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Engineering Human Cells and Tissues Through Pluripotent Stem Cells

Jeffrey R Jones[1] and **Su-Chun Zhang**[1,2]

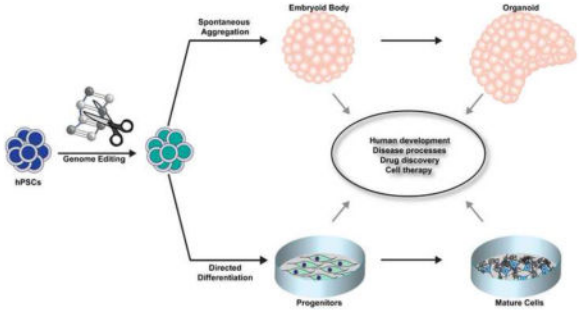
[1]Waisman Center, University of Wisconsin, Madison, WI, 53705

[2]Department of Neuroscience and Department of Neurology, University of Wisconsin, Madison, WI, 53705

Abstract

The utility of human pluripotent stem cells (hPSCs) depends on their ability to produce functional cells and tissues of the body. Two strategies have been developed: directed differentiation of enriched populations of cells that match a regional and functional profile and spontaneous generation of three-dimensional organoids that resemble tissues in the body. Genomic editing of hPSCs and their differentiated cells broadens the use of the hPSC paradigm in studying human cellular function and disease as well as developing therapeutics.

Graphical abstract



Introduction

When the laboratory of James Thomson, taking cues from developmental biology, first successfully isolated and propagated human embryonic stem cells (hESCs) in 1998[1], it heralded the beginning of a new field of science in understanding human development, cellular diversity, and disease. In the years following this discovery, efforts to identify the pluripotent nature (capable of forming all developmental germ layers) of ESCs have yielded a sufficient understanding of the pluripotency network to allow the generation of induced

*Corresponding author: Su-Chun Zhang, suchun.zhang@wisc.edu.

pluripotent stem cells (iPSCs)[2,3]. The use of so-called Yamanaka factors to reprogram somatic cells to iPSCs has allowed the development of tools to study a wide variety of diseases alongside genetically normal samples.

The utility of human pluripotent stem cells (hPSCs, encompassing both hESCs and iPSCs) depends on our ability to guide hPSCs to functional cells and tissues of the human body. Two parallel methods have been developed: directed differentiation to enriched populations of cells that match a regional and functional identity of those normally residing in the human body and spontaneous generation of three-dimensional tissues, called organoids that resemble embryonic tissues. Application of genomic editing in hPSCs and their differentiated cells further enables the use of hPSCs in studying early human development, modeling diseases, and developing therapeutics.

Specification of embryonic germ layers from hPSCs

The pluripotent nature of hPSCs allows for generation of diverse cell types, comprising the whole of the human body. It also presents challenges to directing hPSCs to a particular cellular subtype through temporal and spatial coordination of a wide variety of external stimuli. Elegant manipulation of these cascades has allowed formation of all three germ layers[4–7], regionally patterned cells[8–10] and subtype specific cells[10–13].

Early hPSC differentiation relied on a combination of intrinsic programs and modification of the environment to guide differentiation. After the removal of a feeder layer and aggregation of PSCs to embryoid bodies [4], a default pathway allows for neurectoderm formation and subsequent generation of neuron-producing spheres (neurospheres)[4] under the culture condition that favors the growth of neural cells. Analysis of these 3D-neurosphere structures generated during the neurectoderm phase of differentiation revealed architecture that remarkably resembles a developing neural tube[4]. It has subsequently been established that neurectoderm differentiation from hPSC bears striking similarities to *in vivo* human development not only in terms of morphological structures, but also regarding gene expression and the normal developmental timeline[14].

The default neurulation hypothesis arises from early studies in *Xenopus laevis* by Spemann & Mangold, where it was found that signals coming from an organizing center of mesoderm were sufficient to induce neurulation. Through the work of several labs, it was demonstrated that these signals were bone morphogenetic protein (BMP) antagonists[15–17]. Building upon these studies and combining the evidence with more recent work on hPSCs[18–20], Chambers et al. developed a strategy to use inhibitors of several anaplastic lymphoma kinases (ALKs) and BMP receptors to drive hPSCs into the neural lineage[21]. Activated SMAD was reduced in treated nuclei by 24 hours and subsequent activation of neural genes was observed. The colonies displayed rosette-like morphology and a large proportion of the cells (~80%) expressed PAX6 after one week. The authors demonstrated that these progenitors generate neurons, thus advancing the directed differentiation of hPSCs to the neural lineage by refining the protocol into an increasingly chemically defined and controlled paradigm. The so-called dual SMAD inhibition protocol, or simply 2i, marks a

shift from following intrinsic developmental programs to a more controllable chemically defined environment.

