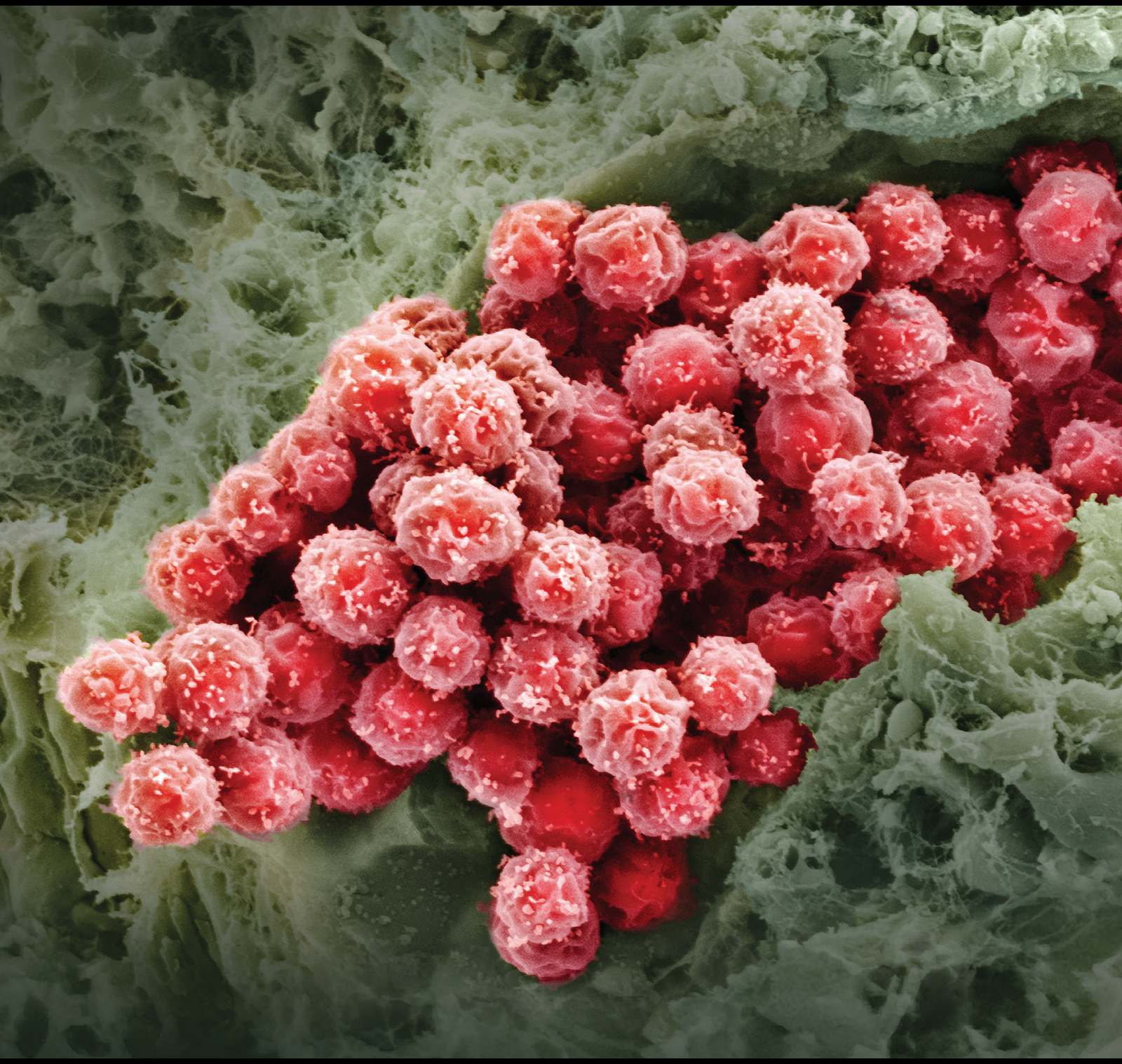
Stem Cells International

iPSCs: From Bench to Clinical Bed

Guest Editors: Yujing Li, Changwon Park, Luciano Vellón, and Xuekun Li



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Editorial Board

Contents

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Editorial

iPSCs: From Bench to Clinical Bed

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Induced pluripotent stem cells (iPSC) have been acknowledged as a milestone in the field of stem cell biology and regenerative medicine, providing an excellent tool to tackle fundamental biological issues regarding reproduction, regeneration, and (de)differentiation at genetic and epigenetic levels, and the valuable cell sources for tissue regeneration, human disease modeling, and drug discovery. Furthermore, iPSC generation with patient-specific somatic cells holds great promise for autologous cell replacement therapy and organ transplantations. Thus, the iPSCs have attracted significant attentions in generation methods, mechanisms of reprogramming, and therapeutical applications. Given a body of achievements in iPSC generation methodology, mechanisms, and clinical application, it is of significance to publish a special issue focused on the topic of iPSCs (from bench to clinical bed). In this special issue, research and review articles are collected, covering the advances in the areas of iPSC generation strategies, molecular mechanisms for reprogramming, iPSC models for human diseases, cell therapy, organ generation, and transplantation.

Starting from the initial discovery of the iPSCs decade ago, significant efforts have been made to develop the protocols with high efficiency and safety to generate clinically relevant cells by employing a variety of (epi)genetic and biochemical approaches. Consequently, some protocols have become practical to significantly enhance the efficiency of iPSCs generation as well as the safety levels of the iPSCs for therapeutic applications. With regard to the progress in the iPSC generation strategy, P. Ji et al. and N. Xie and B. Tang highlight the technical advances in recent years from virus-mediated to virus-free strategies to reactivate the reprogramming factors silenced in the somatic cells and finally give perspective pertaining to the future directions on how to further develop the protocols particularly by high throughput screening to find small molecules and epigenetic modifiers to enhance the iPSC generation efficiency and clinical safety. Although small molecules that have been identified so far have limited effects on enhancement of the somatic reprogramming efficiency, with development of the more efficient screening strategies, it is still expected that appropriate small molecules play important roles in this regard.

Although the human iPSCs (hiPSCs) have been expected to play essential roles in regenerative medicine, the safety of the generated hiPSCs has been a big issue. K.-I. Lee et al. address their own research progress in generation of foot-free and xeno-free iPSCs for clinical therapy purpose by combining the xeno-free/feeder-free culture system and microRNA delivery based mRNA mediated reprogramming. In addition, R. Rungsiwiwut et al. report an important discovery that coculture of hiPSCs combining human foreskin fibroblasts (HFF) with human cord blood-derived serum (hUCS) confers the high pluripotency, differential capacity, and karyotypic stability; even the hiPSCs are cocultured for much extended period, overcoming the instability during the iPSC large scale and long period of culture.

Previously, experiment animals such as mouse and rat serve as the main source for human disease models, while contributing to partial understanding of the pathological

mechanisms, but bearing some fatal shortcomings. The discovery of iPSCs opens a new angle for the development of new models to dissect the pathological mechanisms of the human diseases and to discover new strategies for clinical therapy because the iPSCs from patient tissues could be differentiated into the cell types that recapitulate the identical genome of the patient. Although this strategy is still at a very early stage, pilot achievements have been made. In this special issue, N. Xie and B. Tang highlight the recent advances in iPSC models of human diseases with four strategies: (1) directly reprogramming patient somatic cells to iPSCs, (2) generating humanized mouse chimera with iPSCs injection, (3) three-dimensional structured in vitro models, and (4) iPSC-derived minibrains, respectively. In addition, these authors also review the potential challenges encountered in the practice of these strategies. Meanwhile, J. Kang et al. and W. Zhang et al. further review the progress in the iPSC models of human Parkinson's and Alzheimer's diseases in more detailed ways, respectively, and address the challenges and future directions.

With significant improvement on the iPSC generation methods, more and more mysteries behind the molecular mechanisms that regulate the somatic reprogramming at the level of genetics and epigenetics have been uncovered. Several review articles in this special issue highlight the advances in the understanding of the reprogramming mechanisms. At epigenetic levels, S. Hu and G. Shan summarize the global epigenetic remodeling during the somatic reprogramming, particularly the alteration of the long noncoding RNAs (lncRNAs) expression levels. Meanwhile, P. Ji et al. focus on the reprogramming regulation at epigenetic and nonepigenetic levels. Epigenetically, regulations at levels of chromatin, genomic DNA, and histone macroH2A have been proven to play essential roles in the somatic reprogramming regulation. And with discovery of these epigenetic modifiers as well as chromatic remodelers that significantly regulate the somatic reprogramming, some of these regulators have been believed to function as enhancers for the iPSC generation efficiency and potential drugs for therapeutic applications clinically.

Since the iPSCs can be generated from the same patient, recapitulating the whole identical patient genome, the immune rejection encountered in the conventional transplantation can be avoided by applying the iPSCs-based generation of the organs. Thus, the iPSC-based clinical application in regenerative medicine particularly in cell therapy and organ regeneration as well as transplantation has been attracting more and more attentions. In this special issue, A. J. Orqueda et al. highlight the recent breakthrough particularly in the iPSC-derived generation of miniorganoids such as miniature stomach, 3D gut, minilivers, little lungs, building hearts, tiny eyes, and baby brains derived from 3D culturing of the iPSC-based neuroectoderm and these baby brains could lead to further generation of cerebral cortex, ventral telencephalon, choroid plexus, retinal identities, and so forth. These tries have become the pilot in the regenerative medicine and shed light on the in vitro generation of the human organs, opening a new era for the regenerative medicine.

For iPSC-based cell therapy, several review articles in this special issue highlight the recent promising advances. A. J. Orqueda et al. summarize the impact of insulin-producing pancreatic cells, motor neurons, and retinal cells differentiated from iPSCs in either animal models or patients and discuss the application of CRISP/CAS9 technology in target gene mutation corrections for the patient-specific iPSCs. N. Xie and B. Tang summarize the cell replacement strategies and the potential challenges. J. Kang et al. and N. Xie and B. Tang highlight the effects of the iPSCs-derived motor neurons in ischemic stroke rescue in rat PD models.

Since the conventional pharmaceutical drug screening pipeline confers low efficiency, high cost, and extremely low approval rate of the clinically tested drugs, scientists have been trying better alternatives. Because the patient-derived iPSCs could better emulate the real pathological mechanisms, it is logical to speculate that the patient-derived iPSCs-based drug screening could provide a better platform efficiently and identify the candidates from the large number of chemical compounds. In addition, the iPSCs-based platforms could more efficiently assess cell/tissue specific off-target effects and toxicities. In this special issue, N. Xie and B. Tang summarize the progress made in the iPSCs-mediated drug screens and address the challenges.

In summary, by highlighting the recent advances in iPSC research from methodology to the mechanisms and from bench experiments to the clinical applications, the authors of the research and review articles in this special issue hope that they contribute a shortcut for the readers to get the related information easily, who are dedicated in the research field of iPSC and regenerative medicine.

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Review Article

Modeling Alzheimer's Disease with Induced Pluripotent Stem Cells: Current Challenges and Future Concerns

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Alzheimer's disease (AD) is the most prevalent type of dementia and its pathology is characterized by deposition of extracellular β-amyloid plaques, intracellular neurofibrillary tangles, and extensive neuron loss. While only a few familial AD cases are due to mutations in three causative genes (APP, PSEN1, and PSEN2), the ultimate cause behind the rest of the cases, called sporadic AD, remains unknown. Current animal and cellular models of human AD, which are based on the Aβ and tau hypotheses only, partially resemble the familial AD. As a result, there is a pressing need for the development of new models providing insights into the pathological mechanisms of AD and for the discovery of ways to treat or delay the onset of the disease. Recent preclinical research suggests that stem cells can be used to model AD. Indeed, human induced pluripotent stem cells can be differentiated into disease-relevant cell types that recapitulate the unique genome of a sporadic AD patient or family member. In this review, we will first summarize the current research findings on the genetic and pathological mechanisms of AD. We will then highlight the existing induced pluripotent stem cell models of AD and, lastly, discuss the potential clinical applications in this field.

1. Introduction

Since Alzheimer's disease (AD) was first diagnosed by Dr. Alois Alzheimer in 1906 [1], it has become the most prevalent neurodegenerative disease overall. Over 30 million people had suffered from AD worldwide before 2010, and the count number is estimated to double every 20 years to reach 66 million in 2030 and 115 million in 2050 [2] (http://www.alz.co.uk/research/statistics; accessed October 9, 2012).

Clinically, AD is characterized by gradual memory loss and a progressive learning disability and inability to carry out daily tasks. The main pathological hallmarks of AD are thought to be the deposition of extracellular senile plaques composed of insoluble β-amyloid (Aβ) peptide, the formation of intracellular neurofibrillary tangles, and the loss of cholinergic neurons in the basal forebrain, amygdala, hippocampus, and cortical area. However, only the Aβ and the abnormal truncated and hyperphosphorylated tau hypotheses cannot fully explain all of the symptoms of AD. Indeed, various antiamyloid drugs succeeded in lowering the Aβ levels in the brain but failed to slow down the cognitive decline in the treated patients [3, 4]. Additionally, antitangle drugs, which target the kinases and activators involved in the hyperphosphorylation of tau (including the GSK-3 inhibitors, Tideglusib and the methylthioninium chloride tau aggregation inhibitor, Rember) were successful in phase II clinical trials [5] but showed imprecise efficacy in larger phase II trials.

Due to AD's multifactorial and heterogeneous features, its ultimate etiology of AD is not thoroughly understood. While mutations of Presenilin 1 (*PSEN1*), Presenilin 2 (*PSEN2*), and amyloid precursor protein (*APP*) account for most of the early-onset familial AD cases [6, 7], the etiology of the remaining 95% sporadic AD patients is complicated, which is due to various factors including aging, gender, education, and genotype of apolipoprotein E (*ApoE*) [8]. Therefore, there is a pressing need for the emergence of new technologies and models reflecting the progression of AD in patients,

confirming the disease pathology, and predicting novel or optimal therapeutic strategies.

Since its creation in 2006 by Yamanaka groups, induced pluripotent stem cell (iPSC) is considered as a potential tool for modeling neurodegenerative diseases [9]. By forced expression of certain genes, including Oct 3/4, Sox2, Klf4, and c-Myc, patients' specific somatic cells are reprogrammed towards their pluripotent state. In this manner, iPSCs are generated artificially and regain the ability to convert into any cell type of the three germ layers: mesoderm, ectoderm, and endoderm. Several preclinical studies, by modeling both familial and sporadic AD, have established promising methods to gather insights into the exact cellular mechanisms, potential therapeutic strategies, and personalized treatments for AD. Here we summarize the current research on the pathogenesis and iPSC-based models of AD and highlight the potential future application of these cells.

2. Genetics and Pathology of AD

Given the fact that most AD cases are sporadic and that the disease occurs at an old age, an increasing evidence indicates that the underlying cellular or molecular pathological process may start early and progress throughout one's life. The early-onset, familial AD (FAD) accounts for less than 5% of all AD sufferers, and the late-onset, sporadic AD (SAD) affects the remaining 95% [10] (http://www.molgen.vib-ua.be/ADMutations/). FAD and SAD appear to share the same clinical and pathological process in a way that both types of AD patients exhibit progressive dementia clinically, extracellular Aβ plaques, and intracellular accumulation of phosphorylated tau protein. In general, major achievements of understanding to AD came from the study of the familial AD and mostly from FAD patients with disease-causing mutations.

Genetic factor is considered to be among main contributors to the risk of AD. Mutations in disease-causing genes and disease-risk genes have been identified and linked with either early-onset AD (EOAD) or late-onset AD (LOAD) (Table 1). Usually, EOAD is inherited in an autosomal dominant manner, and by linkage analyses three rare forms of EOAD have been identified to be linked to their causative genes which include one that encodes for the amyloid precursor protein (APP) and two coding for presenilin, PSEN1 and PSEN2. Approximately 50% FAD patients carry mutations in the three causative genes. Among them, mutations in PSEN1 that represent the majority comprise the majority (approximately 70–80%) of the mutations in EOFAD, followed by APP mutations (15–20%) and mutations of PSEN2 accounting for less than 5% [10]. The amyloid cascade hypothesis demonstrates the underlying targets of the three causative genes. In central nervous system (CNS), the APP protein functions as a neuron surface receptor and participant in neurite growth, neuronal adhesion, and axonogenesis. Physically, the APP protein is cleaved by α-, β-, and γ-secretase at three major sites, respectively. The α-secretase (mainly ADAM10, a disintegrin and metalloproteinase 10) mediated cleavage reduces the production of Aβ, while β-secretase (mainly BACE I, β site APP-cleaving enzyme I) and γ-secretase lead to Aβ production [11, 12]. PSEN1 and 2 are transmembrane protein components of the γ-secretase complex involved in Aβ production during APP processing. The Aβ clearance pathway includes Neprilysin, IDE (insulin-degrading enzyme), ECE (endothelin-converting enzyme), and ACE (angiotensin-converting enzyme) [13, 14]. Imbalance between production and degradation of Aβ (e.g., the mutations of APP, PSEN1, and PSEN2) results in its accumulation and aggregation in the brain. The consequences of Aβ accumulation include a series of abnormal cellular responses such as the formation of intracellular neurofibrillary tangles (NFTs) made of abnormal truncated and hyperphosphorylated tau [15, 16], microglial and astrocytic activation, inflammatory response, oxidative stress, mitochondrial dysfunction, and at last neuron loss. Tau is a microtubule-associated protein with function of promoting microtubule assembly and stability that may also be involved in the establishment and maintenance of neuronal polarity, axonal transport, and neurite outgrowth, although there are no known tau mutation in AD. In the AD brain, the principal hallmark of tau pathology is the formation of paired helical filaments (PHFs) and NFTs. Tau hyperphosphorylation is a potent inducer of tau pathology because hyperphosphorylated tau displays an increased propensity to form PHFs. It is possible that Aβ peptides that have initially accumulated in the AD brain could activate some tau kinases to promote tau phosphorylation through insulin or wnt pathway [17, 18]. Among these, GSK3β is identified to be able to phosphorylate tau at several sites to form PHFs in neurofibrillary tangles distributed in AD brain [19]. In some PSEN1 mutation cases, GSK3 also became active with the existence of Aβ peptide [20]. On the other hand, tau is also a substrate for various proteases. Truncations of tau protein at aspartic acid 421 (D421) and glutamic acid 391 (E391) residues by several caspases are associated with NFTs in the brains of AD patients [21, 22]. In vitro Aβ treatment produces a 17 kDa fragment (tau 45–230), and overexpression of it induces neuronal apoptosis [23]. Additionally, Calpains, thrombin, and cathepsins are also involved in tau truncation apart from caspase [24–26]. However, more tau fragments found in AD brain are not well investigated and their production and impact remain to be identified.

Due to the multifactorial and heterogeneous nature of AD, genetic counseling of SAD is empiric and relatively nonspecific. It is often speculated that SAD is the combined action of unknown environmental factors and multiple susceptibility genes. Among them, frequent variations of apolipoprotein E (*APOE*) are the only well-documented association with SAD. APOE is a component of several lipoproteins consisting of 3 isoforms determined by cysteine-to-arginine substitutions at positions 112 and 158 of the amino acid sequence [27]. Individuals with heterozygous APOE ε4 are 4 times more likely to develop AD while homozygous for APOE ε4 are 8 times relative to individuals without APOE ε2 and APOE ε3 allele [28]. In CNS, APOE is thought to facilitate clearance of Aβ, and the APOE ε4 allele seems to have the lower ability to clear Aβ resulting in a high risk of developing AD. APOE ε4 is also identified with smaller gray matter volumes and accelerated brain aging [29, 30].

With the application of Genome-Wide Association Study (GWAS) since 2005, next generation whole exome (WGS),

Table 1: Causative and risk variants of AD.

Gene	Variant	Effect allele frequency	Odds ratio	Function	AD-related pathways
APP	—	—	—	Aβ peptide precursor, neurite outgrowth, adhesion, and axonogenesis	APP processing, produce Aβ
PSEN1	—	—	—	Component of γ-secretase complex that cleaves APP into Aβ fragments	APP amyloidogenic pathway, cleaves APP
PSEN2	—	—	—	Component of γ-secretase complex that cleaves APP into Aβ fragments	APP amyloidogenic pathway, cleaves APP
				Frequent variants	
APOE	—	—	—	Component of lipoproteins, transports lipids and cholesterols, mediates synaptogenesis, synaptic plasticity, and neuroinflammation	Aβ clearance
CR1	rs6656401	0.197	1.18	Bind C3b and C4b, and moderate the activity of the complement system	Aβ clearance
BIN1	rs6733839	0.409	1.22	Participant in Clathrin-mediated endocytosis, intracellular trafficking, apoptosis, and interacting with the microtubule cytoskeleton	Mediate tau toxicity
CD2AP	rs10948363	0.266	1.10	Cytoskeletal organization, endocytosis	Mediate tau toxicity
EPHA1	rs11771145	0.338	0.9	Mediate brain development, particularly axonal guidance	Immune response, neural development
CLU	rs9331896	0.375	0.86	extracellular chaperone, inhibits formation of amyloid fibrils by APP	Aβ clearance
MS4A6A	rs983392	0.403	0.9	Signal transduction	unknown
PLCALM	rs10792832	0.358	0.87	Clathrin-mediated endocytosis	Aβ clearance
CD33	rs3865444	0.307	0.94	Inhibition of cell activity	Aβ42 uptake
HLA-DRB5–HLA-DRB1	rs9271192	0.276	1.11	Histocompatibility antigen, peptide antigen binding	Immune response
PTK2B	rs28834970	0.366	1.10	Induce long term potentiation in hippocampus	Synapse function and neural development
SLC24A4 and RIN3	rs10498633	0.217	0.91	Calcium transport, brain and neural development	Neural development
DSG2	rs8093731	0.017	0.73	Component of intercellular desmosome junctions	unknown
INPP5D	rs35349669	0.488	1.08	Regulate cell proliferation and survival	Immune response
MEF2C	rs190982	0.408	0.93	Participant in hippocampal-dependent learning and memory by suppressing the number of excitatory synapses,and neuronal development and distribution	Neural development, synapse function
NME8	rs2718058	0.373	0.93	Spermatogenesis, ciliary functions	unknown
ZCWPW1 and NYAP1	rs1476679	0.278	0.91	Epigenetic regulation (ZCWPW1); brain and neural development (NYAP1)	Neural development
CELF1	rs10838725	0.316	1.08	mRNA splicing	unknown
FERMT2	rs17125944	0.092	1.14	Cell adhesion, cell shape and Wnt signaling pathway	Mediates tau toxicity
CASS4	rs7274581	0.083	0.88	cell adhesion and cell spreading	Cytoskeleton and axonal transport
SORL1	rs11218343	0.039	0.77	Lipoprotein uptake, APP trafficking to and from Golgi apparatus	APP trafficking
ABCA7	rs115550680	0.09	1.79	lipid metabolism, phagocytosis of apoptotic cells	Aβ clearance
				Rare variants	
ADAM10	Q170H and R181G	—	—	Constitute and regulate α-secretase activities	APP nonamyloidogenic pathway
APP	rs63750847	0.0045	0.24	Inhibit β-secretase activities	APP processing
TREM2	rs75932628	0.0063	2.26	Aβ clearance	Immune response
UNC5C	rs137875858	0.0003298	2.15	Increase susceptibility to neuronal cell death	Inflammatory response
PLD3	rs145999145	0.003077	2.1	APP trafficking and cleavage	APP processing
AKAP9	rs144662445 rs149979685	0.0006298 0.000432	2.75 3.61	unknown	unknown

and whole-genome sequencing (WES) to identify common and rare variations, a series of genes or locus are proposed to increase the risk of AD. These include the identified common risk variation of *CR1*, *CLU* [31, 32], *PICALM* [32], *BIN1* [33], *HLA-DRB5/DRB1* [34], *CD2AP*, *MS4A*, *ABCA7*, *CD33* [35, 36], *INPP5D*, *MEF2C*, *SLC24A4-RIN3*, *CASS4*, *NME8*, *ZCWPW1*, *PTK2B*, *CELF1*, and *SORL1* [34]. Their functions vary from brain development, guiding neural plasticity, cytoskeletal organization, cell apoptosis, and lipid metabolism to Aβ uptake and microtubule cytoskeleton interaction (Reviewed in [37]). While these frequent variations are responsible for risk of AD, rare variation detected from WES/WGS might have larger effect sizes than the common variations. A rare variant of Triggering Receptor Expressed on Myeloid Cells 2 (*TREM2*) gene, the rs75932628 (R47H) mutation was confirmed to increase the age of onset in LOAD patients [38]. Further studies on the *TREM2*-associated risk of AD indicated that it is the recessive loss-of-function mutations in *TREM2* that were responsible for early-onset dementia [39]. Generally speaking, TREM2 is expressed by microglial cells in CNS and was found to be presented with amyloid plaques in the brain of AD mice, suggesting that TREM2 may play a role of Aβ clearance. The presence of *TREM2* R47H variant was also confirmed in population from French [40], Spanish [41], Catalan [42], and Belgian [43]; however, in a study involving 1133 patients and 1157 subjects from China, the R47H variant was not detected [44]. In our study with 360 AD cases and 400 controls of Chinese population, the rs201280312-T (A130V) variant was detected in two of the AD cases [45], suggesting the genetically heterogeneous nature of TREM2 mutations.

