (bio)markers (for example: gap junctions; gray shapes). *Bottom*. Hypoxic conditions may enhance current methods of PSC engineering and DNA editing (shown as X), facilitating the conversion of differentiated cells into PSCs. A hypothetical gradient ranging from hypoxia (> 1% O_2) to ambient (< 21%O_2) is shown below.

1.5. PSC gap junction interactions and communication

Gap junctions facilitate the direct exchange of small cytoplasmic particles between neighboring cells. Gap junction channels form via the interaction and opening of two hemichannel (connexon) structures found on the plasma membranes of adjacent cells. Each connexon is comprised of six

connexin subunits which surround a central pore through which cytoplasmic factors can diffuse intercellularly. Gap junction proteins are found to be expressed in early developing mouse and human blastocysts, with disparate connexin patterns in ICM and TE cells [81–83]. In addition, several studies report an association between changes in connexin expression and PSC engineering to support their use as important lineage biomarkers [84,85].

In the blastocyst, OCT4 was shown to regulate gap junction expression and transport between cells of the ICM and the TE [86]. Moreover, during iPS cell reprogramming connexin-43 expression levels were increased by OCT4, NANOG, and SOX2 reprogramming factors [84,85,87]. These data suggest that in addition to their use as biomarkers, connexin subunits may play an important part in cellular pluripotency. In fact, the presence of connexin channels in the blastula may shed light on some early molecules essential for PSC communication and survival. While cAMP was reported to be one factor transported between stem cell gap junctions, ROSs may be another [86,88,89]. In fact, connexins may be involved in maintaining pluripotential cell integrity by the dilution or removal of ROS from areas of high concentration. Accordingly, a stem cell shift toward more energetically active cells may trigger changes in connexin expression and their protein distribution among cells. The pattern of connexin expression on PSCs might be indicative of highly regulated and healthy environments that are conducive to cellular proliferation and/or differentiation.

1.6. Stem cell properties and their cultivation methods

Inter-laboratory discrepancies in stem cell growth conditions may often impact cellular outcome. Moreover, as pluripotential cells derived from the inner cell mass of embryonic blastocysts tend to form embryoid bodies, or 3-dimensional (3D) cell aggregates, the validity of experiments which employ a standard 2-dimensional culture may also be misleading [90,91]. By the same token, other data indicate that suspension cultures prolong the expansion and maintenance of ESCs and iPSCs in culture [92,93]. In suspension cultures, free-floating stem cells were shown to communicate through paracrine (not juxtacrine) factors with anchoring cells that can serve as ‘feeders’ [94]. Feeder cells, possibly of fibroblast lineage, may also originate from the free floating precursor population [95]. Similar to embryoid bodies, free-floating stem cells in suspension culture could also form 3D spheres [96]. Physically, a sphere characterizes the greatest volume for a given surface area ratio. Biologically, spheroid formation was suggested to enhance “stemness” and cell division [97–99]. Minimizing structural surface area would also decrease (cell) exchange with the external environment, a phenomenon that may closer represent a true naive stem cell state. In addition, tightly-packed cellular clustering within the sphere might increase inter-cellular contacts and communications by gap junctions. This, in turn, would enhance the cellular transfer or excretion of membrane impermeable molecules or metabolites, including peroxides [100–104]. Finally, combined with a reduction in membrane surface area, the presence of a living, respiring cellular parameter would necessarily reduce oxygen levels in the sphere center (Figure 1) [105]. This design would result in an increasingly hypoxic environment for the innermost PSCs.

Hypoxic conditions provide a strong stimulus for gene expression and reprogramming and does not reduce gene transfection efficiency in human and mouse cell types [106]. At the same time, ROS can directly interfere with transcription factor-DNA binding mechanisms and stress pathway signaling in *Xanthomonas campestris* bacteria, required for TALE mediated transcriptional activation [107–109]. Therefore, applying hypoxic conditions to the gene targeting step could be

advantageous, leading to an improved somatic cell reprogramming yield. Experiments examining the effects of oxygen tensions on cellular DNA editing systems and PSC engineering are warranted.

2. Conclusion

While the targeting of cellular gene expression can spur progress in combating human disease, many conditions that contribute to PSC development and growth remain unresolved [110–113]. These include the methods of genetic engineering, as well as the myriad of external conditions that influence stem cell fate [114–117]. Novel studies meant to approach these and other questions remain a crucial step towards the advancement of safe and efficient cell-based therapies.

Conflict of interests

Authors declare that they have no competing interests.

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