Directed differentiation of functionally specialized cells from hPSCs

During embryonic development, coordination of intrinsic program and environmental cues guides the generation of regionally and functionally specialized cell and tissue types, a process called regional patterning. This principle has been instrumental for directed differentiation to specialized cell types. The neuroectoderm or neuroepithelial cells differentiated from hPSCs carry a dorsal forebrain identity[10,18], consistent with early human brain development. However, the initial neuroepithelial cells are specified to a neural identity but not yet committed to a specialized neuronal fate. They are hence called primitive or naïve neuroepithelia or neural stem cells (NSCs)[10]. These primitive NSCs, in response to temporarily and spatially available patterning molecules, differentiate to cells of the ventral forebrain, mid/hindbrain, and the spinal cord (Figure 1).

Decades of work across numerous deuterostomes and planarians have established the critical role of Wnt signaling in patterning the anterior-posterior axis of bilaterally symmetrical organisms[22]. Similarly, Sonic Hedge-Hog (SHH), secreted from the notochord[23](Figure 1), determines the dorsal-ventral axis[23,24]. Cellular patterning requires precise feedback and happens in a graded, temporally and spatially dependent manner. Similarly, differentiation of regionally and functionally specialized neuronal types requires application of specific patterning molecules in a specific concentration at a particular time.

Recapitulating neural patterning, Li et al[10] treated the hPSC-derived (differentiated for 2 weeks) SOX1-expressing neurectoderm with a combination of SHH and retinoic acid (RA) in an attempt to guide stem cells to motor neurons that reside in the ventral spinal cord. Indeed, these cells generated βIII-Tubulin / MAP2 expressing neurons, however regional transcription factors associated with motorneurons (HB9, Lim3, Islet1) were only expressed by about half of the population, with very little HB9 expression observed[10]. This result suggests that these patterning molecules have little effect on the cells that are already fated to the forebrain. When RA was added onto the primitive neuroepithelia at an earlier time point (before SOX1 expression) the resulting cells were positive for all regional transcription factors associated with motor neuron progenitors and developed into post-mitotic, ChAT+, functional motorneurons. This example highlights the importance of coordination between intrinsic properties (e.g., responsiveness to morphogens) of stem cells and environmental factors (patterning molecules). By the same principle, primitive neuroepithelia, in the presence of increasing concentrations of small molecules that activate Wnt signaling, differentiate to progenitors of the midbrain, hindbrain, and spinal cord, which further produce midbrain dopamine neurons[9,25,26], hindbrain serotonin neurons[13], and spinal motor neurons[10]. Similarly, in the presence of increasing concentrations of SHH but absence of caudalizing morphogens, the primitive neuroepithelia retain the anterior identity but differentiate to progenitors of the cerebral cortex, lateral ganglionic eminence(LGE), and medial ganglionic eminence (MGE), which generate cortical glutamate neurons[27,28] striatal medium spiny GABA neurons[29], cortical GABA interneurons[30,31], and basal forebrain cholinergic neurons[30](Figure 1). Thus, by exploiting the intrinsic properties of

stem/progenitor cells and timely application of concentration gradients of morphogens (or small molecules), one can guide hPSCs to regional cells along the entire rostral/caudal and dorsal/ventral axes, making hPSCs a high resolution tool for insight into development, function, and disease for virtually any cell type within the human central nervous system.

Tissue generation from hPSCs

The fact that hPSCs form embryoid bodies and differentiate to early structures of the three germ layers, including neural tube-like structures in suspension culture, suggests that the intrinsic developmental program is retained in the differentiating cells in culture. It would also suggest a possibility of generating tissues or organs from hPSCs when appropriate exogenous substrates are provided in 3D culture systems. This notion has led to the generation of organ-like structures (organoids) *in vitro.*

A striking example of organoids is demonstrated by the work of Eiraku et al.[32]. Using a serum-free embryoid body suspension culture in which primitive neural structure forms[33], the authors tested if the evaginations observed in these neural-organoids may be competent to form organized retinal tissue. Utilizing a Rx-GFP reporter to demarcate early retinal progenitors, the authors transiently applied Activin, which has been shown to promote retinal tissue *in vitro*[33,34]. Activin indeed induced retinal cell generation, surprisingly however, the organized structure of the putative optic cup was not present. The authors then speculated that extracellular matrix proteins may be essential for generation of such architecture. Addition of matrigel to the system stimulated organoid formation which was remarkably similar to development of the eye[32]. These cells not only differentiated to express proper temporal transcription factors, but also structurally changed to an optic cup which eventually formed retinal pigmented epithelia and a stratified neural retina. Though this work was initially conducted using mouse stem cells, the experiment has subsequently been replicated using hPSCs[35]. These studies highlight that though hPSCs possess intrinsic developmental programs, extracellular matrix is important in organ formation.