Other rare variations were identified in genes coding for Netrin receptor, *UNC5C* [46], phospholipase D3 (*PLD3*) [47], ATP-binding cassette transporter (*ABCA1*) [48], and disintegrin and metalloproteinase domain-containing protein 10 (*ADAM10*) [49, 50], although their functional participation in AD occurrence needs further investigation. Elucidating the genetic contribution is a major concern in understanding SAD while there is neither animal models nor proper cell models of SAD to modeling SAD in a dish.

3. iPSCs Reprogramming and the Basis of Alzheimer's Disease Modeling

Both embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs) have the ability to self-renew and differentiate into all three germ layers, thereby endowing us the possibility of reconstructing all types of cells, tissues, and even organs. However the applications for human ES (hES) are limited by several challenging problems such as allogeneic immune rejection, potential tumor formation, and ethical issues concerning the utility of human embryo [51, 52]. Derived from human fibroblast, iPSC was first generated by Yamanaka groups in 2007 [53]. With the similar ability of differentiation with ES cells, but without the concerns of immune rejection problems or ethical issues, iPSC soon gained worldwide attention.

3.1. Introduction of iPSCs Technology. The iPSCs were first generated from mouse fibroblasts through the retroviral-mediated introduction of four transcription factors (OCT4, SOX2, KLF4, and c-MYC) by Takahashi and Yamanaka in 2006 [9]. They found that forced expression of the four extrinsic factors was sufficient to return somatic fibroblasts into a pluripotent state within a few weeks. As soon as a year after this breakthrough, the technique was applied to human fibroblasts [53, 54]. The induced pluripotent stem cells showed similar colony morphology, gene expression, cell surface marker expression, and the ability to self-renew and differentiation as embryonic stem cells (ESC). Since then, more combinations of transcription factors emerged, such as forced expression of OCT4, SOX2, NANOG, and LIN28 mediated via lentiviral vector to reprogram human fibroblasts into an undifferentiated state [55]. One of the concerns for iPSCs methodology is that the insertion of retrovirus vectors into human genome might become a potential threat to troublesome changes such as tumor-genesis. To avoid shortcomings brought by viral vector interaction, nonintegrating viruses have been applied for generation of the iPSCs, including Adenovirus [56] and Sendai Virus [57–60]. However, these methods are either of low efficiency or technological immaturity. Thus, more alternatives for non-integrating methods were invented such as transfection miRNA for transcription factors [61], episomal plasmids, three oriP/EBNA plasmids (a kind of plasmid vector that may express for a long period of time) harboring either an Oct4, Sox2, Nanog, and Klf4, an Oct4, Sox2, and SV40 large Tantigen, or a c-myc and Lin28 combination. This way, human foreskin fibroblasts were reprogrammed into iPSCs as soon as 20 days after transfection [62].

Efforts were also directed at improving the reprogramming efficiency as the original method of reprogramming by Yamanaka achieved an efficiency of only ~0.02% at ~30 days after retroviral transduction [53] and the mRNA expression method mentioned above was able to raise the efficiency to 1.4% within 20 days [61]. It has been found that by shifting the culture condition to 5% O_2 and adding valproic acid into the cell culture medium, the efficiency could be increased to 4.4% [61]. miRNA has been believed to be another promising factor that could increase the reprogramming efficiency with or without Yamanaka factors since some miRNAs are upregulated in both iPSCs and hESCs. For instance, with the presence of four Yamanaka factors, miR-302b and/or miR-372 could increase the efficiency of reprogramming in MRC5 and BJ-1 fibroblasts from 10- to 15-fold compared with the four factors alone [63], while expression of miR302/367 only could transform ~10% of the BJ-1 fibroblasts into iPSCs after 12–14 days after infection [64].

In addition to seeking safer reprogramming factors and improving the reprogramming efficiency, searching for the proper cell sources for reprogramming represents another important strategy. Although skin fibroblasts are considered as the traditional, classical cell source for iPSCs generation, one must undergo a rather invasive procedure for donating samples. The collection of cells from other lower yield sources can be far less invasive, including the collection of mononuclear cells from peripheral blood [65], or hair follicles [66],

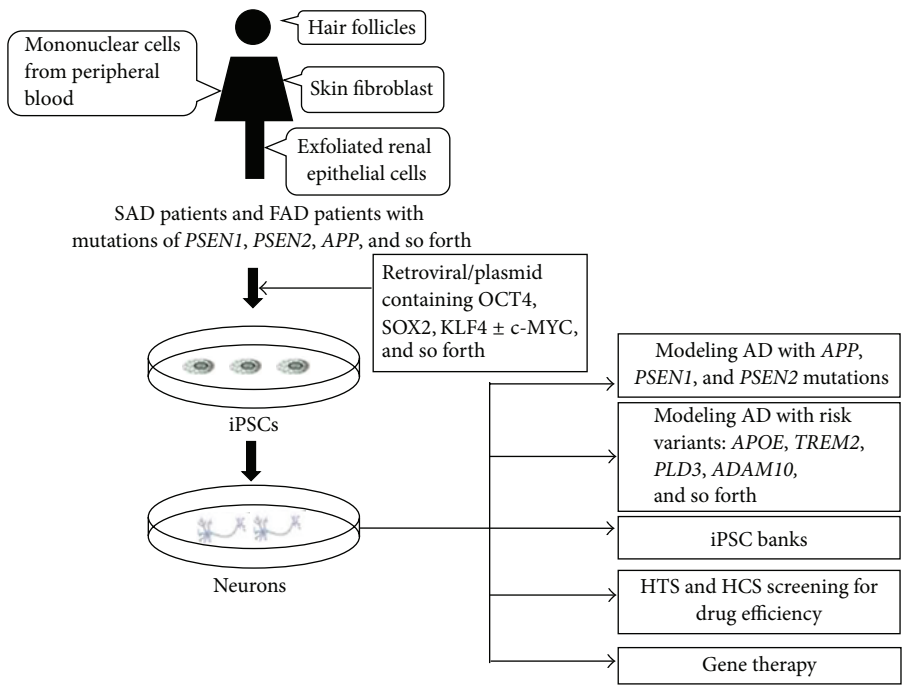

Figure 1: The generation of iPSCs and iPSC-derived neurons from various sources of somatic cells and application of iPSC in Alzheimer's disease.

or even the exfoliated renal epithelial cells from urinary sediments [67]. However, due to the inherent inefficiency of iPSC generation, a large amount of somatic cells is required. Furthermore, the culture potential of the primary cells often varies according to the donors' ages, physical conditions, and long-term drug use. Consequently, it is urgent to decipher the primary underlying cause of the differences between various cell sources and to find an easier method for isolating enough somatic cells in the least invasive manner.

3.2. Application of iPSC in Alzheimer's Disease. In light of the important benefits conferred by their self-renewal and multidirectional differentiation capacity, iPSCs are valued in the context of regenerative medicine and disease modeling, especially for neurodegenerative disorders. Indeed, despite the limitations imposed by the low-efficiency and time-consuming nature of the reprogramming process, iPSCs remain a relevant tool to study the fundamental etiology of neurological diseases and perform high-throughput drug screening for CNS disorders. In fact, several neurological diseases have been modeled using iPSCs, such as monogenic disorders and versions of complex diseases caused by known mutations have been modeled by iPSCs. These disorders include, among others, Parkinson's disease (PD) with SNCA, PINK1, PARK2, GBA1, and LRRK2 mutations [68–70], amyotrophic lateral sclerosis (ALS) with TDP43 mutation [71], Huntington's disease (HD) with HTT mutation [72], and Spinal muscular atrophy (SMA) with SMN1 mutation [73]. Similarly, AD is another slow progressing disease with a poorly understood etiology and a lack of efficient therapeutic strategies. Therefore, it is of the utmost importance for the AD patients' unmet clinical needs that we identify suitable, disease-relevant cell models to solve these problems.

iPSCs can be directionally differentiated into neurons using a specific array of protocols. First, neural stem/progenitor cells are generated from iPSCs with the presence of the neuroectoderm inducer, retinoic acid [74–76]. A similar outcome can also be achieved by inhibiting the bone morphogenetic protein (BMP) and the transforming growth factor-β (TGFβ) superfamily signal transduction pathways [77, 78], both of which are capable of directing epidermal or mesodermal differentiation. Then, these neural stem cells could be further exposed to certain growth factors to direct differentiation into specific neuronal subtypes. To model AD, induced cholinergic neurons can be generated using a combination of Compound E, 2S-2-N-propanamide (Calbiochem), and Compound W, 3,5-bis(4-nitrophenoxy)benzoic acid, to activate specific intracellular signaling pathways targeting the repressor element 1-silencing transcription factor (REST) and its corepressor (CoREST) [79, 80]. These induced neurons are further selected by specific markers expressed on the endogenous neurons. Upon transplantation into animal models of neurodegenerative diseases, these neurons function well and contribute to the recovery of several neurological deficits.

In general, isolated somatic cells undergo a series of reprogramming and neural differentiation procedures to generate a large number of induced AD patient-specific neurons for both research and transplantation purposes (Figure 1). However, there are some hurdles to be overcome before attaining the stage of clinical application. First, even though iPSCs and induced neurons retain the original patients' specific genome, some random DNA alterations and epigenetic

changes cannot be avoided during either the reprogramming or the differentiation processes [81]. The potential alterations in DNA splicing or gene expression may induce clonal heterogeneity within the iPSCs and result in cellular functional changes. Second, neuroglia cells participate in the induction of immune responses and Aβ peptide clearance in AD pathogenesis, and the participation of astrocytes and microglia cells may have an important influence on our understanding and interpretation of the figuring out of the AD-specific cellular phenotypes and drug efficiency. Actually, GWAS analyses have yielded a pattern of common cellular pathways involved in AD patients carrying certain risk variants. In the future, it will not be enough to induce the formation of cholinergic neurons only. Instead, three-dimensional human neural cell culture models will be essential for accurate AD modeling [82]. Third, before reaching the stage of clinical application, it will be essential to determine the optimal and safest iPSC generation and neuron differentiation protocols to use. Indeed, some protocols using integrative viruses and the culture media or feeder cell layers containing animal components constitute potential health threats due to the potential for unwanted immune responses and tumor-genesis.

4. Specific Cellular Phenotypes and Processes in the iPSC-Based Models of AD

Modeling AD using iPSCs was initiated from the modeling of familial cases with mutations in disease-causing genes including *APP*, *PSEN1*, and *PSEN2*. Until now, five out of eight publications reported reprogramming of iPSCs-derived cholinergic neurons from patients with FAD (summarized in Table 2), indicating that modeling AD using iPSCs is still in its infancy. However, these studies, which are seeking to find AD-specific unique cell phenotype and AD-related cellular processes, appear as the first step in gaining insights into the genetic contributions to AD.

4.1. FAD Disease Modeling with iPSC. iPSCs have been generated from patients with several mutations of *PSEN1*, *PSEN2,* and *APP* by five groups. Two groups analyzed the production of Aβ peptides and the accumulation of phosphorylated tau protein. Both *PSEN1* A246E- and *PSEN2* N141I-expressing mutant neurons showed an increased ratio of Aβ42 to Aβ40 compared with control neurons, but the ratio in iPSCs lines was very low, indicating that the secretion of Aβ peptides varies during differentiation. In addition, no accumulation of tau protein was observed in this type of FAD-derived neurons [80]. However, the *PSEN1* A246E mutant was further analyzed during the differentiation and observed an increase in the ratio of Aβ42 to Aβ40 in both fibroblasts, neural progenitor cells (NPCs) and early neurons [83], which is somewhat conflicting with the result of Takuya Yagi groups [80].

Induced neurons that carry a duplication of APP exhibited a higher level of Aβ40 but not Aβ42. In fact, the Aβ42 and Aβ38 levels were completely lower than the detection range of the assay [84]. Through fluorescence-activated cell sorting, researchers were able to isolate a more than 95% pure culture of induced neurons. Purified neurons also exhibited higher levels of phosphorylated tau (Thr 231) and active GSK-3β. Compared with control neurons, RAB5-positive early endosomes were enlarged in the neurons from patients with duplication of *APP*, suggesting that early endosomes may regulate APP processing to result in the increased level of phospho-tau, neurofibrillary tangles, synaptic loss, and apoptosis. Muratore et al. found that iPSCs and neurons harboring the *APP* (V717I) mutation showed a twofold increase in the production of Aβ42 and a slight increase in Aβ40 [85]. The Aβ38 level and the calculated Aβ38/40 ratio were also significantly increased compared with control neurons [85]. Furthermore, FAD neurons secreted a lower ratio of APPsα/APPsβ. The APPsβ production showed a 1.4-fold increase compared with controls [85], suggesting that the V717I mutation may primarily alter the initial epsilon site of cleavage within APP. In another study in patients with APP E693Δ mutation [86], the Aβ oligomers accumulated in the iPSCs-derived neurons and astrocytes with APP E693Δ. The hall markers of ER stress and oxidative stress, including BiP, cleaved caspase-4, PRDX4-coding antioxidant protein peroxiredoxin-4, and ROS levels, were also increased in the FAD neurons. Therefore, there was a possibility that the intracellular Aβ oligomers may provoke an antioxidant stress response resulting in increased ROS levels. These results supported the hypothesis that oxidative stress participates in the pathogenesis of AD.

4.2. SAD Disease Modeling with iPSCs. Primary cells from SAD patients have also been used for reprogramming studies and were mostly compared with iPSC originating from cells donated by FAD patients. By researching into the SAD case, Hossini et al. were able to draw an AD-related protein interaction network composed of *APP* and GSK3β among others [87]. In Israel et al.'s study, relative to nondemented controls, both iPSCs and neurons generated from mutation of the *APP* gene and SAD patients showed elevated levels of Aβ peptides, hyperphosphorylation of tau, and GSK3β. While neurons from only one of two SAD patients exhibited increased levels of intracellular Aβ aggregates, similar to the cells derived from the *APP*-E693Δ FAD patients. It is possible that some underlying de novo acquired genes may also participate in the pathogenesis of SAD cases, reflecting the inherent variability of iPSCs. A recent gene expression study in neurons derived from an 82-year-old SAD patient revealed significant gene expression changes between primary cells and induced neurons [87]. The iPSCs technique offers an opportunity to study the underlying molecular events leading to SAD without interference of environmental contributions, allowing the identification of novel AD-associated networks of regulated genes. However, one concern related to the heterogeneous nature of SAD is that the iPSCs-derived neurons from AD patients without inheritance need to expand until cells are produced to enable statistically meaningful analyses.

4.3. Using iPSC-Derived Models to Screen Novel Drugs for AD. Novel treatments targeting the amyloid cascade, APP processing, and ER stress have been tested on the iPSC-derived models of both FAD and SAD. Some of them

Table 2: Human somatic cell reprogramming-based neuronal models of Alzheimer's disease.

Disease	Genetic defect	Outcome	Drug test	Reference
Azheimer's disease	PSEN1 A264E; PSEN2 N141I	Increase secretion of Aβ1–42 in neurons with mutations	γ-secretase inhibitors	Yagi et al., 2011 [80]
	Duplication of *APP*; Sporadic	Increase secretion of Aβ1–42 and phosphorylated tau (Thr231) in neurons with mutations	β-secretase inhibitors	Israel et al., 2012 [84]
	APP E693Δ; APPV717L; Sporadic	Increase of intracellular Aβ olimgo in neurons with APPE693Δ;	DHA	Kondo et al., 2013 [86]
	Asymptomatic and symptomatic APP V717I	Increase secretion of Aβ1–42 and Aβ1–38 in neurons with mutations	Aβ antibody	Muratore et al., 2014 [85]
	PSEN1 A246E; PSEN1 M146L	Gene expression differences between neurons with mutations of *PSEN1* and controls	no	Sproul et al., 2014 [83]
	Sporadic	Changes in gene expression as well as the inducible subunits of the proteasome complex associated with AD in AD-iPS derived neuronal cells	γ-secretase inhibitors	Hossini et al., 2015 [87]

exhibited significant efficiency on the cell-based models and may become candidate drugs to cure AD patients in the future. γ-secretase inhibitors were first screened in iPSCs and neurons carrying *PSEN1* A246E and *PSEN2* N141I mutations [80]. In the presence of compound E, a potent γ-secretase inhibitor, both Aβ42 and Aβ40 decreased sharply in a dose-dependent manner in FAD neurons. Another γ-secretase substrate, the Notch intracellular domain, was also inhibited in a dose-dependent manner, suggesting that both *PSEN1* and *PSEN2* iPSCs-derived neurons respond to the γ-secretase inhibitor treatment. In Muratore et al.'s study, both FAD neurons with *APP* V717I mutation and SAD neurons responded to DAPT, another γ-secretase inhibitor, and the induced neurons exhibited an inhibited production of Aβ38, 40, and 42 when treated with 5 μm DAPT for 48 hours [85]. Aβ antibody that binds and sequesters the Aβ peptides was able to prevent the increase in the total tau levels in *APP* V717I neurons, suggesting a crosstalk between the amyloid cascade and tau hyperphosphorylation in the AD brains [85]. However, in the clinical trials, although the antibody succeeded at lowering the levels of Aβ peptides, it failed at slowing down the progression of the cognitive impairment. Docosahexaenoic acid (DHA) has been reported to improve ER stress or to inhibit ROS generation [88]. In *APP*-E693Δ neurons, DHA could significantly decrease the BiP protein, cleaved caspase-4, and peroxiredoxin-4 levels, as well as reduce the ROS production, ER stress, and oxidative stress markers. As a consequence, the neurons with an *APP*-E693Δ mutation survived longer after DHA treatment for 16 days compared with the SAD and control neurons [86]. Considering that the levels of Aβ oligomers, which trigger the ER and oxidative stress, remained unchanged, this indicates that DHA treatment may be considered as a symptom relief invention but not as a preventive/curative therapy. Taken together, these results show that iPSCs-derived models allow screening for proper treatment strategies under specific individual genomes.

5. Challenges and Concerns

The potential applications of the reprogramming technology provide a promising approach to generate accurate human cell models of neurodegenerative disorders. To a certain extent, it now becomes possible to aim at recapitulating AD "in a dish." While iPSCs-derived cell models allowed the identification of factors associated with the disease phenotypes and the screening of various novel potential therapies, for example, the β- and γ-secretase inhibitors on the basis of the amyloid cascade hypothesis, there are still more discoveries to be made by using iPSC-derived models of AD (Figure 1).

5.1. Investigation of Novel Mutations in APP, PSEN1, and PSEN2. So far the iPSCs generated from fibroblasts with several FAD mutations, including *PSEN1* A264E, L166P and M146L, *PSEN2* N141I, *APP* V717I, E693Δ, and duplication of *APP* are summarized in Table 2. Compared with neurons induced from control fibroblasts, FAD presenilin and *APP* mutant neurons exhibited not only an increased Aβ42/40 ratios, an elevated level of phosphorylated tau protein, and an increased activated GSK3β. Furthermore, they also displayed abnormal endosomes, indicating a novel dysregulated pathway alongside of APP processing and tau phosphorylation. Therefore, more iPSCs with additional FAD mutations should be investigated to confirm the existing conclusions and help identify other underlying mechanisms behind the disparate phenotypes of the patients.

5.2. Investigation of AD Risk Variants Identified by GWAS, WES, and WGS. Whole-genome sequencing and large scale genome-wide association studies aimed at elucidating the factors that result in SAD has brought to light a variety of rare and frequent variants that may predispose or to protect from AD. However, the processing of these data represents a major challenge. For example, screening for these variants in diverse disease populations is already planned, but the related underlying molecular and biochemical mechanisms as well as the impact of the different genetic variants on the neuronal phenotype and AD risk still remain an unsolved puzzle. Actually, several common cellular pathways are associated with variations identified as GWAS, such as inflammation and immune response, endocytosis, and lipid metabolism. The application of the iPSCs technology to study those potential AD risk variants maybe an efficient way to identify the ultimate impact of these genes on the pathology of SAD, to distinguish between the real and the false positive variations and to find out novel pathways associated with the pathogenesis of AD.

5.3. Construction of the iPSCs Bank for AD. Although cord blood bank has been initiated in many countries, given the expensive procedure and the limited availability at the moment of birth only, iPSCs bank would be a promising way alternative to the cord blood bank to restore and recycle cells from patients with different phenotype and mutations because the iPSCs technology could generate a self-renew, stable progenitor population. Public banks of diseased fibroblasts from patients with genetic mutations responsible for certain neurodegenerative disorders already exist. The existence of these banks allows for categorizing sporadic cases and familial cases with different mutations for personalized medicine purposes, investigating the typical phenotype of each individual's unique genetic background, and may ultimately provide a potential treatment means for regenerative medicine.

5.4. Novel Drug Testing: High-Throughput Screening (HTS) and High Content Screening (HCT). Since iPSCs could retain the patients' genotype and enable us to recapitulate AD in a dish, neurons derived from the disease-specific iPSCs have been used to test several candidate drugs, such as the γ- and β-secretase inhibitors and Aβ antibody. The five studies based on iPSCs-derived models of AD patients harboring the mutations of PSEN1, PSEN2, and APP point mutation as well as APP duplication, respectively, all report decreasing Aβ peptide levels in iPSCs-derived neurons treated with a γ-secretase inhibitor, an Aβ antibody, and DHA. The reduction in phosphorylated tau and GSK-3β levels was observed in the neurons treated with a β-secretase inhibitor. Likewise, the nonsteroidal anti-inflammatory drug (NSAID)

sulindac sulfide has been proposed to become one of the novel strategies for AD therapy by inhibition of the Aβ production [89]. The stem cell-derived neurons expressing wild-type *PSEN1* treated with NSAID exhibited a decrease in Aβ42 level. However the therapeutic effect was absent in cells harboring the *PSEN1* L166P mutation [90]. These studies constitute the primary attempts at screening potential treatment strategies in human genotype- or disease-specific cells. Using iPSCs, HTS, and HCT could allow for rapid analysis for thousands of compounds and disease hallmarks, as well as various cellular contexts affecting drug efficiency, or chemical toxicity, for example. Indeed, by accessing the induced neurons from a cohort of patients and controls in a 96-well format, researchers could rapidly analyze a substantial number of drugs and chemicals for endpoints such as Aβ peptides levels or phosphorylated tau levels. Using the HTS technique as a prescreening method would streamline the time-consuming preclinical animal studies and potentially reduce the number of failing clinical trials. After target drugs or compounds identification using HTS, HCS would enable the subsequent analysis of relevant cellular signals and pathways. Highly efficient screening based on iPSC-derived neurons could become a routine drug discovery pathway.

5.5. Gene Therapy and iPSCs Transplantation. The potential iPSCs-based regeneration medicine and gene therapy on AD include gene correction and iPSCs induced neurons transplantation. Gene correction has already been conducted in HD by using homologous recombination in the iPSCs stage, leading to normalized pathogenic HD signaling pathways, including cadherin, TGF-β, BDNF, and caspase activation in neural stem cells [72]. On the basis of the cellular endogenous recombination mechanism, zinc-finger nucleases (ZFNs) and tal-effector nucleases (TALENs) are emerging as engineered nucleases usable to modify individual genomes [91, 92]. First, a wild-type nucleotide sequence binds into the FOK1 nuclease fused to arrayed domains triggering a DNA double-strand break. Then, the endogenous recombination machinery emerged induces DNA homologous recombination and nonhomologous end-joining. Finally, the disease-causing mutations are corrected into the wild-type genomic sequence. Genetically corrected iPSCs or induced neurons transplantation in CNS is still at the stage of animal testing. In 1985, researchers successfully transplanted embryonic cholinergic neurons into an AD rat model. The procedure resulted in memory improvement, suggesting that cholinergic cells transplantation may induce functional recovery in the rodent brains [93]. With the generation of the first iPSCs in 2006, the idea of autologous transplantation emerged with the advantage of reducing immunoreactions usually associated with heterologous transplantation. A recent study on the autologous transplantation of iPSC-derived dopamine neurons in a cynomolgus monkey (CM) PD model demonstrated that after iPSC-derived dopamine neurons injection on one side of midbrain, the CM exhibited motor improvement on the transplanted side without a need for immunosuppression. This study indicated a progression that is a step closer to human clinical applications [94]. However, safety is an essential problem concerning insertional mutagenesis and tumorigenesis prior to clinical use. The amount of induced neurons required for functional improvement is another hurdle, an improvement in the iPSCs/neurons generation protocols is indispensable to get equivalent or greater neuron survival.

After all, the iPSCs technology provides a potential therapy for monogenic disorders, as to AD patients with mutations in *APP*, *PSEN1*, and *PSEN2*, shedding light on ultimate therapy of FAD by correcting these mutation. This strategy also holds great promise for complex diseases like SAD. For example, correcting the mutations of risk genes selectively enables a direct comparison between the iPSC lines derived from WT and mutant cells under the same genome circumstance, significantly reducing the intrinsic genome variability existing in different patients.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Weiwei Zhang, Miaojin Zhou, and Tao Zhou conceived and wrote the paper, the table, and the imaging; Bin Jiao and Lu Shen critically edited the paper.

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Research Article

Extracellular Matrix-Dependent Generation of Integration- and Xeno-Free iPS Cells Using a Modified mRNA Transfection Method

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Human induced pluripotent stem cells (iPS cells) hold great promise in the field of regenerative medicine, especially immune-compatible cell therapy. The most important safety-related issues that must be resolved before the clinical use of iPS cells include the generation of "footprint-free" and "xeno-free" iPS cells. In this study, we sought to examine whether an extracellular matrix- (ECM-) based xeno-free culture system that we recently established could be used together with a microRNA-enhanced mRNA reprogramming method for the generation of clinically safe iPS cells. The notable features of this method are the use of a xeno-free/feeder-free culture system for the generation and expansion of iPS cells rather than the conventional labor-intensive culture systems using human feeder cells or human feeder-conditioned medium and the enhancement of mRNA-mediated reprogramming via the delivery of microRNAs. Strikingly, we observed the early appearance of iPS cell colonies (~11 days), substantial reprogramming efficiency (~0.2–0.3%), and a high percentage of ESC-like colonies among the total colonies (~87.5%), indicating enhanced kinetics and reprogramming efficiency. Therefore, the combined method established in this study provides a valuable platform for the generation and expansion of clinically safe (i.e., integration- and xeno-free) iPS cells, facilitating immune-matched cell therapy in the near future.

1. Introduction

The discovery of induced pluripotent stem cells (iPS cells) has opened a new avenue for patient-specific and immune-compatible cell replacement therapy [1].

The initial approaches used to introduce reprogrammed genes to human fibroblasts relied on retroviral or lentiviral vectors, which caused undesired random insertion of transgenes into chromosomes [2, 3]. The chromosomal integration of transgenes by these viral vectors potentially causes tumor formation depending on the insertion sites, as clearly demonstrated in previous gene therapy trials for X-linked severe combined immunodeficiency [4–6]. Furthermore, the integrated transgenes may be continuously expressed after reprogramming due to incomplete silencing or, in some cases, may elicit full expression resulting from reactivation.

Therefore, methodologies for generating iPS cells without chromosomal integration of exogenous reprogrammed genes have been evolving rapidly. These methods include episomal plasmid transfection [7–9], Sendai virus-mediated gene delivery [10], and mRNA transfection [11].

Among these three integration-free methods, the mRNA transfection method displays several unique advantages. For example, in contrast to episomal plasmid transfection, mRNA transfection completely avoids the possibility of chromosomal integration. In addition, unlike both episomal plasmid transfection and Sendai viral infection, mRNA transfection does not require prolonged passaging to remove lingering exogenous gene expression due to the short half-life of the introduced mRNAs. However, the requirement of 17 consecutive daily transfections of mRNAs [11, 12] is highly laborious, which potentially limits the utility of this method for producing Good Manufacturing Practice- (GMP-) grade iPS cells for cell therapy. Therefore, it is desirable to establish a more efficient and convenient method to generate iPS cells using mRNAs.

Another important issue to consider regarding the clinical application of iPS cells is the generation and expansion of these cells under strictly xeno-free conditions. Xeno-free culture prevents xenopathogen transmission and immune complications caused by non-human antigens [13, 14]. To perform mRNA-mediated reprogramming, the initial and subsequent studies used human feeder cells, and human neonatal fibroblast- (NuFF-) conditioned medium [11, 12, 15, 16]. Although these methods used xeno-free conditions during reprogramming, the preparation of human feeder cells or human feeder-conditioned medium is cumbersome and labor-intensive. Therefore, there has been great demand for the establishment of a simpler and more convenient mRNA-mediated reprogramming protocol for cell replacement therapy.

In this study, we sought to establish such a method by combining our previously established extracellular matrix- (ECM-) based xeno-free/feeder-free human pluripotent stem cell (hPSC) culture system [17] with an improved mRNA-mediated reprogramming protocol. Because clinically safe iPS cells are required for cell replacement therapy, this study provides a useful platform that facilitates future cell therapeutic approaches using iPS cells.

2. Materials and Methods

2.1. Cell Culture. The study was approved by the Ethical Committee of the CHA University Bundang CHA Hospital, Republic of Korea (application number: KNC12005). Human adult dermal fibroblasts (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in DMEM (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen) and 1x penicillin/streptomycin (P/S) (all from Invitrogen, Carlsbad, CA, USA).

Human iPS cells were cultured on vitronectin XF (Primorigen Biosciences, Madison, USA) coated culture dishes using our recently established xeno-free/feeder-free hPSC culture medium with minor modifications [17]. Briefly, the medium consisted of DMEM/F12, 15% KnockOut SR XenoFree CTS, 1x nonessential amino acids (NEAA), 1x GlutaMAX, 0.1 mM β-mercaptoethanol, 1x P/S (all from Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF) (CHA Biotech Co., Daejeon, Korea), 10 nM trichostatin A (TSA) (Sigma-Aldrich, St. Louis, MO, USA), 5 uM Gö6983 (Tocris, Ellisville, MO, USA), and 1 mM dorsomorphin dihydrochloride (Tocris).

2.2. The Generation of Integration- and Xeno-Free iPS Cells. The modified mRNA-based reprogramming reagents used in this study were a kind gift from Stemgent, Inc. (Cambridge, MA, USA). This system consisted of a microRNA cocktail solution (20 uM) containing microRNAs that perform reprogramming functions, including mir302a-d and mir367, and an mRNA cocktail containing mRNAs for Oct4, Sox2, Klf4, c-Myc, and Lin28 at a molar stoichiometry of 3 : 1 : 1 : 1 : 1, respectively. Each mRNA was included at a concentration of 100 ng/μL. The generation of iPS cells using the reagents was performed according to the protocol provided by Stemgent, Inc., with minor modifications [11].

To generate iPS cells, first, human adult dermal fibroblasts were seeded at a density of 5×10^4 cells per well in a 6-well dish precoated with vitronectin XF (10 μg/mL). The next day, the culture medium was replaced with our xeno-free/feeder-free hPSC culture medium 2 hours before transfection. Recombinant B18R protein (working concentration, 200 ng/μL) (eBioscience, Inc., San Diego, CA, USA), a type 1 interferon inhibitor, was added at this time point to promote cell viability after RNA transfection. A Stemfect RNA Transfection Kit (Stemgent, Inc.) was used for RNA transfection: 1 μg of the mRNA cocktail and/or 3.5 μL of a microRNA cocktail (20 μM) was mixed with Stemfect Transfection Buffer in one tube (total volume of 50 μL), and 4 μL of the Stemfect RNA Transfection Reagent was added to the Stemfect Transfection Buffer in a second tube (total volume of 50 μL). The mixture in the second tube was added to the first tube, followed by gentle pipetting of the total 100 μL volume of the combined solution. After incubating the RNA-liposome complex for 15 minutes at room temperature, the mixture was added to the medium in a drop-wise manner with gentle shaking of the dish to ensure uniform distribution of the RNA-liposome complex in the wells. After incubation of the cells for 4 hours, the medium was replaced with 2 mL of fresh xeno-free/feeder-free hPSC culture medium. mRNA transfection was conducted for 11 consecutive days beginning on day 2, and microRNA transfection was performed on days 1 and 5 after cell seeding (twice only). The appearance of iPS cell colonies was monitored every day, and an individual colony was selected at approximately days 20–22 via mechanical methods for further clonal expansion.

2.3. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated using a NucleoSpin RNA II Kit (Macherey-NagelGmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan), and qRT-PCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and a StepOnePlus Real-Time PCR System (Applied Biosystems) under the following conditions: 40 cycles of DNA denaturation at 95°C for 5 seconds, DNA annealing with each primer pair at 55–63°C for 30 seconds, and polymerization at 72°C for 30 seconds. The human β-actin gene was used as a normalization control. The primers used in the qRT-PCR experiments are listed in Supplementary Table 1 available online at http://dx.doi.org/10.1155/2016/6853081.

2.4. In Vitro Differentiation Assay. To test their pluripotency, the iPS cell colonies were mechanically detached and cultured in suspension in Petri dishes (SPL Lifesciences, Pocheon, Korea) in embryoid body (EB) medium (DMEM/F12, 10% KnockOut SR XenoFree CTS, 1x NEAA, 1x P/S, and 0.1 mM β-mercaptoethanol; all from Invitrogen). After 5–10 days of 3-dimensional culturing, the EBs were attached to Matrigel (BD Biosciences, Bedford, MA, USA) coated slides and

further cultured for 15 days in differentiation medium (DMEM/F12 supplemented with 1% NEAA, 1x P/S, 0.1 mM β-mercaptoethanol, and 10% FBS for endoderm and mesoderm or DMEM/F12 supplemented with 1x NEAA, 1% P/S, 0.1 mM β-mercaptoethanol, 1x N2 supplement, and 10 ng of bFGF for ectoderm). Several representative markers specific for derivatives of these three germ layers were used for immunostaining after differentiation. The antibodies used in this study are listed in Supplementary Table 2.

2.5. Teratoma Formation. Approximately 2×10^6 iPS cells per mouse were injected intramuscularly into the thigh of NOD/SCID mice, and the tumor masses were dissected at 9–12 weeks after injection. The presence of all three germ layer structures in the tumor masses was examined after staining with hematoxylin and eosin.

2.6. Karyotype Analysis. G-banding analysis of the iPS cells harvested from a T-25 flask was performed at SamKwang Medical Laboratories (Smlab, Seoul, Korea).

2.7. DNA Fingerprinting. To confirm that the iPS cells did indeed originate from the fibroblasts used for iPS cell generation, DNA fingerprints of the genomic DNA from the iPS cells and the fibroblasts were compared at the Korea Gene Information Center (Seoul, Korea).

2.8. Bisulfite Sequencing. The genomic DNA from human fibroblasts, H9-hESCs, and iPS cells was extracted using an Exgene Tissue SV kit (GeneAll Biotechnology, Seoul, Korea), and the DNAs were treated with an EpiTech Bisulfite Kit (Qiagen GmbH, Hilden, Germany) for bisulfite conversion according to the manufacturer's instructions. The "CpG-rich" promoter regions of the human Oct4 and Nanog genes were amplified using the specific PCR primers listed in S1 Table. The amplified PCR products were subcloned into the TA cloning vector (RBC Bioscience Corp., New Taipei City, Taiwan) and were subjected to sequencing analysis.

2.9. Global Gene Expression Profiling. Total RNA samples were prepared using a NucleoSpin RNA II Kit (Mahcerey-Nagel GmbH & Co. KG, Duren, Germany) according to the manufacturer's protocol. The profiling of global gene expression was performed at Macrogen, Inc. (Seoul, Korea) using 2 μg of total RNA and the HumanHT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA, USA). Microarray data was deposited in a public repository such as gene expression omnibus (GEO, series record GSE68035).

3. Results

3.1. Generation of Xeno- and Footprint-Free iPS Cells via RNA Transfection in an ECM-Based Feeder-Independent Culture System. In our experiment, we sought to generate xeno-free and integration-free iPS cells by combining our ECM (vitronectin) based xeno-free/feeder-free hPSC culture system with an mRNA-mediated reprogramming method. The mRNA reprogramming method used in this study was modified such that the reprogramming efficiency was enhanced using a microRNA cocktail. The microRNA cocktail was transfected into fibroblasts twice, on days 1 and 5 after cell seeding. A mixture of Oct4, Sox2, Klf4, c-Myc, and Lin28 mRNAs was transfected into the fibroblasts for 11 consecutive days beginning on day 2 after seeding. The phenomenon of the mesenchymal-to-epithelial transition (MET) was apparent between days 3 and 5 (Figure 1(b), top row, left two panels). The initial signs of ESC-like colonies (tightly packed cell clumps with clear borders and are composed of cells with high nucleus-to-cytoplasm ratio and notable nucleoli) began to appear on day 11 (Figure 1(b), top row, the most right panel), although they became more evident by day 13 (Figure 1(b), bottom row, the most left panel) after cell seeding. If the cells became too dense during days 14–16, they were split into 3 dishes. Each ESC-like colony was selected approximately on days 20–22 and was cultured using our vitronectin-based xeno-free/feeder-free hPSC culture system for expansion. The efficiency of ESC-like colony formation was approximately 0.2–0.3% (Figure 1(c)), and strikingly, the percentage of the ESC-like colonies among the total colonies was remarkably high (~87.5%) (Figure 1(d)). Among the ESC-like colonies that we selected, two clones, referred to as mRNA-iPSC2 and mRNA-iPSC11, were used for further experimentation. DNA fingerprint analysis confirmed that these iPS cells were derived from the original fibroblasts used for iPS cell generation rather than from contamination by other pluripotent stem cells (Supplementary Table 3).

Taken together, our results showed the high efficiency and accelerated kinetics of our reprogramming process.

3.2. iPS Cells Derived from Fibroblasts Displayed Typical Characteristics of Pluripotent Stem Cells. The iPS cells that were passaged multiple times positively stained for alkaline phosphatase, an early marker of undifferentiated cells (Figure 2). All of the colonies exhibited sharp boundaries and rounded shapes, indicating their undifferentiated state. To more closely examine whether these iPS cells expressed markers of undifferentiated cells, immunostaining was performed for several antigens specific for PSCs. Oct4, Sox2, SSEA4, Tra-1-60, and Tra1-81 were robustly expressed in the iPS cells that had been passaged 15 times in our ECM-based xeno-free/feeder-free hPSC culture system (Figure 2). To examine the specificity of antibodies used in this study, we performed immunostaining of hESC-derived neural precursors with antibodies against some pluripotency markers such as Oct4 and Tra1-60 (Supplementary Figure 1).

Next, we examined the expression levels of several markers of undifferentiated cells in two iPS cell lines generated in this study, along with hESCs and urine-derived iPS cells (UNFiPSC1) as positive controls. In addition, hESC-derived EBs were used as negative controls (Figure 3(a)). The mRNA-iPSCs expressed Oct4, Nanog, Sox2, DNMT3B, Zic3, and REX1 as abundantly as hESCs. In contrast, the mRNA-iPSCs minimally expressed representative markers for the ectoderm (NCAM, Nestin, and Pax6), mesoderm (FoxF1, Hand1, and Gata2), and endoderm (AFP and Gata6) lineages (Figures 3(b)–3(d)).

We further analyzed the expression patterns of mRNA-iPSCs using a DNA microarray. Both scatterplot and heatmap

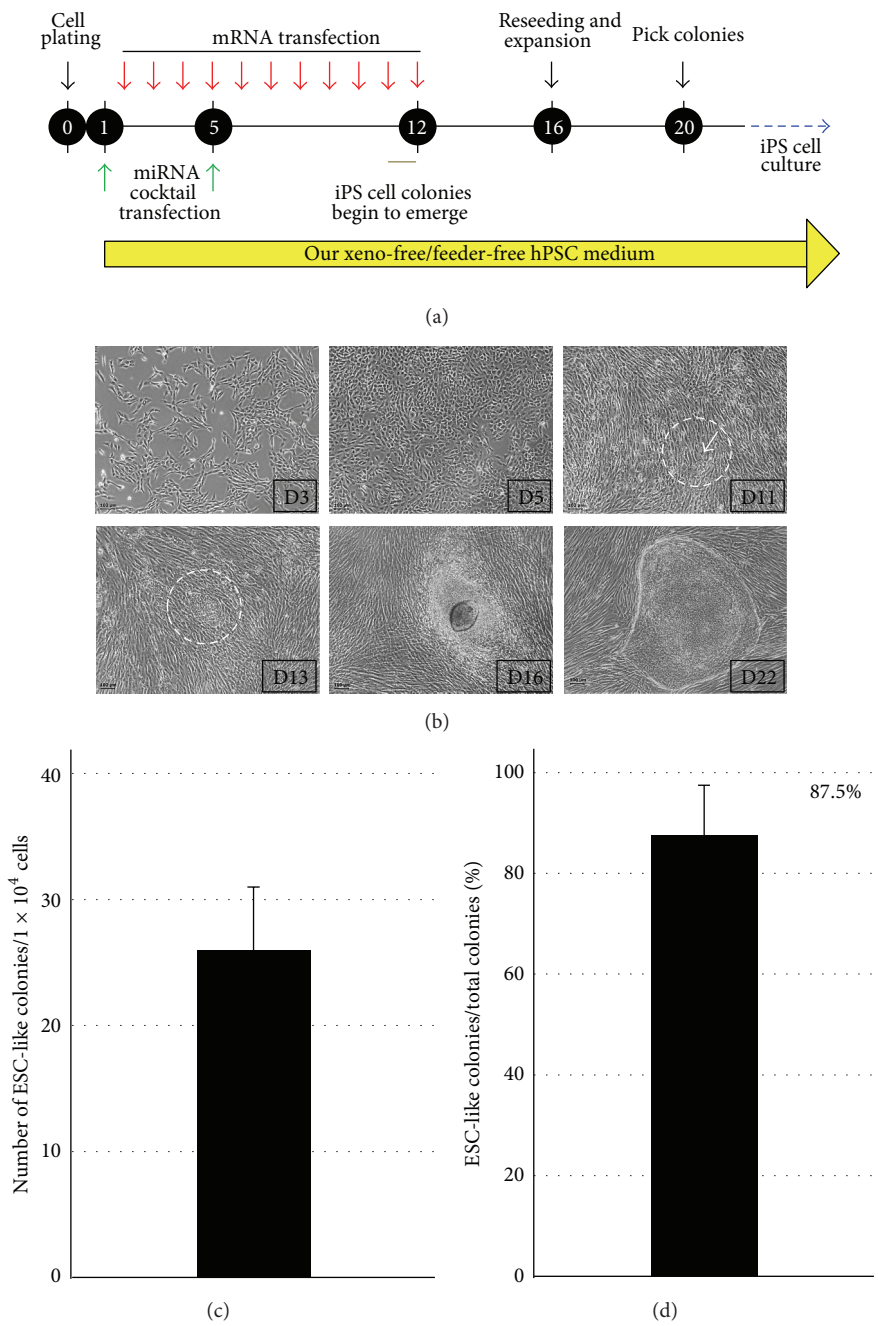


Figure 1: RNA-mediated generation of iPS cells using an ECM-based xeno-free/feeder-free hPSC medium. (a) A schematic representation of the experimental design. Human adult dermal fibroblasts were seeded on 6-well plates (5×10^4 cells per well) and were subjected to multiple transfections as shown in the schedule. (b) The ESC-like colonies began to appear on day 11 after cell seeding (see the tip of the arrow at the center of the white circle, *top right panel*). The number within the square at the right bottom corner of each panel indicates the number of days passed after cell seeding. Scale bar: 100 μm. (c) The graph shows the number of ESC-like colonies generated per 10,000 fibroblasts initially seeded. (d) The percentage of ESC-like colonies among the total colonies is presented in graph format.

analyses of the genome-wide gene expression profiles demonstrated that the gene expression pattern of the mRNA-iPSCs was very similar to that of hESCs (Figures 4(a) and 4(c)). However, the gene expression pattern of mRNA-iPSCs was significantly different from that of the fibroblasts from which the mRNA-iPSCs had originated (Figures 4(b) and 4(c)). The list of human ESC-enriched genes and fibroblast-enriched genes is presented in Supplementary Table 4.