During cerebral cortical development, NSCs first organize into the inner and out-most layers, from which neural progenitors migrate along radial glia, differentiating to neurons, and form cortical layers in an inside-out fashion at a specified time frame[36]. Such a (intrinsically) timed generation of layer-specific neurons has been replicated in monolayer cultures[37]. Organoids that resemble the layered cortex of the human brain have also been generated from hPSCs[38–40]. Such organoid systems of the brain have shown the ability to produce deep and superficial cortical neurons[39] along with astrocytes[40] in a developmental paradigm which temporally follows human development. Furthermore, for the first time these organoids showed functional neuronal integration[40]. This powerful model system has already been utilized in addressing human diseases. In this case, the authors demonstrate that microcephaly patient derived iPSCs contain less radial glial progenitors compared to wild-type controls[38]. The authors conclude that in the organoid model of microcephaly, progenitor pools such as radial glia prematurely generate neurons thus leading to a smaller overall organoid. Studies such as these mark the beginning of an era when complex organs such as the human brain can be studied in an *in vitro* system.

Genetic engineering empowers applications of hPSCs

Invaluable information has been learned through the use of genetic modification of animal model systems. Along the line, effective engineering of human genome would substantially empower hPSCs as a research tool and potentially as a source for therapeutics. Traditionally, tools for genetic modification were restricted to transfection of copious amounts of episomal vectors or the use of various viruses. Such techniques work well to answer many biological questions, but often yield false positives due to cellular responses to foreign DNA/RNA[41,42], over expression of proteins that may result in off-target interactions, and unknown locations or copy numbers of viral integration[43]. Recently, the development of the clustered regularly-interspaced short palindromic repeats (CRISPR)[44,45] method (and transcription activator-like effector nuclease (TALEN)[46] to a lesser extent) has potential to be a monumental advance in cellular biology, especially when the implications for use with hPSCs are considered. CRISPR now offers the ability to specifically alter DNA loci to tag endogenous proteins, induce or correct genetic mutations, and/or conditionally knock-out genes.

Genetic correction and rescue of human disease-causing mutations has already been demonstrated by several groups[47,48]. These disease-corrected lines offer an isogenic background for comparison, thus removing the false positives often present when comparing RNA and protein levels from cells derived from over-expression or genetically different cell lines. Intestinal organoids generated from cystic fibrosis patient iPSCs with homozygous CFTR mutations exhibit reduced responsiveness to cAMP-induced swelling. Upon genetic correction of the CFTR gene via CRISPR, wild-type CFTR gene expression was restored[47]. Consequently, functional assays demonstrated an increase in basal organoid swelling as well as forskolin-induced swelling that was absent in the uncorrected lines[47]. Such studies illustrate the power of genome engineering of hPSCs in revealing disease phenotypes accurately and in a simplified manner.

The combination of specific genetic engineering tools with hPSCs has already demonstrated proof of concept for translational applications. To circumvent potential histocompatibility issues in blood platelet donation, Feng et al.[49] knocked out the β-2-microglobulin (β2M) gene in human iPSCs. The β2M knockout hPSCs were differentiated to megakaryocytes (MKs). These mature MKs were further found to produce platelets, respond to a standard blood clotting assay, and were functional upon infusion into mice. Excitingly, the MK progenitor cells could be cryopreserved until required for rapid expansion and maturation, offering tantalizing potential for future use in blood transfusions.

Genetic engineering also enables building of a functional switch into hPSCs so that the function of differentiated cells may be tuned up or down. As a proof of concept, Chen Y et al. engineered tunable hPSC lines that express designer receptors exclusively activated by designer drugs (DREADDs) in the AAVS1 locus[50]. They then differentiated the transgenic hPSCs to midbrain dopamine neuron progenitors and transplanted the cells into a mouse model of Parkinson’s disease. When the animals transplanted with cells expressing the excitatory DREADD are treated with a designer drug, the functional recovery is enhanced. In contrast, when the animals grafted with cells expressing the inhibitory DREADD are

treated with a designer drug, the functional recovery is abolished. This study not only provides mechanistic insights into the way by which grafted cells work but also suggests possibility of tuning up or down the function of grafted cells to achieve optimal therapeutic outcomes.