PluriTest, a bioinformatic assay of pluripotency based on gene expression profiles, showed that the mRNA-iPSCs are

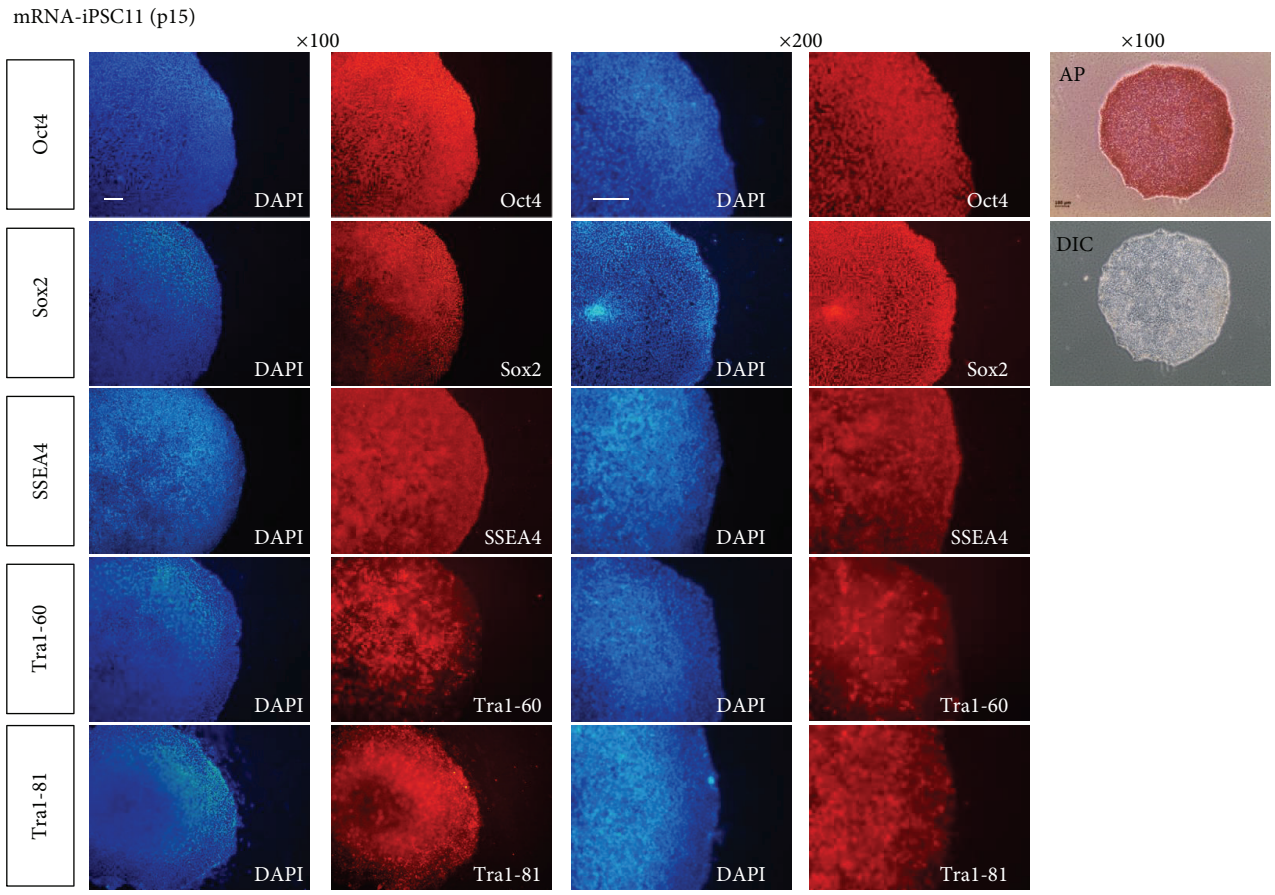


Figure 2: Immunostaining of the iPS cells for several pluripotent cell markers. The mRNA-iPSCs generated and cultured for 15 passages in our xeno-free/feeder-free hPSC system positively immunostained for the pluripotent cell markers Oct4, Sox2, SSEA4, Tra1-60, and Tra1-81. In addition, the colonies were stained with the commonly used undifferentiated cell marker alkaline phosphatase (*top right panel*).

pluripotent, similar to hESCs and other iPS cells, including UNFiPSC1 and ANFiPSC1 that have been established previously [18] (Figures 4(d) and 4(e)). Hierarchical clustering analysis of the gene expression data indicated that the mRNA-iPSC2 line was more similar to hESCs than to the fibroblasts from which this cell line was derived (Figure 4(f)).

3.3. Analysis of the Methylation Pattern of the Promoter Regions of Pluripotency-Associated Genes. We performed bisulfite sequencing analysis to examine the methylation patterns of the cytosine guanine dinucleotides (CpGs) in the promoters of two representative pluripotency-associated genes, Oct4 and Nanog. It is well documented that promoter methylation inversely correlates with gene expression [19]. The methylation of the promoter The DNA methylation patterns of the Oct4 and Nanog gene promoter regions of the mRNA-iPSCs were similar to those of the hESCs but not of the original fibroblasts (Figure 5(a)). This result is consistent with the high expression of the Oct4 and Nanog genes in both mRNA-iPSCs and hESCs (Figure 3(a)).

Cytogenetic analysis of Giemsa-banded metaphase mRNA-iPSCs showed no gross abnormality in the chromosomes, even after prolonged passaging up to passage 35 (Figure 5(b)).

3.4. Pluripotency of the mRNA-iPSCs Both In Vitro and In Vivo. To examine whether the mRNA-iPSCs acquired pluripotency, the cells were spontaneously differentiated into the derivatives of the three germ layers *in vitro*. To this end, EBs were generated first, followed by the formation of adherent cultures on Matrigel-coated dishes in medium containing 10% FBS. When analyzed 15 days after differentiation on Matrigel, clear expression of representative markers for ectoderm (Nestin and Class III β-tubulin (Tuj1)), mesoderm (smooth muscle actin (SMA) and platelet endothelial cell adhesion molecule (PECAM)), and endoderm (alpha-fetoprotein (AFP) and FoxA2) was detected (Figure 6(a)).

When transplanted into NOD/SCID mice, the mRNA-iPSCs developed into teratomas consisting of various cell types derived from all three germ layers (secretory epithelium (ectoderm), cartilage (mesoderm), and gut (E4) epithelium (endoderm)) (Figure 6(b)).

Taken together, these results clearly demonstrate that the mRNA-iPSCs generated and cultured in our

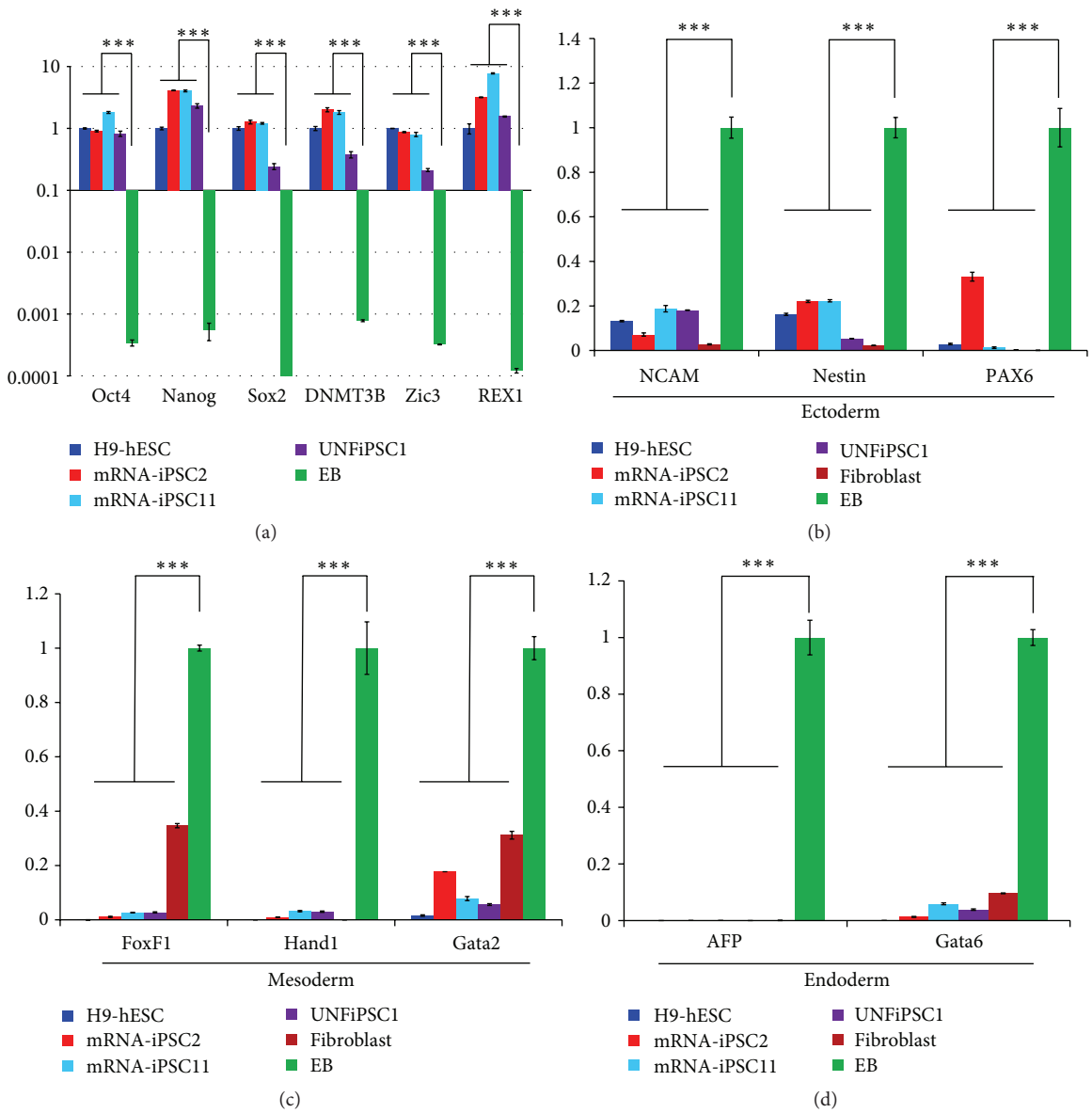


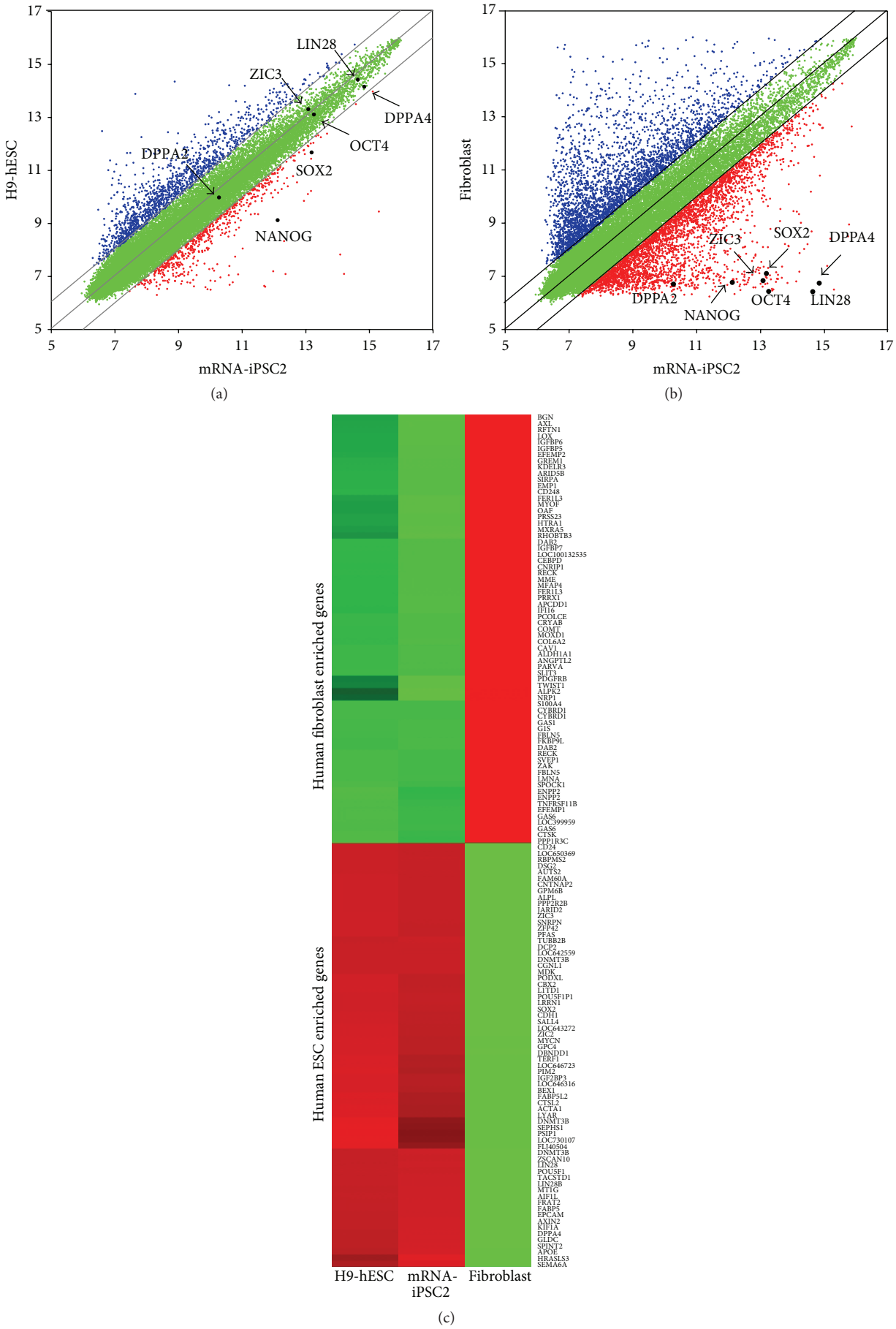
Figure 3: qRT-PCR analyses of the iPS cells. (a) The mRNA-iPSCs were analyzed for the expression of multiple representative lineage-specific markers. H9-hESCs and urine-derived iPS cells generated using the episomal plasmid method (UNFiPSC1) [18] were used as positive controls, and EBs were used as negative controls. $^{***}p < 0.01$. (b–d) The expression levels of representative markers of derivatives of ectoderm (NCAM, Nestin, and Pax6) (b), mesoderm (FoxF1, Hand1, and Gata2) (c), and endoderm (AFP and GATA6) (d) were examined via qRT-PCR. In these experiments, H9-hESCs and UNFiPSC1 were used as controls for undifferentiated cells, whereas EBs and fibroblasts were used as controls for differentiated cells. $^{***}p < 0.01$.

xeno-free and feeder-free hPSC culture system exhibited pluripotency.

4. Discussion

A human iPS cell-based clinical trial targeting retinal disease is currently underway [20], and several trials are expected to start within the foreseeable future. Therefore, the need for generating clinically compliant safe iPS cells continues to increase.

Among the methods for generating iPS cells without chromosome integration, mRNA transfection displays important advantages, such as the complete avoidance of chromosomal integration. Unlike the episomal transfection method, using the mRNA transfection method there is no need to examine the insertion of transgenes into the chromosome. In addition, mRNA transfection enables the



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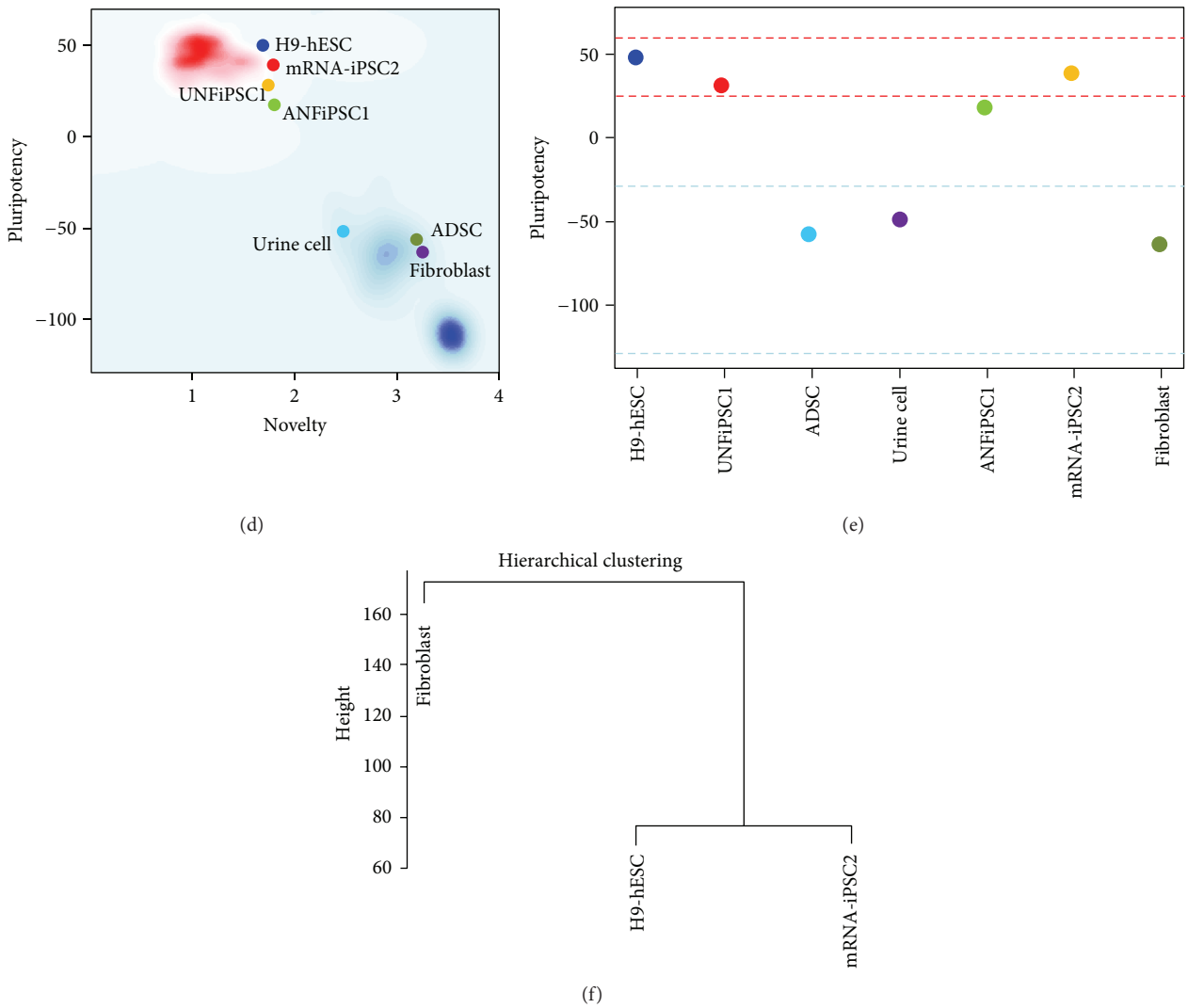

Figure 4: Genome-wide gene expression profiling of the mRNA-iPSCs. (a-b) Scatterplot analysis showed that the global gene expression pattern of mRNA-iPSCs is similar to that of H9-hESCs (a). However, the gene expression patterns are clearly different between mRNA-iPSCs and their parental cells, fibroblasts (b). (c) Heatmap analysis indicated that the expression patterns of 100 hESC-enriched genes and 100 human fibroblast-enriched genes (Supplementary Table 4) in mRNA-iPSCs were similar to those in H9-hESCs but not fibroblasts. (d-e) PluriTest analysis showed that mRNA-iPSCs and other previously established iPS cells (UNFiPSC1 and ANiPSC1) [18] were clustered with H9-hESCs in the pluripotent group, whereas fibroblasts and other primary cells were clustered in the nonpluripotent group. (f) Gene profiling-based hierarchical clustering analysis demonstrated the close association of the mRNA-iPSCs with H9-hESCs but not with the primary cells (fibroblasts).

rapid removal of exogenous genetic material from the cells after reprogramming, which reduces the unwanted negative effects of lingering transgenes.

However, this method is associated with certain problematic issues that must be resolved before its clinical application: (1) the original protocol for mRNA-mediated reprogramming required 17 daily transfections for complete reprogramming [11], which is highly labor-intensive; (2) the initial and subsequent protocols used either human feeder cells (i.e., human neonatal foreskin fibroblasts (NuFF)) [11] or NuFF-conditioned medium during the reprogramming stage [12, 15, 21, 22]. Using human feeder cells or NuFF-conditioned medium is both costly and laborious, requiring the repeated preparation of postmitotic human feeder cells and the collection of human feeder-conditioned medium, respectively; and (3) most of the mRNA reprogramming protocols reported to date have used different culture conditions for reprogramming and for expansion of the generated iPS cells. For example, Warren et al. used NuFF feeder cells during reprogramming and used mouse embryonic fibroblasts (MEFs) for expansion of the iPS cells [11]. More recent protocols for mRNA reprogramming adopted NuFF-conditioned medium during the reprogramming stage, followed by using either human feeder cells [21] or feeder-free defined medium [12] to culture the iPS cells. Using different culture systems for the reprogramming and expansion of iPS

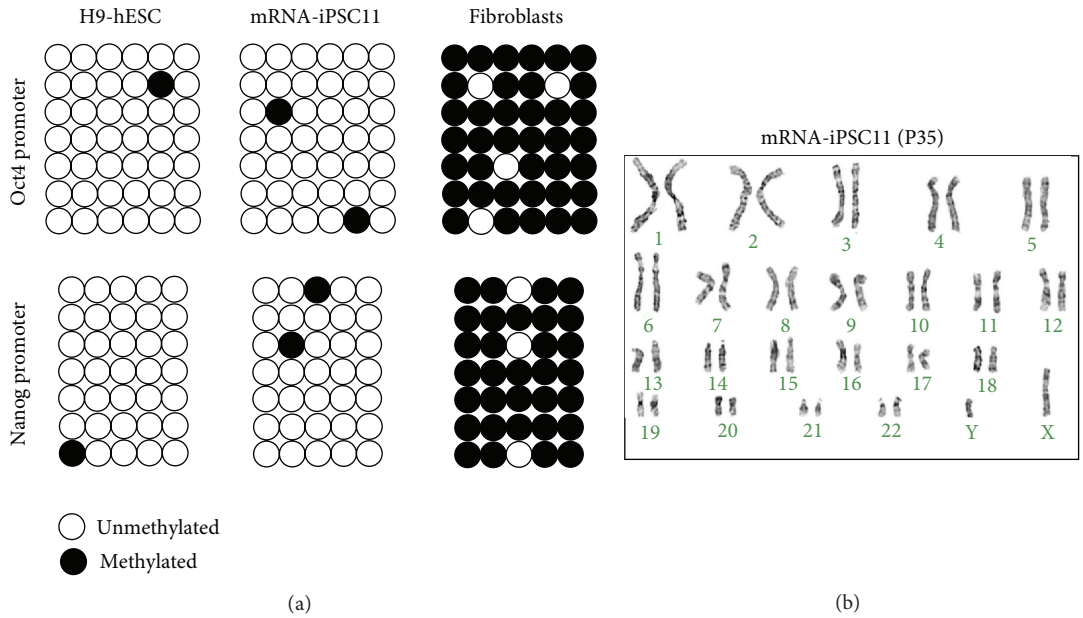

Figure 5: Analyses of the methylation in the Oct4 and Nanog promoters and of chromosomal abnormality in the mRNA-iPSCs. (a) The bisulfite sequencing data indicated that the promoters of the *Oct4* and *Nanog* genes in the mRNA-iPSCs were largely demethylated, similar to the methylation status of these promoters in hESCs. In contrast, their original cells, fibroblasts, were hypermethylated at these promoters. (b) G-banding analysis showed that no apparent chromosomal abnormality was generated during reprogramming and extended culture (for 35 passages) of the mRNA-iPSCs in our ECM-based xeno-free/feeder-free hPSC culture system.

cells is inconvenient and cumbersome, prompting the search for a culture system that can be used throughout the entire process of iPS cell generation (i.e., both the reprogramming stage and the propagation of generated iPS cells).

In this study, we established a simpler and more convenient method for the generation and propagation of xeno-free and integration-free iPS cells by combining an mRNA transfection method with our previously developed xeno-free and feeder-free hPSC culture system. These two systems functioned compatibly during both the generation and expansion of xeno-free iPS cells. The mRNA transfection method used in this study was improved from the original protocol by adding two transfections of a microRNA cocktail during the reprogramming stage. These two transfections with the microRNA cocktail were considered to accelerate the kinetics and efficiency of reprogramming. Consistent with this concept, we detected ESC-like colonies beginning at 11 days after seeding the cells, which is faster than the results reported by Warren et al. (~day 17) [11]. The total number of daily mRNA transfections required for reprogramming was reduced from 17 [11] to 11 due to the transfection of the microRNA cocktail. Furthermore, we found that the percentage of ESC-like colonies among the total colonies was approximately 87.5%, which was significantly higher than previously reported results [2, 9]. Taken together, our results showed that a mixture of microRNAs facilitated the reprogramming process and, thus, rendered the mRNA-mediated generation of iPS cells as more labor- and time-effective.

Another important feature of our method is the use of the same (or very similar) xeno-free/feeder-free hPSC culture medium for both reprogramming and propagation of iPS cells, which makes the iPS cell generation and expansion procedures simpler and more convenient. The simplicity of our method will advance the production of good manufacturing practice (GMP) grade xeno-free iPS cells for cell therapy.

The efficiency of iPS cell generation using an mRNA reprogramming method has been reported to be significantly variable (0.04–4.4%) [11, 12, 22], which may be at least partially caused by the innate properties of the somatic cells used for reprogramming, the transfection efficiency, the number of reprogramming factors introduced, the oxygen concentration during culturing (5% vs. 20%), and the culture media/conditions adopted for reprogramming [11, 12, 22]. For example, the reprogramming efficiency, which was 1.89% on feeder cells, dropped to 0.22% when a Matrigel-based feeder-free culture condition was used [22]. In our study, we consistently obtained a reprogramming efficiency of ~0.2–0.3% under normoxic conditions (20% O_2) when our xeno-free/feeder-free hPSC culture system was adopted for the reprogramming of human adult dermal fibroblasts.

The medium used in this study was a completely defined, xeno-free, and feeder-free hPSC culture medium. Here, we demonstrated that this medium was highly compatible with a modified mRNA transfection method for the generation and expansion of iPS cells. Therefore, this combination of an ECM-based xeno-free/feeder-free hPSC culture system with an improved version of the mRNA transfection method provides a rapid, efficient, and labor-effective platform for the generation of footprint-free and xeno-free iPS cells for safe clinical applications.

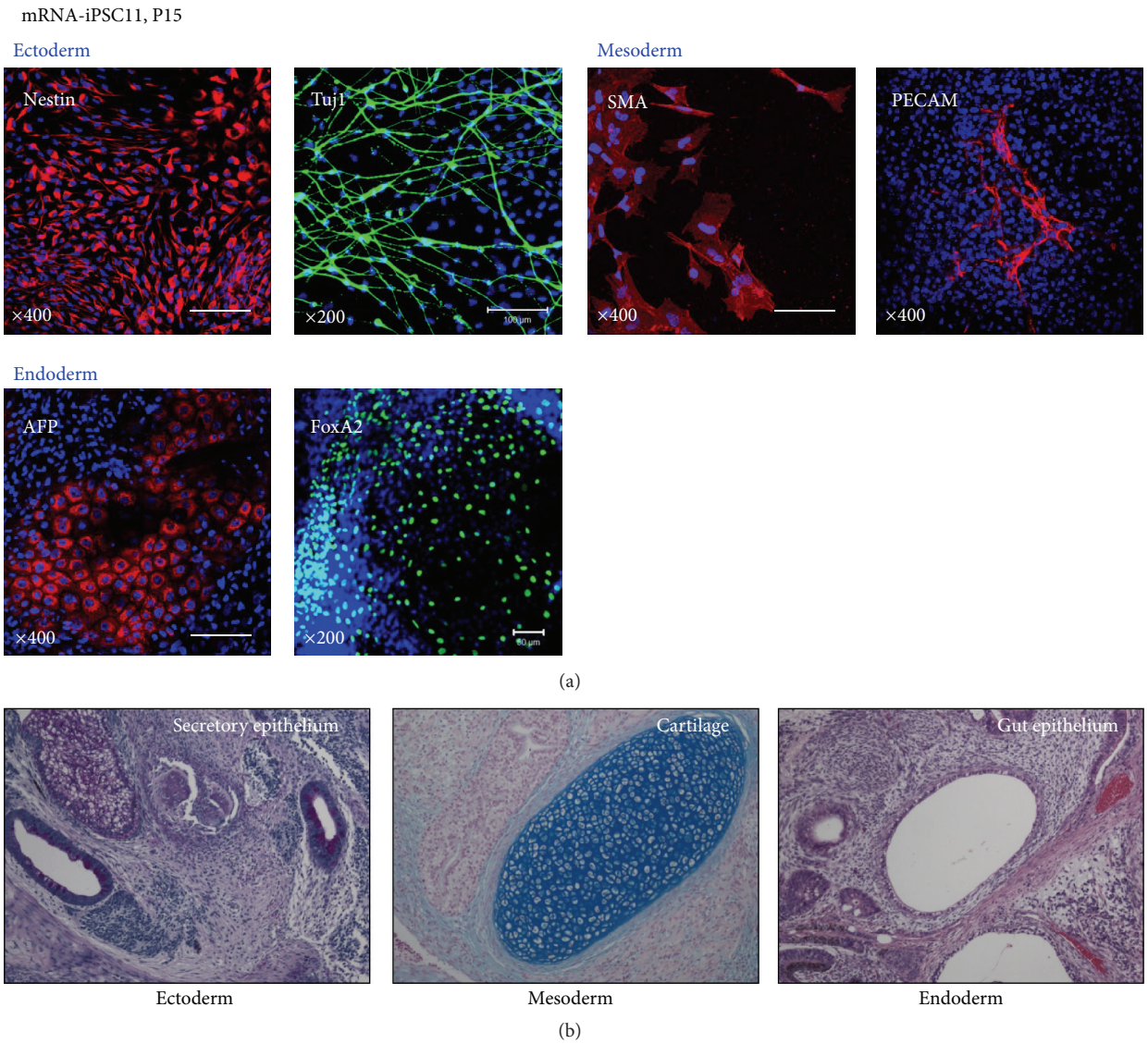

Figure 6: Analyses of pluripotency *in vitro* and *in vivo*. The mRNA-iPSCs were subjected to spontaneous differentiation via EB culturing, and the expression levels of representative markers of the ectoderm (Nestin and Class III β-tubulin (Tuj1)), mesoderm (SMA and PECAM), and endoderm (AFP and FoxA2) lineages were examined. Scale bar: 100 μm. (b) Teratomas including structures and derivatives of the three germ layers were found at 3-4 months after intramuscular administration of the mRNA-iPSCs to NOD/SCID mice.

5. Conclusions

Here, we report that therapeutically safe (i.e., xeno-free and footprint-free) iPS cells can be efficiently generated and expanded by combining our previously developed ECM-based xeno-free/feeder-free hPSC culture system with an improved mRNA reprogramming method, facilitating the use of a labor- and time-effective platform for future iPS cell-mediated cell therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Kang-In Lee and Seo-Young Lee have equal contribution to this paper.

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Review Article

The Progress of Induced Pluripotent Stem Cells as Models of Parkinson's Disease

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In recent years, induced pluripotent stem cells (iPSCs) were widely used for investigating the mechanisms of Parkinson's disease (PD). Somatic cells from patients with *SNCA* (α-synuclein), *LRRK2* (leucine-rich repeat kinase 2), *PINK1* (PTEN induced putative kinase 1), *Parkin* mutations, and at-risk individuals carrying *GBA* (β-glucocerebrosidase) mutations have been successfully induced to iPSCs and subsequently differentiated into dopaminergic (DA) neurons. Importantly, some PD-related cell phenotypes, including α-synuclein aggregation, mitophagy, damaged mitochondrial DNA, and mitochondrial dysfunction, have been described in these iPSCs models, which further investigated the pathogenesis of PD. In 2007, Takahashi et al. and Vodyanik et al. generated iPSCs from human somatic cells for the first time. Since then, patients derived iPSCs were applied for disease modeling, drug discovery and screening, autologous cell replacement therapy, and other biological applications. iPSC research has now become a hot topic in a wide range of fields. This review summarizes the recent progress of PD patients derived iPSC models in pathogenic mechanism investigation and potential clinical applications, especially their promising strategy in pharmacological study and DA neurons transplantation therapy. However, the challenges of iPSC transplantation still exist, and it has a long way to go before it can be used in clinical application.

1. Introduction

Induced pluripotent stem cells (iPSCs) are similar to human embryonic stem cells in their morphology, self-renewing capacity, and differentiation potential to any cell types. In 2007, Takahashi et al. and Vodyanik et al. induced adult human somatic cells into iPSCs by transferring a series of specific transcript factors (Oct4, Sox2, Klf4, c-Myc or Oct4, Sox2, Nanog, Lin28) [1, 2], which represented a new method to generate disease-specific pluripotent stem cells from the patients. From then on, the researches of iPSCs have come into a new milestone. Takahashi and Vodyanik generated iPSCs with retroviruses carrying transcription factors and oncogene c-Myc, which raise the risk of tumorigenicity and other side effects. Also, low efficiency of vector expression may limit the differentiation potential of the iPSCs. To overcome the potential safety risk and low efficiency, the protocols of iPSCs reprogramming and DA neuron generation have constantly been refined. Safer and more effective methods using nonintegrating vectors, synthetic modified mRNA, and small molecules (for SB431542, PD0325901, and Thiazovivin) instead of transgene integration have been attempted in many studies, which can directly activate the expression of transcription factors in iPSC generation through different ways. Furthermore, the course of generation can be controlled in a strict way and reduce the uncertainty risk of genetic alteration and transformation to the greatest extent. The new methods not only saved time but also improved reprogramming efficiency and safety [3–7], which provided an alternative strategy for disease modeling and clinical study.

2. Parkinson's Disease

Parkinson's disease (PD) is the second most common late-onset neurodegenerative disorder, clinically characterized

by a series of motor symptoms such as bradykinesia, resting tremor, rigidity, and postural instability as a result of dopaminergic (DA) neuron degeneration in the substantia nigra pars compacta (SNpc), accompanied with sleep disorders, cognitive decline and other nonmotor symptoms [8]. PD is a complex disease with the combination of environmental exposures and genetic factors. About 10% of PD patients have a positive family history and a series of genes such as *SNCA*, *LRRK2*, *VPS35*, *Parkin*, *PINK1*, *DJ-1*, *PLA2G6*, and *ATP13A2* were cloned in familial PD patients [9]. The pathogenesis of PD is still elusive; mitochondria dysfunction, α-synuclein accumulation, mitophagy, and oxidative stress were thought to play an important role in the occurrence of PD [8].

Over the past hundred years, the therapeutic methods for PD were gradually and steadily developed. Dopaminergic therapy is the most effective symptomatic treatment drug for PD, while deep brain stimulation (DBS), entering into a new era of human neural-network modulation, improved the life quality of patients greatly [10]. Since the discovery by Takahashi and Vodyanik, iPSCs generated from patient's somatic cells have opened up a new avenue to exploring the mechanisms of PD and developing the stem cell-based personalized therapeutic strategy.

3. iPSCs Models for PD

Over the years, iPSCs derived from the patients and then differentiated into disease-relevant cell types have been widely used to mimic the phenotype of diseases and have achieved great progress in pathogenic mechanism studies. In terms of nervous system disorders, patient-derived iPSCs have been used to differentiate into motor neurons, astrocytes, DA neurons, or other cell types of the affected diseases. iPSCs generated from Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), spinal muscular atrophy (SMA), and PD patients with genetic mutations were successfully differentiated into neuronal cells, and disease-related pathologic phenotypes have been identified in these cells. Furthermore, patient iPSC-derived neuronal cells offer a direct insight into the early-stage and progressive pathologic alternations in disease, further recapitulating the molecular pathogenesis of diseases [11–14].

Since PD is a kind of disease with progressive degeneration of dopaminergic (DA) neurons, iPSC differentiated DA neurons seem to be the appropriate model to decipher physiological and pathological mechanisms of PD. As is described above, iPSCs can be generated by specific transcription factors, viral vector, or other small molecules and are then differentiated into DA neurons. The methods of DA neurons differentiation include targeted differentiation and direct lineage conversion. Targeted differentiation strategy is similar to embryonic stem cell (ESC) induction, which has been widely used to differentiate DA neurons, and the protocol has been constantly modified or refined. In 2009, Chambers and colleagues demonstrated that, with the combination of Noggin and SB431542, two inhibitors of SMAD signaling in differentiation and the efficiency of iPSC differentiation could be improved, which allowed for the complete induction into neuronal cells [15]. The second strategy for obtaining DA neurons is the direct lineage conversion. Somatic cells can be first induced into neural precursor cells (iNPCs), which are then differentiated into astrocytes and DA neurons. More importantly, as the generation of iNPCs is a gradual process, the donor transcription factors are silenced over time, which lowers the risk of tumors. Direct lineage conversion provides a new source of human cells for stem cell based replacement therapy and holds promise for application in drug discovery and screening [16, 17].

In 2009, Soldner et al. generated iPSCs from sporadic PD patients by using modified lentiviruses carrying loxP sites flanking the integrated provirus for the first time. This strategy not only improved the efficiency of reprogramming but also allowed the removal of the transgene sequences to generate iPSCs free of reprogramming factors [14]. Over the past few years, somatic cells from patients with *SNCA*, *LRRK2*, *PINK1*, and *Parkin* mutations and at-risk individuals carrying *GBA* mutations have been successfully induced to iPSCs and differentiated into DA neurons. With the help of these techniques, the pathogenesis of PD has become clearer.

3.1. SNCA. *SNCA* encodes α-synuclein protein. Missense mutations (such as A53T, E46K, and A30P) and genomic multiplications (duplication and triplication) of *SNCA* were reported in many autosomal dominant PD patients. Patients with *SNCA* mutations were characterized by a loss of DA neurons in SNpc and α-synuclein accumulation in neurons—the pathological hallmark of PD [8]. However, the definitive pathogenic mechanism caused by *SNCA* mutations is still elusive, although it is widely believed that α-synuclein aggregation and cellular toxicity may contribute to the course of neuronal degeneration [18, 19].

In 2011, Soldner et al. generated iPSCs from two early onset PD patients with A53T and E46K mutations by combining zinc-finger nuclease- (ZFN-) mediated genome editing and iPSC technology, but the phenotypic changes of these cells were not reported [20]. The accumulation of α-synuclein in DA neurons is a common pathological change in PD patients, which also exist in iPSC generated DA neurons derived from patients with *SNCA* triplication. In 2011, Devine and Byers reported that PD patients with α-synuclein triplication (AST) and unaffected controls showed no difference in ectopic expression of α-synuclein. When differentiated into DA neurons, the quantity of α-synuclein was doubled in AST neurons compared with neurons from the controls. Furthermore, AST neurons were more sensitive to peroxide induced oxidative stress, further substantiating the role of α-synuclein accumulation and oxidative stress in PD [21, 22]. Also, their findings were consistent with a previous study in blood and brain tissue from the patient with *SNCA* triplication, which showed higher levels of α-synuclein compared with controls [23].

3.2. LRRK2. *LRRK2* is a member of the leucine-rich repeat kinase family, encoding a protein that has GTPase and kinase functions. The dysfunction of *LRRK2* was reported to be associated with impaired dendritic neuronal arborization and autophagy [24, 25]. *LRRK2* mutations are the most common

cause of familial PD. Mutations including N1437H, R1441C, and G2019S of *LRRK2* were reported in autosomal dominant PD patients, and G2019S is by far the most common mutation, especially prevalent among Ashkenazi Jews [8, 26].

iPSCs derived from PD patients with G2019S mutation were generated in many groups. Oxidative stress, α-synuclein accumulation, autophagy, and damaged mitochondrial DNA were reported in these iPSC-derived DA neurons and the pathogenesis of PD was further investigated. Compared with unaffected DA neurons, the G2019S-iPSC-derived DA neurons were more sensitive to oxidative stress (such as hydrogen peroxide, MG-132) or proteasomal stress-induced apoptosis, and they also exhibited an increased α-synuclein protein level after long-term cultivation [27, 28]. In addition to its role in oxidative stress and α-synuclein protein accumulation, impaired autophagic clearance and morphological alterations (including reduced numbers of neurites and neurite arborization) can be seen in these DA neurons [29]. More importantly, another study showed damaged mitochondrial DNA (mtDNA) in G2019S-iPSC-derived DA neurons, and the damage can be reversed by zinc finger nuclease-mediated repair. It suggested that mtDNA damage might be induced by *LRRK2* mutations, and oxidative stress, α-synuclein accumulation, and impaired autophagy together with damaged mtDNA may play interactive roles in the course of PD [30].

3.3. PINK1. *PINK1* encodes a mitochondria-targeted kinase, which can protect neuronal cells from stress-induced mitochondrial dysfunction [8]. In 2013, Rakovic et al. demonstrated that iPSCs derived from *PINK1* mutation carriers showed a deficiency of endogenous *Parkin* levels to initiate mitophagy upon loss of the mitochondrial membrane potential due to ubiquitination dysfunction, suggesting that *PINK1* may play an overlapping role with *Parkin* in mitophagy [31]. In 2011, Seibler et al. reported that mutant *PINK1* iPSC-derived DA neurons showed impaired recruitment of *Parkin* to mitochondria upon depolarization, increased mitochondrial copy number, and upregulation of PGC-1α. More importantly, another study reported that lentiviral overexpression of wild type *PINK1* in these DA neurons was able to restore the translocation of *Parkin* to mitochondria, further validating the association between *PINK1* and *Parkin* [32]. In addition, these cells showed a decreased mitochondrial membrane potential and mitochondrial complex I activity, which was consistent with the results of previous studies from other groups [33, 34]. Therefore, Seibler and Rakovic generated a valuable cellular model closely resembling the phenotype reported in PD patients and highlighted the importance of *PINK1* mutation-caused mitochondrial dysfunction in pathogenesis for PD.

3.4. Parkin. *Parkin* encodes a component of the E3 ubiquitin-ligase complex, which mediates the targeting of substrate proteins for proteasomal degradation. Besides a concerted role with PINK1 in mitophagy and oxidative stress, *Parkin* is also associated with dopamine homoeostasis. iPSC-derived DA neurons from patients with *Parkin* mutation showed decreased DA uptake and increased spontaneous DA release. There was also an increased level of reactive oxygen species (ROS) in these neurons as a result of mitochondrial dysfunction, which indicates that *Parkin* can enhance the precision of DA neurotransmission and suppress the oxidation of DA [35, 36]. Furthermore, these cells exhibited similar pathological changes seen in G2019S-iPSC-derived neurons, including the accumulation of α-synuclein and its correlation with Lewy body formation [35].

In addition, mutant *Parkin* iPSC-derived DA neurons showed a reduced neurite length and complexity due to destabilization of microtubules, which could be rescued by overexpressing wild type *Parkin* in these neurons. Their findings supported that microtubule stabilization maintains the morphological complexity in neurons, and the dysfunction of *Parkin* damages not only the morphology of DA neurons but also neuron survival [37].