Conclusions

By following developmental principles, we are able to consciously direct and pattern hPSCs to virtually any cell types. Pharmacologic manipulation of signaling pathways via small synthetic molecules further enables devising strategies to guide hPSC differentiation in an increasingly defined and controlled manner. A major hurdle in using hPSC-derived cells for functional evaluation and disease modeling is that the *in vitro* produced human cells resemble those in embryos and require a substantial amount of time in culture to reach maturation. Hence, a strategy to speed up the functional maturation of hPSC-differentiated cells is needed. This is particularly true for cells that are born late in development, such as glial cells and cortical interneurons. Efforts are being made to accelerate the maturation by expressing transcription factors that are associated with post-mitotic cells (e.g., Ngn2) or aging[51,52]. However, these transgenic approaches may bias the cell fate or causes pathological changes. A pharmacologic approach, as developed for cellular differentiation over the past decade, would be a better alternative.

Another issue is the generation of a consistent population of cells in a large quantity. This is particularly important for high throughput analysis and for future clinical application. Current approaches to expand progenitors with conventional mitogens almost invariably yield mixed cell populations. For example, neural progenitors expanded by FGF2 resultsin generation of different types of neurons and glial cells depending on time in culture. Similarly, a specified progenitor, such as dopamine neuronal progenitors, when expanded by FGF2, will quickly lose the ability to produce dopamine neurons. Efforts are being made to expand a progenitor pool while maintaining identity and differentiation potency, such as spinal motor neuron progenitors[53], by regulating its fate choice, preventing alternative fates, and modulating relevant epigenetic machineries. Such a strategy is essential for materializing the translation of human stem cell biology.

Inconsistency is also a bottleneck for generation of organoids from hPSCs. While some organoids can be routinely produced in a reasonably simple manner, the variation among individual organoids within a single culture is so large that it makes it nearly impossible for high throughput analysis. Engineering a defined substrate with controlled morphogen gradients could reduce variations. An alternative is to take advantage of our ability to generate defined populations of progenitors and print an organoid in a 3D manner. This approach may be particularly helpful in producing organoids that are composed of cells from different germ layers.

Directed differentiation of hPSCs to functionally specialized cells and tissues under defined conditions, together with precise genetic modification, is rapidly paving the way for future applications in medicine. Mechanistic insights gained from *in vitro* modeling of human development and disease may help elucidate human specific pathways for refined screening

assays and more effective therapeutics. While the use of hPSCs in cell replacement therapies needs further development, the technology exists to make it a reality in the not too distant future.

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Highlights

- Human stem cells are differentiated to functional cells by regulating developmental pathways.
- Stem cells form primitive tissue organoids following intrinsic developmental program.
- Genomic editing expands potential use of human stem cells.

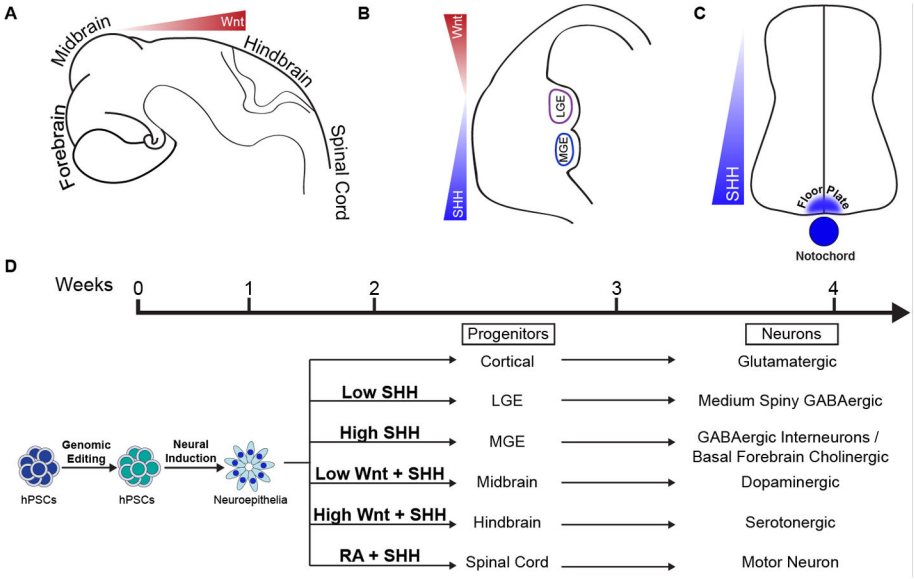

Figure 1. Summary of regional patterning and neuronal subtype generation

A. Sagittal view of developing mammalian nervous system illustrating morphogen gradient along the rostral-caudal axis. **B**. Cross section view of developing brain showing the lateral and medial ganglionic eminence (LGE & MGE), illustrating morphogen gradient along the dorsal-ventral axis. **C**. Cross section of the spinal cord showing notochord; the source of SHH in the developing nervous system. **D**. Summary of in vitro directed differentiation paradigm for subtypes of progenitors and neurons in response to morphogens.