3.5. GBA. *GBA* encodes a lysosomal membrane protein β-glucocerebrosidase (also known as acid β-glucosidase), the mutation of which results in accumulation of glycolipid substrates in lysosomes, leading to an autosomal recessive lysosomal storage disorder—Gaucher disease [38].

Mutations in *GBA* were thought to be a risk factor for PD in different ethnic groups [39, 40]. Glucocerebrosidase deficiency and lysosomal dysfunction were thought to be an important pathogenic mechanism for PD [41]. In 2012, Panicker et al. reported that iPSC-derived DA neurons from patients with Gaucher disease showed a high level of α-synuclein protein and decreased clearance ability in macrophages due to glucocerebrosidase (GCase) deficiency. On the contrary, the overexpression of α-synuclein inhibits the intracellular trafficking of GCase, which can decrease the activity of lysosomal GCase [42, 43]. The findings suggested a bidirectional effect between a-synuclein accumulation and GCase deficiency, further supporting the important role of α-synuclein neurotoxicity and autophagy-lysosomal pathways in the process of PD occurrence.

Though iPSCs derived from patients with causative or at-risk mutations have successfully modeled PD and further illustrated the pathogenic pathway of the disease, the pathogenesis of neurodegeneration in PD remains elusive. Further studies of iPSC-derived DA neurons from patients with other genetic mutations (such as *VPS35*, *DJ-1*, and *PLA2G6*) are needed to model PD and elucidate the pathogenesis.

4. Potential Clinical Applications of iPSCs

Taking all described above together, patient-derived iPSCs seem to be an ideal model to recapitulate the disease-related phenotypes and the pathological changes of diseases, as these cells are able to differentiate into any cell types of human body for disease modeling and mechanism exploring. Indeed, iPSCs have served as potential cell tools for clinical applications, some of which even achieved promising results. iPSCs derived from PD patients were applied for drug discovery, replacement therapy, or other biological applications, aiming at realizing personalized treatment and transforming biomedical research into clinical application.

4.1. iPSCs Models in Drug Discovery. In 2012, Cooper et al. generated DA neurons from individuals carrying PINK1 Q456X, LRRK2 G2019S, and R1441C mutations. They found that these cells were more vulnerable to PD associated chemical toxins valinomycin and concanamycin A. Moreover, the mutant PINK1 iPSC-derived neurons showed increased mitochondrial reactive oxygen species (mROS) concentrations when a low concentration of valinomycin was added. Accompanied with the increased mROS in these neurons, the level of glutathione (GSH), an important antioxidant to prevent damage caused by mROS concentrations, was decreased. Importantly, damage induced by these chemical toxins could be rescued by coenzyme Q10, rapamycin, and GW5074 (a kind of LRRK2 kinase inhibitor). Their results suggested that iPSC-derived cells are an ideal model for pharmacological study, and that coenzyme Q10, rapamycin, and GW5074 may save the damaged DA neurons and prevent them from progressive degeneration [44, 45].

4.2. iPSCs for Cell Replacement Therapy. Stem cell based therapy for PD can be traced back to three decades ago; from then on, scientists have been striving to advance the therapy and have got varying results for that. In 2008, Mendez et al. reported that PD patients who had DA neurons implanted from fetal midbrain cell suspensions lived 14 years without pathology. Recently, another group reported long-term clinical outcomes for fetal mesencephalic tissue (rich in dopaminergic neuroblasts) transplantation in two PD patients, showing an improvement of their motor symptoms free of any pharmacological dopaminergic therapy. Their findings proved that DA neurons transplantation might offer a long-term symptomatic relief in PD patients [46, 47]. On the other hand, some significant side effects, such as graft-induced dyskinesia and dystonia, occurred in patients who received fetal nigral transplantation [48, 49]. Considering these adverse effects, the use of stem cell-derived DA neuron transplantation in PD patients remains controversial.

After Takahashi et al. and Vodyanik et al. induced adult human somatic cells into iPSCs with a series of transcription factors (Oct4, Sox2, Klf4, c-Myc or Oct4, Sox2, Nanog, and Lin28), the transplantation of DA neurons for PD patients had become more feasible and easily operable. iPSCs can be obtained from human somatic cells, which avoids the ethical problems of applying human embryos for study. However, the risk of tumorigenicity and other unpredictable adverse effects were raised due to viral vector insertions and c-Myc oncogene reactivation [50].

In recent years, many studies have reported that transplanted iPSC-derived neurons were able to increase regeneration and functional recovery in ischemic stroke rat model [51, 52]. Notably, an improvement in motor ability was found in a PD rat model after the transplantation of human derived induced neural stem cells (iNSCs) into the striatum of the rats. In addition, in vivo study proved that these iNSCs were able to survive and differentiate into DA neurons, suggesting that iPSC-derived neuron transplantation can replace the lost neuronal cells and rescue the damaged function of neurons [53, 54]. Putting the adverse effects aside, patients' somatic cell-derived iPSC transplantation may be a potential personalized cell strategy to treat PD or other degenerative diseases in future with little or no immune reaction.

5. Challenges and Future Directions

As described above, iPSCs derived from patients with different genetic mutations or carrying at-risk mutations are an ideal model for studying the pathophysiological mechanisms underlying PD. Its potential applications in drug discovery and cell replacement therapy, will support an improved life quality of the patients.

Despite the fact that iPSC technology is still ongoing and has been greatly improved to accelerate the development of clinical trials, there still exist several challenges and limitations in iPSC transplantation for PD. Firstly, iPSCs are induced by viral vector insertion of transcription factors, which is accompanied with tumorigenicity or other adverse effects. Safer and more effective transduction methods have been attempted in many studies, successful application of these new methods will not only save time but also improve reprogramming efficiency and safety [3–7]. Until now, there is no study reported using iPSC-derived neurons for transplantation in PD patient. Secondly, because our human is an integrated complex system, different cell types can play an interactive role with each other. Though iPSCs can model PD, it would be difficult for these cells to reveal the exact pathophysiology status of human. Last but not least, as PD is a neurodegenerative disease, whether the transplanted neurons will function as expected for long term is still unknown. Moreover, ethical issues before transplantation should also be taken into consideration [55]. Taken together, further investigations are required for iPSC-derived DA neuron transplantation in rodents and nonhuman primates to evaluate the long-term clinical benefits and potential adverse effects. The road toward clinical application of iPSC-based therapy is promising, but we still have a long way to go.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

LncRNAs in Stem Cells

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Noncoding RNAs are critical regulatory factors in essentially all forms of life. Stem cells occupy a special position in cell biology and Biomedicine, and emerging results show that multiple ncRNAs play essential roles in stem cells. We discuss some of the known ncRNAs in stem cells such as embryonic stem cells, induced pluripotent stem cells, mesenchymal stem cells, adult stem cells, and cancer stem cells with a focus on long ncRNAs. Roles and functional mechanisms of these lncRNAs are summarized, and insights into current and future studies are presented.

1. Introduction

Less than 2% of human genome is composed of protein-coding genes, but transcription is widespread and corresponding to more than ~80% of the genome [1, 2]. With the development of deep sequencing and bioinformatics, a large number of noncoding RNAs (ncRNAs) are identified in eukaryotic cells [3, 4]. NcRNAs greater than 200 nucleotides are grouped as long noncoding RNAs. Majority of lncRNAs are intergenic transcripts and sense or antisense to other transcripts. Similar to mRNAs, most lncRNAs are Pol II transcripts which have a poly-A tail and 5′ cap. But compared to protein-coding genes, lncRNAs show lower evolutionary conservation and lower expression level. LncRNAs are predominantly localized in nucleus [5–7]. LncRNAs have been associated with significant roles in diverse biological events such as chromatin modifications, transcriptional regulation, and posttranscriptional regulation [8–10].

Stem cells are undifferentiated cells that can either self-renew or differentiate into specialized cells. Stem cells are of great interest to both basic and biomedical research due to their unique properties in cell biology [11–13]. In recent years, researchers are paying more and more attention to roles of ncRNAs in stem cells.

In this review, we will discuss some of the known roles of ncRNAs in stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), adult stem cells, and cancer stem cells. Table 1 gives a brief summary to key properties of each stem cell type. The focus is on lncRNAs, and for roles of small ncRNAs such as microRNAs and piRNAs in stem cells, readers are referred to other articles [14–17].

2. LncRNAs in ESCs

Mohamed et al. performed a genome-wide screen to identify lncRNAs transcriptionally regulated by Oct4 and Nanog in mouse ESCs (mESCs) by combining the mESC transcriptome with the chromatin immunoprecipitation genomic location maps of Oct4 and Nanog [18]. Two conserved lncRNAs AK028326 and AK141205 were further confirmed to be directly activated by Oct4 or suppressed by Nanog, respectively. In fact, AK028326 might be a coactivator of Oct4. Later, a systematic survey for lincRNAs, which are a subclass of lncRNAs transcribed from intergenic regions of coding genes, was conducted with several murine cell lines including ESCs [19]. More than a thousand lincRNAs were identified, and many of them might possess putative functions in the pluripotency or proliferation of ESCs. Transcription of some lincRNAs was also regulated by key transcription factors such as Sox2, Oct4, and Nanog in ESCs.

In human ESCs (hESCs), multiple ESC specific lncRNAs were identified with microarray assays [20]. Some of these

Table 1: Key properties of each stem cell type.

Stem cell	Key properties
Embryonic stem cell	Derived from the blastocyst stage early mammalian embryos and has the ability to differentiate into any cell type and propagate
Induced pluripotent stem cell	A type of pluripotent stem cell that can be generated directly from adult cells; classic four specific genes encoding transcription factors (Oct4, Sox2, cMyc, and Klf) could convert adult cells into iPSCs, holding great promise in the field of regenerative medicine because of indefinite propagation
Mesenchymal stem cell	Multipotent stromal cells that can differentiate into a variety of cell types including osteoblasts, chondrocytes, myocytes, and adipocytes
Adult stem cell	Undifferentiated cells, which are found throughout the body after development, can divide or self-renew indefinitely, and generate all the cell types of the organ from which they originate
Cancer stem cell	Cancer cells that possess the ability to give rise to all cell types found in particular cancer samples and can generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types

lncRNAs were involved in pluripotency maintenance and interacted with SOX2 and SUZ12 (a subunit of PRC2 complex). A subset of the identified lncRNAs was involved in neurogenesis of ESCs, and these lncRNAs potentially played neurogenic roles via physical interaction with important epigenetic factors such as REST and SUZ12. One of the lncRNAs identified in this study, rhabdomyosarcoma 2-associated transcript (RMST), was further investigated in a later study [21, 22]. RMST interacted directly with and served as a transcriptional coregulator of SOX2 to modulate the transcription of a number of SOX2 downstream genes. Recently, the long terminal repeats of human specific endogenous retrovirus subfamily H (HERVH), which is transposable elements expressed preferentially in hESCs, was found to function as nuclear lncRNAs with associations with OCT4, coactivators such as p300 and Mediator subunits [23].

A recent report showed that pluripotency-associated transcription factor SOX2 has an overlapping long noncoding transcript SOX2OT, which was highly expressed in embryonic stem cells and downregulated upon the induction of differentiation [24]. SOX2OT had a potential role in modulating pluripotency through the regulation of *SOX2* expression.

Another lincRNA, linc-RoR might function as microRNA sponge to prevent mRNA of some key transcription factors in hESCs from microRNA mediated regulation [25]. Thus, this lncRNA played a role in ESC maintenance and differentiation in the cytoplasm.

These results implicated that lncRNAs could modulate the pluripotency and differentiation of ESCs via intervening the transcriptional and epigenetic regulatory networks in the nucleus or tuning the microRNA functions in the cytoplasm. Other cytoplasmic and nuclear roles of lncRNAs in ESCs are waiting for further investigations.

3. LncRNAs in iPSCs

The conversion of lineage-committed cells to iPSCs is a process called reprogramming. In a 2010 paper, Rinn Lab characterized the transcriptional reorganization of lincRNAs and identified a vast number of lincRNAs associating with reprogramming of human iPSCs (hiPSCs) [26]. These lincRNAs were overexpressed in both hESCs and hiPSCs but showed an elevated level in iPSCs compared to ESCs, suggesting that their activation might promote the iPSCs. One of the lincRNAs named lincRNA-RoR for "regulator of reprogramming" was regulated by pluripotency transcription factors such as OCT4, SOX2, and NANOG. Knockdown of lincRNA-RoR resulted in an inhibition in reprogramming. Interestingly, linc-RoR was later found to function as a competing endogenous RNA in hESCs as already discussed.

By analyzing single-cell transcriptomes during somatic cell reprogramming, more than 400 hundred lncRNAs were characterized that were dynamically expressed at defined stages of reprogramming [27]. During reprogramming, sets of relevant lncRNAs were activated to modulate signaling pathways, repress lineage-specific genes, and regulate metabolic gene expression.

The reprogramming process is accompanied by global epigenetic remodeling [28]. The lncRNA *Xist* responsible for X chromosome inactivation (XCI) has been investigated as a classic epigenetic regulator in the context of reprogramming [29, 30]. Female mESCs have low *Xist* expression and two active X chromosomes. Successfully reprogrammed female murine iPSCs (miPSCs) have undergone X chromosome reactivation and have the same feature in *Xist* expression as mESCs. Upon differentiation, female mESCs and miPSCs initiate *Xist* expression and XCI. Surprisingly, hESCs and hiPSCs can be divided into three classes based on their different states of *Xist* expression. Class I hESCs and hiPSCs behave just like mESCs and miPSCs. Class II human pluripotent stem cells express *Xist* and exhibit random XCI even before differentiation. Class III cells have lost *XIST* expression but have already initiated XCI, regardless of whether they are in the undifferentiated or differentiating conditions [31].

It seems that lncRNAs play essential roles in the reprogramming process. A better understanding in lncRNAs and more broadly ncRNAs in the context of iPSCs would certainly benefit future biomedical research aiming to utilize iPSCs in the clinic.

4. LncRNAs in MSCs and the Mesodermal Lineage

Mesenchyme is connective tissue derived from the mesoderm during animal development. MSCs are first identified in the bone marrow (BM) stroma. BM MSCs provide microenvironment for hematopoietic stem cells and can also differentiate into various mesodermal lineages. Later, MSCs are found in many tissues such as placenta, umbilical cord blood, adipose tissue, muscle, corneal stroma, and the dental pulp of deciduous baby teeth. These MSCs with different origins all possess property to differentiate into mesodermal lineages. Thus, MSCs are an important source of multipotent cells with great potential of clinic applications. LncRNAs in MSCs have significant regulatory roles and potentially can be used as biomarkers for disease or therapeutic targets. Wang et al. performed differential expression profiles of lncRNAs and mRNAs of undifferentiated versus chondrodifferentiated human BM MSCs using microarray in 2015 [32]. Some of the identified lncRNAs could be important regulators in chondrogenic differentiation. The same group identified lncRNAs with differential expression and putative functions during the osteogenic differentiation of human BM MSCs [33]. A total of 1,206 differentially expressed lncRNAs were identified, and among them 687 were upregulated and 519 downregulated, more than twofold.

Some lncRNAs are functionally required during adipogenesis [34]. Brown adipose tissue (BAT) and white adipose tissue (WAT) are differentiated from MSCs in adipose tissues. Sun et al. performed RNA sequencing and identified 175 lncRNAs in total from *in vitro* cultured brown and white preadipocytes, *in vitro* differentiated mature brown and white adipocytes, and primary brown and white mature adipocytes directly isolated from mice [34]. These lncRNAs were up- or downregulated during differentiation of both brown and white adipocytes. Activation of some transcriptional factors and cofactors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (CEBPα) is the hallmark of brown adipocyte differentiation. These lncRNAs have promoters with binding of PPARγ and/or CEBPα key transcription factors. RNAi-mediated loss-of-function identified the top 20 lncRNAs that may be involved in the proper differentiation of adipocyte precursors. Later in 2014, substantially more lncRNAs were identified and may be functionally required during proper adipogenesis [35]. Zhao and colleagues identified inducible lncRNAs involved in brown fat development and thermogenesis using transcriptional profiling in adipose tissues and cells during brown adipocyte differentiation [36]. Twenty-one lncRNAs were found to be enriched in BAT, and among them AK038898 significantly impaired brown adipogenesis upon RNAi knocking down. This lncRNA was then renamed brown fat lncRNA 1 (Blnc1). The expression of Blnc1 was highly induced during brown and beige adipogenesis. The induction was correlated with the stimulation of the thermogenic gene program and in consistency with the expression of key thermogenic markers such as Ucp1 in adipose tissues. Blnc1 is primarily localized in the nucleus and forms a ribonucleoprotein complex with EBF2, which is a member of the EBF family of transcription factors that has been implicated in the control of adipogenesis [37]. Blnc1 itself is also a target of EBF2, thereby forming a feed-forward regulatory loop to regulate adipocyte gene expression. Knowing the distinct roles and functional mechanisms of lncRNAs in adipogenesis have great values in developing new approaches to fight obesity and other related pathological consequences.

LncRNAs also regulate cardiomyocyte differentiation. Zhu et al. identified multiple lncRNAs involved in the development of the lateral mesoderm and in the differentiation of cardiomyocytes with P19 cells, which were isolated from embryo-derived mouse teratocarcinoma and could differentiate into cardiacmyocytes [38]. Forty differentially expressed lncRNAs, 28 upregulated and 12 downregulated, were identified using microarrays. In another research, Matkovich and colleagues used genome-wide sequencing and improved bioinformatics to measure dynamic lncRNA regulation during the transition between embryo and adult mouse hearts and identified numerous cardiac-specific lncRNAs [39]. Analyses indicated a broad role of regulated cardiac lncRNAs as modulators of key cardiac transcriptional pathways. Two lncRNAs, Fendrr and Braveheart, were later found to be involved in the development of the lateral mesoderm in the heart and the differentiation of cardiac myocytes, respectively [40, 41]. Fendrr was discovered in a survey of differentially expressed lncRNAs in six different tissues dissected from early somite-stage of mouse embryos by using RNA-seq and ChIP-seq [40]. Knocking out Fendrr by homologous recombination was embryonic lethal. The ChIP data showed that Fendrr interacts directly with the PRC2 and/or TrxG/MLL complexes in mouse embryos, indicating that it acts as a modulator of chromatin signatures defining gene activity in embryonic development. Braveheart also plays roles as an epigenetic factor [41]. This lncRNA is required for the progression of nascent mesoderm towards a cardiac fate by interacting with SUZ12, a component of PRC2 to alter cardiomyocyte differentiation and retain the cardiac phenotype in neonatal cardiomyocyte [41]. Cardiovascular diseases are currently the main cause of morbidity worldwide, and researches on these lncRNAs are critical to achieving a better understanding of heart development and designing new ways for the diagnosis and treatment of cardiac related diseases.

Several important lncRNAs play critical roles in myogenesis [42]. Linc-MD1 is a muscle-specific long noncoding RNA expressed during early phases of muscle differentiation. It promotes the switch of muscle differentiation from early to later stages by acting as a miR-133 and miR-135 sponge [43]. What is more interesting is that linc-MD1 is also the primary transcript of miR-133b [44]. The process of linc-MD1 as a pri-microRNA by Drosha is controlled by HuR. The binding of HuR to linc-MD1 suppresses Drosha cleavage of linc-MD1 and leads to accumulation of linc-MD1, which further inhibits the functions of miR-133 as a sponge so that this microRNA could not target the HuR mRNA. Thus, HuR and linc-MD1 form an elegant feed-forward positive

loop [45]. Another lncRNA YAM-1 affects myogenesis via epigenetic transcriptional activation [42]. The transcription factor Yin Yang 1 (YY1) regulates expression multiple genes in myoblasts, and YAM-1 is positively regulated by YY1. YAM-1 expression is downregulated during myogenesis and its expression inhibits myoblast differentiation. YAM-1 regulates the transcription of miR-715 in *cis* and miR-715 is a microRNA that targets Wnt7b, an important signaling modulator of myogenesis [42].

5. LncRNAs in Adult Stem Cells

A series of studies focused on lncRNAs in adult stem cells such as hematopoietic stem cells and adult neural stem cells. The milestone discovery of lincRNA HOTAIR in 2007 was carried out with primary human fibroblasts, which are a kind of somatic stem cell [45]. In this and following researches, HOTAIR was found to bind both the polycomb repressive complex 2 (PRC2) and the LSD1/CoREST/REST complex and thus to modulate histone modifications on target genes [45, 46]. The lncRNA ANCR (antidifferentiation noncoding RNA) was also characterized by epidermal progenitors versus differentiating cells [47]. Knocking down ANCR alone led to rapid expression of differentiation genes. ANCR was thought to be essential for the undifferentiated state, although the exact molecular mechanism requires further investigation [47]. On the other hand, an lncRNA called terminal differentiation-induced noncoding RNA (TINCR) promoted epidermal differentiation [48]. TINCR had a very low level in epidermal stem cells, but it was dramatically induced upon differentiation. As a cytoplasmic ncRNA, TINCR could interact with mRNAs of many differentiation genes such as KRT80 and RNA-binding protein STAU1 to mediate mRNA stabilization through binding TINCR box.

In a systematic analysis of adult neural stem cell lineage in the mouse subventricular zone for lncRNA with potential roles in adult neurogenesis, Ramos et al. identified two lncRNAs Six3os and Dlx1as playing crucial roles in the lineage specification of adult stem cells [49]. The same lab later characterized a neural-specific lncRNA named Pnky, which localized in the nucleus of human and mouse neural stem cells [50]. With knockdown of Pnky, neuronal differentiation increased both *in vitro* and *in vivo*. Pnky interacted with the splicing regulator PTBP1 and thus regulated the expression and alternative splicing.

Recently, Luo et al. identified 159 unannotated lncRNAs enriched in hematopoietic stem cells with RNA sequencing. Knocking down two of these lncRNAs showed effects on self-renewal and lineage commitment of hematopoietic stem cells [51]. Interestingly, the genomic binding sites of one lncRNA overlapped significantly with binding sites of a key hematopoietic transcription factor E2A, indicating this lncRNA might be a cofactor of the transcription factor.

It is obvious that lncRNAs possess vital roles in a variety of somatic stem cells with diverse mechanisms. Considering the large number of lncRNAs identified, we still have a long way to understand the full range of roles of these regulators in adult stem cells as well as in the other eukaryotic cells.

6. LncRNAs in Cancer Stem Cells

Cancer stem cells (CSCs) play critical roles in tumor initiation, progression, metastasis, chemoresistance, and recurrence [52, 53]. Some studies showed that lncRNAs might be involved in regulating stem cell signaling in CSCs.

A novel lncRNA HIF2PUT (hypoxia-inducible factor-2α promoter upstream transcript) was identified in a kind of CSCs, the osteosarcoma stem cells [54]. HIF2PUT expression levels were positively correlated with its parent gene *HIF-2α* in osteosarcoma tissues, indicating a regulatory role of HIF2PUT through HIF-2α. Knocking down HIF2PUT enhanced the proliferation, migration, and self-renewal of osteosarcoma stem cells while overexpression of HIF2PUT decreased the proliferation, migration, and self-renewal [54]. In liver CSCs, lncTCF7 was identified, and it recruited the SWI/SNF complex and further activated the Wnt signaling to promote self-renewal of liver CSCs and tumor propagation [55].

The lncRNA MALAT-1 (metastasis-associated lung adenocarcinoma transcript 1) was found to be upregulated in CSCs, and high expression levels of this lncRNA positively correlated with the proportion of CSCs in pancreatic cancer cells [10, 56, 57]. MALAT-1 might serve as an oncogenic lncRNA in pancreatic cancer by promoting epithelial-mesenchymal transition and stimulating the expression of self-renewal factors such as Sox2.

An lncRNA H19 known for more than two decades also functions in cancer and cancer stem cells [58, 59]. H19 is an imprinting gene in both mice and human [60, 61]. H19 RNA was identified as a tumor suppressor and was involved in Wilms' tumor long time ago in early 1990s [62, 63]. Despite the fact that H19 is one of the first identified, most abundant and conserved ncRNAs in mammals, its actually physiological functions and functional mechanism have been elusive for a long time. In 2007 and then in 2012, compelling lines of evidence showed that H19 is the primary transcript of a microRNA miR-675 [64, 65]. Overexpression of miR-675 in embryonic and extraembryonic cell lines reduced proliferation. Just like the linc-MD1 (the primary transcript of miR-133b), the processing of H19 into miR-675 is also regulated by HuR. What makes H19 more intriguing is the finding that H19 could be sponge of let-7 and miR-106a (a miR-17-5p family member) [66, 67]. HOTAIR with high expression level was always involved in various cancers as well as CSCs via promoting tumorigenesis, cell proliferation, or tumor metastasis and could be a novel epigenetic molecular target for therapeutics [68, 69]. Linc00617 may be another potential therapeutic target for breast cancer because of functioning as a key regulator of EMT and promotes cancer progression and metastasis via activating the transcription of Sox2 [70].

Knowing the roles of lncRNAs in tumor cells and especially in CSCs would help the development of tumor

Table 2: Regulatory roles of lncRNAs in different stem cells.

LncRNA	Stem cell types	Regulations and functions
AK028326	ESCs	Transcriptionally regulated by Oct4 and Nanog
AK141205	ESCs	A coactivator of Oct4
RMST	ESCs	Involved in pluripotency maintenance and interacting with SOX2 and SUZ12
HERVH	ESCs	Function as nuclear lncRNAs with associations with OCT4, coactivators such as p300 and Mediator subunits
SOX2OT	ESCs	Modulate pluripotency through the regulation of SOX2 expression
Linc-RoR	ESCs, iPSCs	Function as microRNA sponge to prevent mRNA of some key transcription factors
Xist	ESCs, iPSCs	An epigenetic regulator of X chromosome inactivation
Blnc1	MSCs	Highly induced during brown and beige adipogenesis and form a feed-forward regulatory loop with EBF2 to regulate adipocyte gene expression
Fendrr	MSCs	Interact directly with the PRC2 and/or TrxG/MLL complexes and act as a modulator of chromatin signatures defining gene activity in embryonic development
Braveheart	MSCs	An epigenetic factor, required for the progression of nascent mesoderm towards a cardiac fate by interacting with SUZ12
Linc-MD1	MSCs	Promote the switch of muscle differentiation from early to later stages by acting as miR-133 and miR-135 sponge
YAM-1	MSCs	Affect myogenesis via epigenetic transcriptional activation
HOTAIR	ASCs, CSCs	Bind both the polycomb repressive complex 2 (PRC2) and the LSD1/CoREST/REST complex and thus modulate histone modifications on target genes
ANCR	ASCs	Essential for the undifferentiated state
TINCR	ASCs	Interact with mRNAs of many differentiation genes such as KRT80 and RNA-binding protein STAU1 to mediate mRNA stabilization through binding TINCR box
Six3os	ASCs	Play crucial roles in the lineage specification of adult stem cells
Dlx1as	ASCs	Play crucial roles in the lineage specification of adult stem cells
Pnky	ASCs	Interact with the splicing regulator PTBP1 and regulate the expression and alternative splicing
HIF2PUT	CSCs	A regulatory role through HIF-2α
lncTCF7	CSCs	Recruit the SWI/SNF complex and further activated the Wnt signaling to promote self-renewal of liver CSCs and tumor propagation
MALAT-1	CSCs	Serve as an oncogenic lncRNA in pancreatic cancer by promoting epithelial-mesenchymal transition and stimulating the expression of self-renewal factors such as Sox2
H19	CSCs	Primary transcript of a microRNA miR-675; processing of H19 into miR-675 is also regulated by HuR
Linc00617	CSCs	Function as a key regulator of EMT and promote cancer progression and metastasis via activating the transcription of Sox2

diagnosis and therapeutics [71]. So far, lncRNA research in cancer and CSCs is just emerging, and more efforts are required to push the field forward. Table 2 lists lncRNAs, their regulatory roles, and stem cells where they exert functions.

7. Conclusions and Perspectives

LncRNAs and other ncRNAs play substantial roles in diverse stem cell types. Here we have summarized some of the lncRNAs in five classes of stem cells, although we are not trying to summarize all the known lncRNAs in all kinds of stem cells and have to miss a lot of researches about lncRNAs in multiple other somatic and germline stem cells. LncRNAs could participate in the biology of stem cells with diverse mechanisms by serving as transcriptional coactivator or corepressor, modular scaffold for chromatin modifiers, factors of tuning splicing, primary transcripts of microRNAs, competing endogenous RNAs of microRNAs, and so on (Figure 1). We need to keep in mind that the functions and mechanisms of a vast majority of lncRNAs in stem cells remain unknown. The pervasive transcription of the genome and the growing inventory of ncRNAs with newly identified ncRNAs such as circRNAs and EIciRNAs also make a call for enormous future efforts to investigate ncRNAs in stem cells [72–77]. Nevertheless, the field of ncRNA research in stem cells has emerged and has been making progress towards a systematic understanding of stem cell biology. Researches in this area would eventually bring benefit to the understanding of lncRNAs and Biomedicine.

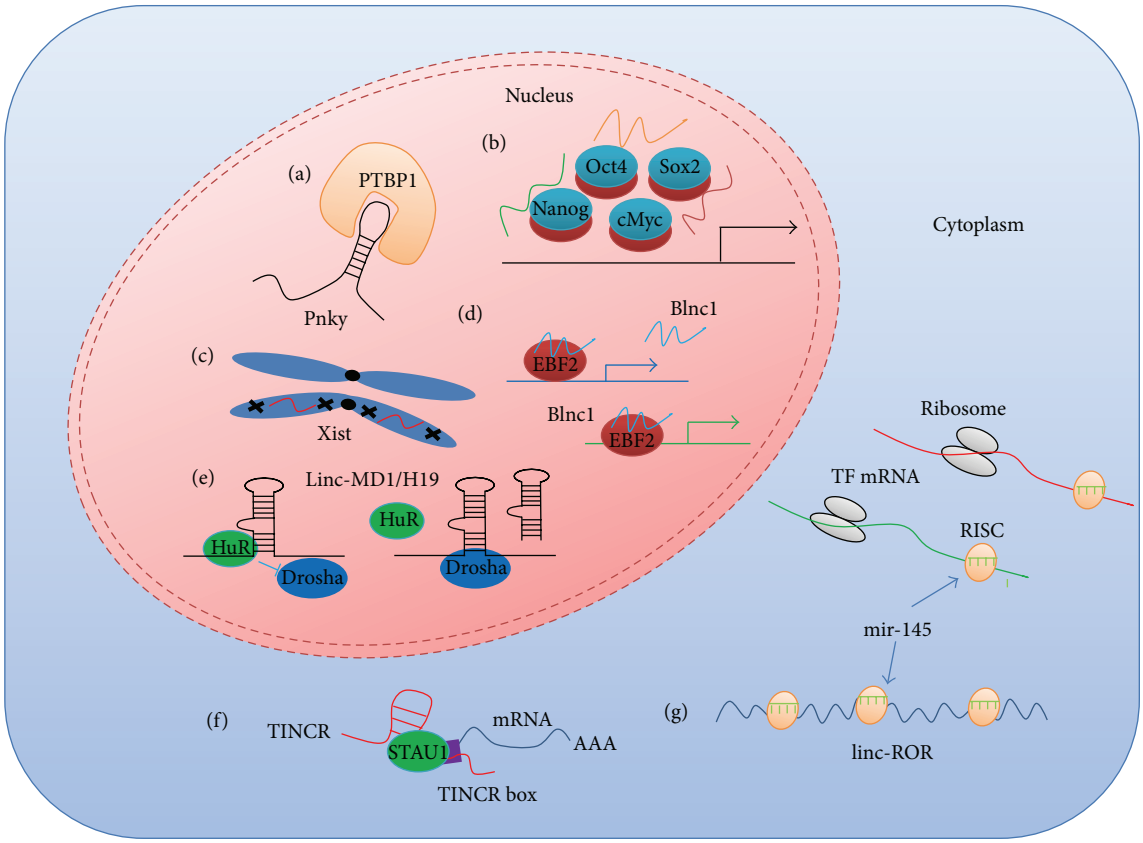

Figure 1: Some known functional mechanisms of lncRNAs in stem cells. (a) Pnky localizes to the nucleus and interacts with the mRNA splicing regulator PTBP1 to regulate splicing of key transcripts; (b) some lncRNAs serve as cofactors of key transcription factors (TF) to regulate gene transcription; (c) Xist as an epigenetic regulator of X chromosome inactivation plays roles in female ESCs and iPSCs; (d) Blnc1 is activated by EBF2 and functions as a coactivator of EBF2; (e) linc-MD1 is the primary transcript of miR-133b and H19 is the primary transcript of miR-675, and the Drosha processing of both is regulated by HuR; (f) TINCR interacts with STAU1 and multiple mRNAs via an RNA motif called TINCR box; (g) linc-ROR functions as a mir-145 sponge.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

iPSCs: A Minireview from Bench to Bed, including Organoids and the CRISPR System

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When Dolly the sheep was born, the first probe into an adult mammalian genome traveling back in time and generating a whole new animal appeared. Ten years later, the reprogramming process became a defined method of producing induced pluripotent stem cells (iPSCs) through the overexpression of four transcription factors. iPSCs are capable of originating virtually all types of cells and tissues, including a whole new animal. The reprogramming strategies based on patient-derived cells should make the development of clinical applications of cell based therapy much more straightforward. Here, we analyze the current state, opportunities, and challenges of iPSCs from bench to bed, including organoids and the CRISPR system.

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells with a high self-renewal rate; they are derived from the inner cell mass of preimplantation embryos and can be differentiated into almost all cell types. The study of genes that are expressed in ESCs led to the identification of cell pluripotency-associated genes [1, 2]. In 2006, Professor Yamanaka's group isolated these candidate genes and introduced them in mouse fibroblasts cultured *in vitro* using viral vectors. Thus, the induced expression of these genes reprogrammed transfected cells, which became pluripotent and acquired ESC-associated morphology and gene expression pattern. These cells also gained the capacity to originate endodermal, mesodermal, and ectodermal tissues and differentiate into neural and cardiac cells [3]. For the first time, it was shown that it is possible to induce pluripotency *in vitro* in somatic adult cells with definite factors. These cells were first developed in mice and named induced pluripotent stem cells (iPSCs); subsequent research led to the development of human iPSCs, with clear impacts on therapeutic applications [4].

2. Genes and Mechanisms of Induced Pluripotency

Differentiated cells exhibit methylation and acetylation patterns that regulate gene expression and influence their potential development. Thus, in differentiated cells, most gene promoters are hypermethylated (typically associated with silenced chromatin), while, in stem cells, the opposite occurs, with most promoters being hypomethylated (typically associated with active chromatin) [5]. The differential pattern of methylation, acetylation, and ubiquitination of genes and histones is known as epigenetics. As discussed later, epigenetics involves transcription factors that are coded by genes used to obtain iPSCs, as well as proteins controlling the activation and repression of gene expression through the binding to the promoters of thousands of genes.

In the first study of iPSCs, 24 genes coding transcription factors related to ESCs were introduced into mouse fibroblasts [3]. The expression of those factors led to cell reprogramming, reaching a pluripotency state similar to that of ESCs. Surprisingly, the results show that the induced

expression of only four factors was necessary for reprogramming pluripotency: OCT4, SOX2, c-MYC, and KLF4, commonly named Yamanaka's factors. The mechanism of action of these factors in iPSCs has been only partially elucidated.

Reprogramming is governed by slightly different mechanisms according to cell type. OCT4, SOX2, and NANOG constitute the core transcriptional regulatory circuitry that allows pluripotency and self-renewal in human ESCs [2]. However, the addition of the *NANOG* gene is dispensable for the generation of human iPSCs [4]. OCT4 and SOX2 usually have actions on the same human ESC promoters, probably cooperatively. Thus, the OCT4/SOX2 complex is believed to act as a key regulator that controls the expression of developmental genes. In addition, several NANOG binding sites are found in the same sequences, such as OCT4/SOX2 complex binding sites, indicating the likely complexity of their interrelationship in gene regulation. Thus, OCT4 directly binds 623 promoters related to several protein-coding genes. SOX2 and NANOG have been found in association with 1271 and 1687 promoters, respectively. Of that number, 353 are regulated by OCT4, SOX2, and NANOG altogether [2]. Surprisingly, the autoregulation of *OCT4*, *SOX2,* and *NANOG* by self-binding to their own promoters has also been reported. Together, these observations suggest that OCT4, SOX2, and NANOG promote pluripotency, cell self-renewal, and suppression of cell differentiation programs.

The KLF4 and c-MYC transcription factors are also used to obtain iPSCs. The high expression of *Klf4* and *c-Myc* has been identified in tumors; thus, these genes are related to cell proliferation and self-renewal. In mammalian genomes, there are more than 25,000 putative c-MYC binding sites, highlighting its role [6]. Meanwhile, KLF4 suppresses the *p53* gene, and because the p53 protein inhibits *NANOG* gene during ESC differentiation, KLF4 could function by activating *NANOG* through *p53* inhibition. Another factor that is commonly used to obtain iPSCs is LIN28, which is related to the negative regulation of the processing of microRNAs that act on the cell differentiation of ESCs [2]. This effect on microRNA repression has also been proposed for c-MYC.

Previous studies cited here have demonstrated the mode of action of transcription factors in gene regulation but do not explain how these factors modify chromatin remodeling. However, different reports have gradually provided significant data to begin uncovering these mechanisms. For example, OCT4 binding to specific targets has been associated with the ability to recruit p300 histone acetyltransferase, showing the interrelationship between transcription factors and modifying histones proteins [5]. In addition, c-MYC has been shown to be associated with the p300 complex [7] and is believed to act on the global acetylation of histones, allowing, for example, OCT4 and SOX2 to bind to their targets [3]. It is well known that several levels in the control of gene expression exist; among these levels, the initiation of transcription involves chromatin architecture and the access of transcription factors to the target site. Thus, DNA hypermethylation catalyzed by DNA methyltransferases in promoters usually silences chromatin and inhibits transcription, while the hypomethylated status commonly associates with actively expressing chromatin. On the other hand, DNA-associated histones may be acetylated and/or methylated, driving different patterns of expression that highly vary according to the residues, histones involved, and multiplicity of amino acids modified. For example, lysine 4 from histone 3 can be trimethylated, generally leading to an active status of chromatin, while lysine 27 from histone 3 can be trimethylated, leading to inactivating chromatin. The acetylation of histones is catalyzed by histone acetyltransferases (HATs), which activate chromatin; in contrast, deacetylation is mediated by histone deacetylases (HDACs), which silence the chromatin.

Altogether, these results allow researchers to develop new strategies to artificially induce cell pluripotency reprogramming. Some recent reports indicate that only 3 factors (OCT4/SOX2/NANOG) are needed to derive human iPSCs from somatic adults cells [8]. Additionally, only 2 factors are needed when the histone deacetylase inhibitor, valproic acid, is added [9]. The effects of c-MYC can be partially compensated for by valproic acid, demonstrating that c-MYC modifies histone acetylation. Valproic acid can replace KLF4 functions in human cell reprogramming, demonstrating that c-MYC and KLF4 may affect similar mechanisms of control. Interestingly, recent studies suggest that OCT4, which was previously believed to be irreplaceable, can be substituted in some cell types by chemically inhibiting G9a histone methyltransferase [5]. These results drive the development of a plethora of next-generation pluripotency inducers, such as pharmacological drugs (discussed below). Notably, unraveling pluripotency reprogramming mechanisms will provide data to better understand cell regulation and related conditions, such as cancer and cell ageing.

3. Methods of Producing iPSCs

iPSCs were first obtained through the transfection of mice and human fibroblasts with viral vectors [3, 4]. This procedure is based on the capacity of retroviral and adenoviral vectors to efficiently introduce genes inside the nucleus. Viral vectors lack pathogenic genes from viruses and only possess information for the packaging and integration of the transgene in the host genome. In addition, these vectors contain reprogramming genes (usually *OCT4*, *SOX2*, *KLF4,* and *c-MYC*) with strong downstream promoters leading to high levels of expression.

There are advantages and disadvantages when these vectors are used: on the one hand, they are characterized by a high efficiency of integration (60 to 80% in the case of retroviruses) and can be used in cells with low mitotic rate or even in nondividing cells (lentiviruses). On the other hand, there is a strong requirement for biosafety protocols when handling viral vectors, and they have low potential in clinical trials.

With the aim of avoiding the integration of foreign genes into the human genomes because of known ethical issues, assays with iPSCs have begun to use new strategies based on the expression of pluripotency genes without being

integrated. Thus, the use of circular plasmids and messenger RNA (mRNA) emerged; circular plasmids are 10 kbp molecules that are closed by covalent bonds. These molecules are easily handled and can be driven inside cells through liposomes or cell pores that are generated by electroporation. One study showed that iPSCs can be obtained from human fibroblasts cultured *in vitro* using a circular plasmid coding OCT4, SOX2, LIN28, NANOG, and Green Fluorescent Protein (GFP) reporter genes [10]. Two weeks after transfection, green cells started acquiring ESC-like features and stopped expressing GFP. Later, PCR genetic analyses revealed that once iPSC lines were established, there were no more traces of the plasmid containing foreign genes, suggesting the following: (i) plasmids were not integrated in the human genome and were lost during successive mitotic divisions and (ii) the expression of foreign transcription factors was only necessary during the first steps of cell differentiation, leading then to the expression of endogenous transcription factors.

More recently, human fibroblasts were reprogrammed with mRNA coding the main 4 factors [11]. The authors demonstrated the inefficiency of this methodology because of the need for 5 cycles of reprogramming with mRNA encoding the 4 transcription factors to achieve iPSCs. However, this strategy presents a major advantage in a context of approaching the bench to the bed: it does not require DNA sequences and does not modify genetic host cells. Consequently, it is expected that these alternative technologies will gain high relevance for future clinical applications.

Some years ago, the exogenous expression of pluripotency-associated factors (at least OCT4) was believed to be indispensable for establishing pluripotency [12]. However, a chemical reprogramming strategy emerged with great potential use in generating functional and desirable cell types, excluding genetic manipulation, which limits clinical applications [13]. This type of reprogramming is based on cell-permeable and nonimmunogenic small molecules, which are often easily synthesized and more cost-effective; interestingly, their effects rely on reversible inhibition or the activation of specific protein functions. In this context, the identification of small molecules driving the reprogramming of mouse embryonic fibroblasts has recently been reported [14]. Thus, the glycogen synthase kinase 3 inhibitor CHIR, the transforming growth factor-β inhibitor 616452, the cAMP agonist Forskolin, and the S-adenosylhomocysteine hydrolase inhibitor DZNep were identified in a small-molecule library and demonstrated induced expression levels of most pluripotency marker genes and growth, with a doubling time similar to that of ESCs. Importantly, the DNA methylation state and histone modifications at *OCT4* and *NANOG* promoters in chemically iPSCs were similar to those in ESCs; in addition, differentiation into tissues of all three germ layers was observed when reprogrammed cells were injected into immunodeficient mice, showing fully reprogramming ability.

In this line of evidence, other pathways involved in reprogramming include MEK and transforming growth factor-β. One study showed that reprogramming can also be achieved with chemical inhibitors of the MEK and transforming growth factor-β pathways (PD0325901 and SB431542, resp.) [15]. In this study, the specificity of different primary cell cultures to both inhibitors was reported; iPSCs were generated only from the head-derived primary culture of mouse embryonic cells, while primary cell cultures that were derived from the liver, side-body skin, and tail-tip of the embryos showed no reprogramming.

Unfortunately, until now, small-molecule strategies have shown little efficiency in reprogramming. For example, a CHIR/616452/Forskolin/DZNep combination generated iPSCs from mouse somatic cells at a frequency of up to 0.2% [14]. In addition, an estimated efficiency of the reprogramming of 4 iPS-like colonies per 1.7×10^6 starting cells has been reported, of which 40% of clones were alkaline-phosphatase-positive, a characteristic feature of ESCs [15]. As a consequence, great efforts have been made to obtain a higher efficiency in reprogramming strategies. One study recently showed an almost 100% efficiency in reprogramming somatic cells. By means of genetic depletion of the core member of the nucleosome remodeling and deacetylation complex *MBD3*, *OCT4/SOX2/KLF4/c-MYC* transgene delivery, inhibition of ERK1/2 and GSK3-β, and stimulation with leukemia inhibitory factor (LIF), pluripotency was achieved in mouse embryonic fibroblasts, which showed similar genome-wide chromatin mapping for H3K27me3, H3K4me3, and H3K27ac histone markers, genome-wide DNA methylation mapping, and expression of key endogenous pluripotency markers to that of ESCs [16].

4. Identification and Characterization of iPSCs

At first glance, iPSCs are very similar to ESCs: they form flat colonies with regular borders, divide at similar rates, self-renew (allowing a great number of passages *in vitro*), and exhibit long nucleoli and a limited cytoplasm. Although morphological evidence is mandatory, characterization requires other techniques [17], such as those based on the analysis of immunofluorescence, the expression pattern, and the capacity for the generation of different tissue types (pluripotency).

One of the first tests that were developed to determine cell pluripotency is the alkaline phosphatase activity assay because this enzyme is active in ESCs and iPSCs. The telomerase-coding gene *hTERT* is another gene that is typically expressed in these types of cells; this enzyme lengthens chromosome ends (telomeres), assuring indefinite cell division. On the other hand, immunocytochemical techniques are utilized to fluorescently label membrane and intracellular proteins, as well as transcription factors such as OCT4, SOX2, and NANOG, which are associated with ESCs. For these techniques, cells must be fixed, permeabilized, and incubated with specific antibodies against OCT4, SOX2, NANOG, and so forth; subsequent observation of samples through fluorescence microscopy allows for the detection of these proteins. These genes can also be assayed by western blotting or real-time PCR following retrotranscription.

Importantly, obtained iPSCs must be demonstrated to originate the 3 germ layers [1, 3, 17]. One method of choice is suspension culture forming embryoid bodies [18, 19]. Embryoid bodies are round cell clusters that are obtained when iPSCs are cultured in suspension, transferred to

gelatin-coated plates, and cultured afterwards until the appearance of adherent cells of endo-, meso-, and ectodermal origin. The formation of teratomas is another assay that is frequently used to study the pluripotency of iPSCs [20]. Teratomas are tumors containing a variety of cell types, which can originate a number of tissues, for example, cartilage, skin, hair, and even nails. These tumors are usually generated by the subcutaneous injection of iPSCs in immunosuppressed mice.

Chimera formation is another test for the pluripotency of iPSCs that can only be assayed in animals and nonhuman primates. Chimeras are made by the addition of iPSCs to embryos that were previously obtained by fertilization; after chimeric embryos are transferred to the receptor females in which they will develop, the contribution of iPSCs to the different tissues of the newborn animals is analyzed. However, iPSCs generate live chimeras in mice only with great difficulties [3].

Finally, despite global gene expression and methylation and acetylation markers to identify iPSCs, it is now well established that these patterns may deeply differ from those of ESCs, highlighting the differences between both types of cells [4, 21–24].

5. Miniorganoids

Recent breakthroughs in 3-dimensional (3D) organoid cultures of many organ systems have led to new *in vitro* physiologically complex models. Perhaps in this new scenario, the engineering of human organs will take greater advantage of iPSCs, furthering the study of human development and disease transplantation. The coculturing of more than one iPSC-derived cell type to make complex autologous *bona fide* organs for transplantation medicine may be achievable in the near future. Meanwhile, iPSCs have demonstrated the ability to generate organoids that are capable of making functional 3D structures for *in vitro* disease modeling and drug screening.

5.1. Miniature Stomach. Gastric diseases, including peptic ulcer disease and gastric cancer, affect 10% of the world's population and are largely due to chronic *Helicobacter pylori* infection. In a recent report, the authors showed that the temporal manipulation of several signaling pathways (FGF, WNT, BMP, retinoic acid, and EGF) and 3D growth are sufficient to generate human gastric organoids (3D human gastric tissue *in vitro*) from human iPSCs [25]. The organoids formed a primitive gastric gland, surface cells, antral mucous cells, and a diverse amount of gastric endocrine cells. Interestingly, these organoids were successfully used for modeling *H. pylori* infection.

5.2. Growing a Gut. A robust and efficient process to direct the differentiation of human iPSCs into 3D gut organoids has also been created [26]. The resulting intestinal 3D-cultured tissue presented a cellular composition similar to that of fetal intestine, expressed intestinal stem cell markers, and presented absorptive and secretory functions. The epithelium contained functional enterocytes, as well as goblet, Paneth, and enteroendocrine cells. In addition, the interaction between the human iPSCs-derived intestinal organoids and *Salmonella enterica* populations has been explored [27], demonstrating that iPSC-derived organoids are promising models of intestinal epithelium.

5.3. Minilivers. Recently, the generation of vascularized and functional human liver from human iPSCs through the transplantation of liver buds that were created *in vitro* was reported [28]. Interestingly, specified hepatic cells self-organized into 3D iPSC-derived organoids. Immunostaining and gene expression analyses revealed similarities between iPSC-derived organoids and *in vivo* liver buds. In addition, human vasculatures in iPSC-derived organoid transplants became functional by connecting to the host vessels. The highly metabolic iPSC-derived tissue performed liver-specific functions, such as protein production and human-specific drug metabolism, without recipient liver replacement; in addition, the mesenteric transplantation of these organoids rescued a drug-induced lethal liver failure model, demonstrating for the first time the generation of a functional human organ from iPSCs.

5.4. Little Lungs. A recent report has demonstrated the stepwise differentiation of human iPSCs into lung organoids. After the manipulation of the signaling pathways that are involved in development, iPSCs generated lung organoids consisting of organized compartments and showing structural features similar to those of the native lungs. In addition, the lung organoids possessed an upper airway-like epithelium with basal and immature ciliated cells that were surrounded by smooth muscle and myofibroblasts, as well as an alveolar-like domain. Based on global transcriptional profiles, the authors demonstrated that lung organoids are remarkably similar to human fetal lungs [29].

5.5. Baby Brains. Cerebral organoids have been made by the 3D culturing of neuroectoderm derived from human iPSCs [30, 31]. This method can give rise to a developing cerebral cortex, ventral telencephalon, choroid plexus, and retinal identities, among others, within 1-2 months. Furthermore, because organoids can be maintained for more than 1 year in long-term culture, they also have the potential to model later events, such as neuronal maturation and survival.

5.6. Building Hearts. The ability to create whole functional hearts by means of tissue bioengineering has proven elusive. The closest result to this bioengineering task has been the engineering of heart constructs by repopulating decellularized mice hearts with human iPSC-derived multipotential cardiovascular progenitor cells [32]. The authors reported that the seeded cells migrated, proliferated, and differentiated *in situ* into cardiomyocytes, smooth muscle, and endothelium. After 20 days of perfusion, the engineered heart tissues exhibited spontaneous contractions, generated mechanical force, and were responsive to drugs. These novel results will benefit the study of early heart formation and contribute to the search of applications in preclinical testing.

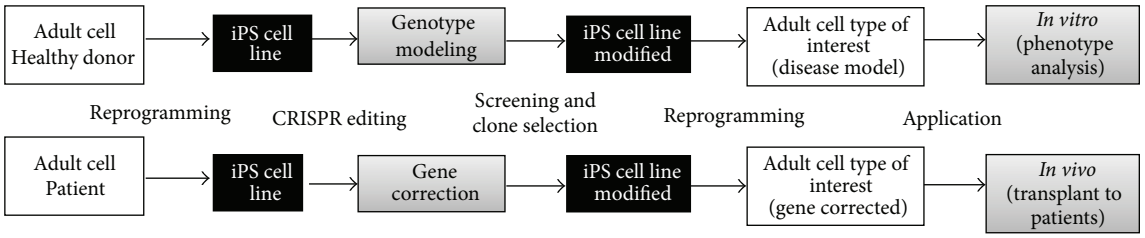

Figure 1: Methodology of gene editing for modeling or cell therapy based on iPSC and CRISPR/Cas9 technology.

Table 1: Miniorganoids that have been obtained from iPSCs and associated disease modeling.

Miniorganoids	Disease modeled	References
Stomach	*Helicobacter pylori* infection	[25]
Gut	*Salmonella enterica* infection	[26, 27]
Liver	Lethal liver failure	[28]
Lungs	Cystic fibrosis	[29]
Brain	Alzheimer disease and Rhett Syndrome	[30, 31]
Heart	Cardiac failure	[32]
Eyes	Age-related macular degeneration	[33, 34]

5.7. Tiny Eyes. Under defined culture conditions, iPSCs have been used to generate optic vesicle-like structures that generate retinal cell types [33] that are suitable for *in vitro* studies and disease modeling [34]. Thus, iPSCs were differentiated into 3D optical vesicles and later started expressing markers of intercellular communication. These 3D optical vesicles contained multiple neuroretinal cell types and spontaneously formed primitive laminae, reminiscent of the developing retina, demonstrating the capacity of iPSCs to self-assemble into rudimentary neuroretinal structures. As a proof-of-principle, the authors examined the role of a key transcription factor, visual system homeobox 2 (VSX2), using iPSC-derived optic vesicle-like structures that were obtained from a patient with microphthalmia caused by an R200Q mutation in the VSX2 homeodomain region. iPSC-derived vesicles have emerged as a versatile model system to study retinal development at stages that were not previously accessible in humans. Finally, the use of retinal pigmented epithelial cells that were derived from iPSCs to treat eye disease is currently being evaluated in clinical trials (see below).

Table 1 summarizes the miniorganoids that have been obtained up to now and the associated modeling of diseases.

In summary, iPSC technology has driven the creation of 3D structures that resemble tissues from the eye, gut, liver, lung, stomach, and brain, among others. These engineered organoids mimic some structures and functions of real organs, increase knowledge of human development, provide novel tools for drug-screening platforms, and serve as disease models that would eventually replace animal models. Consequently, it is possible to envision the use of personalized iPSC-derived organoids in clinical trials. However, the generation of complex 3D organs *in vitro* remains a major challenge for translational studies.

6. Impact on Cell Therapy

The discovery of the reprogramming and differentiation of human adult cells has generated great expectations because of potential therapeutic applications. iPSCs have a major advantage in regenerative therapies because they can be obtained from the same patient, avoiding immune rejection when transplanted. Additionally, culturing iPSCs under restrictive conditions can lead to differentiation to specific cell types (Table 2).

The first attempts came from animal model studies, providing promising results. There have been several assays reporting the therapeutic use of iPSCs in preclinical trials assayed in animals, mainly mice. This technology has led to the reversal of hyperglycemia in diabetic mice [35]. Then, assays in animals presented evidence supporting the potential application of iPSCs in cell therapy. This and other recently achieved goals are shown in Table 3.

Studies in human samples have also produced interesting results. For example, the differentiation of iPSCs into insulin-producing pancreatic cells has been reported [35, 41]. This *in vitro* differentiation requires culture media with proper growth factors and chemicals, such as activin-A [42] and sodium butyrate [43]. After 3 weeks under these conditions, cells start clustering in pancreatic-like islets and secreting insulin after glucose stimulation. The results of these studies demonstrate that pancreatic cells can be obtained from the skin of diabetic patients.

Another promising example is the formation of human motor neurons from iPSCs [48]; in this report, the authors showed that motor neurons can be obtained when iPSCs are cultured under retinoic acid treatment and challenged with Sonic Hedgehog pathway agonists and neurotrophic factors. As a result, iPSC-derived motor neurons express typical molecular markers and are electrically active, similar to those obtained from human ESCs.

The transplantation of iPSCs for cell therapy in humans should overcome the following obstacles: (i) avoidance of the integration of foreign DNA in the human genome (alternative methodologies have been described above, e.g., using plasmids, mRNA, or small molecules); (ii) avoidance of the risky use of oncogenes during the induction of pluripotency

Table 2: Obtaining and differentiating human iPSCs.

Precursor cells	Method of pluripotent stem cell induction	Type of cell-like or tissue-like produced	Reference
Human fibroblasts	Retrovirus	Neural and cardiac	[3]
Human fibroblasts	Retrovirus	Pancreatic islet	[34]
Human fibroblasts	Plasmid	Hepatic and cardiac	[44]
Commercial human cells	Without data	Retina	[45]
Thalassemia patient fibroblasts	Retrovirus	Hematopoietic	[46]
Human fibroblast	mRNA	iPSCs	[47]

Table 3: Therapeutic use of iPSCs in mice.

Precursor cells	Method of transfection	Type of cell or tissue produced	Results	Reference
Diabetic murine fibroblasts	Retrovirus	β-pancreatic	Reversion of hyperglycemia	[35]
Hemophilic murine fibroblasts	Retrovirus	iPSCs	Phenotypic reversion of hemophilia A	[36]
Embryonic fibroblasts	Lentivirus	Neurons	Partial reversion of damaged spinal chord	[37]
Humans Parkinson fibroblasts	Lentivirus	Clusters of neurons	Reduction of Parkinson disease in rats	[38]
Anemic mice fibroblasts	Retrovirus	Hematopoietic progenitors	Reversion of anemia	[39]
Human fibroblasts	Retrovirus	Pancreatic beta cells	Reversion of hyperglycemia	[40]

(e.g., *Klf4* and *c-Myc*); and (iii) replacement of animal-origin products in the media to avoid possible zoonoses.

First Pilot Study. Age-related macular degeneration is one of the most common causes of visual impairment in the elderly, and protocols for the formation of human retinal pigment from iPSCs have been previously developed, including the use of scaffolds [49, 50]. Similarly, nonhuman retinal pigment has been obtained from iPSCs [51]. Importantly, one study showed that iPSC-derived retinal pigment epithelium resembles native retinal pigment epithelium according to the similar expression of typical retinal pigment epithelium markers, the formation of tight junctions with the polarized secretion of growth factors, phagocytic ability, and gene expression patterns [50]. In addition, in this report, these authors demonstrated that transplanted autologous nonhuman primate iPSC-derived retinal pigment epithelium cell sheets show no immune rejection or tumor formation. Along the same line of evidence, it has been shown in an immunodeficient mice model that iPSC-derived retinal pigment epithelium has negligible tumorigenic potential [52].

The treatment of age-related macular degeneration has recently witnessed great advances in a first pilot study. The first attempt to study the safety and feasibility of transplanted retinal pigment epithelium cell sheets in patients with exudative age-related macular degeneration took place in Japan. Autologous iPSC-derived retinal pigment epithelium cell sheets were transplanted into an elderly age-related macular degeneration volunteer. iPSCs and subsequent retinal pigment epithelium cell sheets were produced and validated at a certified clinical grade before transplantation, leading to the posterior analysis of the functional integration of retinal sheets and potential adverse reactions. Unfortunately, a later analysis revealed 6 mutations in the patient's cells, one of them in an oncogene, although associated with low risk tumorigenesis. Tt is believed that these mutations occurred when iPSCs were manipulated, during their isolation or differentiation [53].

7. Application of iPSCs and CRISPR/Cas9 Technology

Although the application of iPSC technology in humans has increased in frequency as a promising regenerative therapy, a new field based on the *in vitro* use of human iPSCs in diseases modeling has emerged due to the capacity to test new chemicals or patient-specific treatments (as discussed above). The possibility of using human cells that are patient specific has high potential. iPSCs proliferate indefinitely *in vitro* and can be differentiated to almost any cell type of the human body, such as cardiomyocytes, nerve cells, or insulin-producing pancreatic cells, providing excellent foundations as human models for testing drug efficiency or toxicity [54].

In addition to patient-specific treatments, there is great interest in developing human iPSC lines with a particular genotype characteristic of certain diseases to understand different pathogeneses. The use of cells from patients who carry the altered genetic background can be considered an adequate option to establish a desired iPSC line. However, it cannot be ruled out that other differences between healthy control and disease genotypes may drive phenotypic differences [55]. Fortunately, new technologies based on iPSCs and CRISPR/Cas9 gene editing have emerged as important tools due to their capacities of generating a cell phenotype with a specific gene failure to be studied *in vitro* using an isogenic cell line as a control [56].

CRISPR/Cas9 is a defense system in which bacteria and archaea degrade viral DNA by means of an RNA probe that is complementary to a target sequence and a nuclease protein

(Cas9) [57]. The use of CRISPR/Cas9-derived biotechnology became a practical RNA-guided platform for targeting and cutting any specific DNA loci by simply specifying a 20 nt targeting sequence within its RNA probe [58–60]. The adaptation of this system for use in eukaryotic cells has resulted in a milestone in the history of genetic engineering because CRISPR/Cas9 technology is cheaper and easier to use than its predecessor techniques involving TALENs and Zinc fingers [61].

In terms of the special capabilities of gene editing for disease modeling, CRISPR/Cas9 technology allows the knock-out of one or more genes at once [62], as well as the knock-in of specific alleles in iPSCs that are associated with different diseases, utilizing single-stranded DNA oligonucleotides as templates for homology-based repair [63]. Previously, gene-editing approaches employed randomly integrating viruses with concomitant issues of insertion mutagenesis, inaccurate gene dosage, and gene silencing, which are inconvenient for clinical application [64]. It must be noted that CRISPR/Cas9 editing occurs at a specific DNA locus of interest, avoiding viral vectors-associated random insertion. In terms of its application in cellular models, the success of the combination of iPSCs and CRISPR/Cas9 technologies in *in vitro* modeling lays in (i) the ability of CRISPR/Cas9 to rapidly and precisely edit genes; (ii) the capacity of iPSCs to proliferate indefinitely, allowing a highly efficient selection of clones carrying the gene modification; and (iii) the capacity of this cell type to reprogram into the desired cell phenotype after DNA editing. The progress has been so high that a great number of diseases have been recently modeled from specific mutations, such as immunodeficiency centromeric region instability and facial anomalies syndrome [64] and pancreatic cancer [65].

Likewise, it is worth mentioning that, in iPSCs from patients with mutated genes, CRISPR/Cas9 editing allows the knock-in of corrected alleles. Based on this background, several groups have proposed, in addition to modeling diseases, taking iPSCs to a new paradigm in cell therapy: the correction of genetic material *in vitro* before transplantation into patients, thus restoring lost functions in specific tissues. The main candidates for *in vitro* gene correction are monogenic diseases, such as β-thalassemia and hemophilia A. β-Thalassemia is a genetic disorder that is caused by mutations in the human hemoglobin beta (*HBB*) gene. CRISPR/Cas9 technology efficiently corrected the *HBB* mutations in patient-derived iPSCs, and when these cells differentiated into erythroblasts using a monolayer culture, gene-corrected iPSCs restored the expression of *HBB* and reduced the reactive oxygen production compared to the parental iPSCs line [66, 67]. Hemophilia A is an X-linked genetic disorder that is caused by mutations in the *F8* gene, which encodes blood coagulation factor VIII. Almost half of all severe hemophilia A cases result from chromosomal inversions. Interestingly, CRISPR/Cas9 nucleases were used to revert these chromosomal segments back to wild type in a mouse iPSC line that expressed the *F8* gene and functionally rescued factor VIII deficiency [68]. More importantly, gene editing excluded the modifications of potential off-targets (nonspecific sequences), which is an advantage compared to gene therapy based on viral vectors, which integrate randomly into multiple sites.

While this strategy exhibits the same difficulties as standard reprogramming in translational medicine, an important advantage arises due to the restoration of lost functions in patients, making it an attractive scenario for the development of new protocols that guarantee higher levels of biosafety. Figure 1 shows a general scheme of the methodology of iPSC and CRISPR/Cas9 technology that are used in gene editing for modeling or cell therapy.

8. Concluding Remarks

The mechanisms of action that are involved in cell reprogramming to pluripotency have begun to be elucidated, and future research will definitively focus on them. Although iPSCs resemble ESCs (e.g., they share the proliferation rate, self-renew, express the same molecular markers, and can differentiate into several cell types), the patterns of global gene expression and methylation differ deeply. Such differences currently concern the scientific community. However, iPSCs have already reached the clinical research phase thanks to exponential advances in the understanding of methylomes, proteomics, analysis of single cells, and so forth, technologies that previously seemed distant but have become reality.

Despite the main objective of iPSCs being clinical application, technologies based on miniorganoids are gaining interest. The possibility of obtaining *in vitro* organ-like structures drives a plethora of "clinical trials in Petri dishes" and promises the production of any desired organ in the lab for later transplantation. In addition, the association of iPSCs with CRISPR/Cas9 may lead to a unique combination of gene and cell therapies.

As shown in this minireview, the study of iPSCs not only began with great expectations, but is also accomplishing them every day.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Application of Human iPSCs in Neurological Diseases: From Bench to Bedside

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In principle, induced pluripotent stem cells (iPSCs) are generated from somatic cells by reprogramming and gaining the capacity to self-renew indefinitely as well as the ability to differentiate into cells of different lineages. Human iPSCs have absolute advantages over human embryonic stem cells (ESCs) and animal models in disease modeling, drug screening, and cell replacement therapy. Since Takahashi and Yamanaka first described in 2007 that iPSCs can be generated from human adult somatic cells by retroviral transduction of the four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, disease specific iPSC lines have sprung up worldwide like bamboo shoots after a spring rain, making iPSC one of the hottest and fastest moving topics in modern science. The craze for iPSCs has spread throughout main branches of clinical medicine, covering neurology, hematology, cardiology, endocrinology, hepatology, ophthalmology, and so on. Here in this paper, we will focus on the clinical application of human iPSCs in disease modeling, drug screening, and cell replacement therapy for neurological diseases.

1. Introduction

In principle, induced pluripotent stem cells (iPSCs) are generated from human somatic cells by reprogramming and gaining the capacity to self-renew indefinitely as well as the ability to differentiate into cells of different lineages. Human iPSCs have absolute advantages over human embryonic stem cells (ESCs). On the one hand, as an unlimited therapeutic source, human iPSCs overcome the ethical issues faced by human ESCs, for human adult somatic tissue is much more accessible than embryonic tissue both technically and ethically. On the other hand, as patients derived stem cells, human iPSCs can better recapitulate disease phenotype and pathological process without having the interspecies difference existing in animal models. Since Takahashi and Yamanaka first described in 2007 that iPSCs can be generated from adult somatic cells by retroviral transduction of the four transcription factors Oct3/4, Sox2, Klf, Klf4, and c-Myc [1, 2], disease specific human iPSCs lines have sprung up worldwide like bamboo shoots after a spring rain, making iPSCs one of the hottest and fastest moving fields in modern science. The craze for iPSCs has spread throughout main branches of clinical medicine, covering neurology [3–6], hematology [7, 8], cardiology [9, 10], endocrinology [11, 12], hepatology [13], ophthalmology [14, 15], and so forth. Here we will focus on the clinical applications of human iPSCs in neurological diseases' modeling, drug screening, and cell replacement therapy (Figure 1).

2. Overall Disease Modeling Strategies and Challenges

The first strategy would be reprogramming patients somatic cells directly to iPSCs. As long as there is accessible patient somatic tissue, a disease specific iPSC line can be gained using suitable reprogramming methods, whether it is a neurogenetic disorder with defined genetic reasons or a sporadic disease with no defined genetic reasons. Up to date, neurological disease models using this strategy have included fragile X syndrome (FXS) [6], Rett syndrome [16], Down syndrome [17], Parkinson's disease (PD) [4, 18–22], Alzheimer's disease [5, 23–27], and schizophrenia (SCZH) [28, 29]. Significant progresses have been made by using this iPSC based strategy although it is at a very early stage. For example, some general neurological disease phenotypes have been revealed, such as synaptic deficiency, inadequate neuronal maturation, abnormal response to oxidative stressors, and mitochondrial

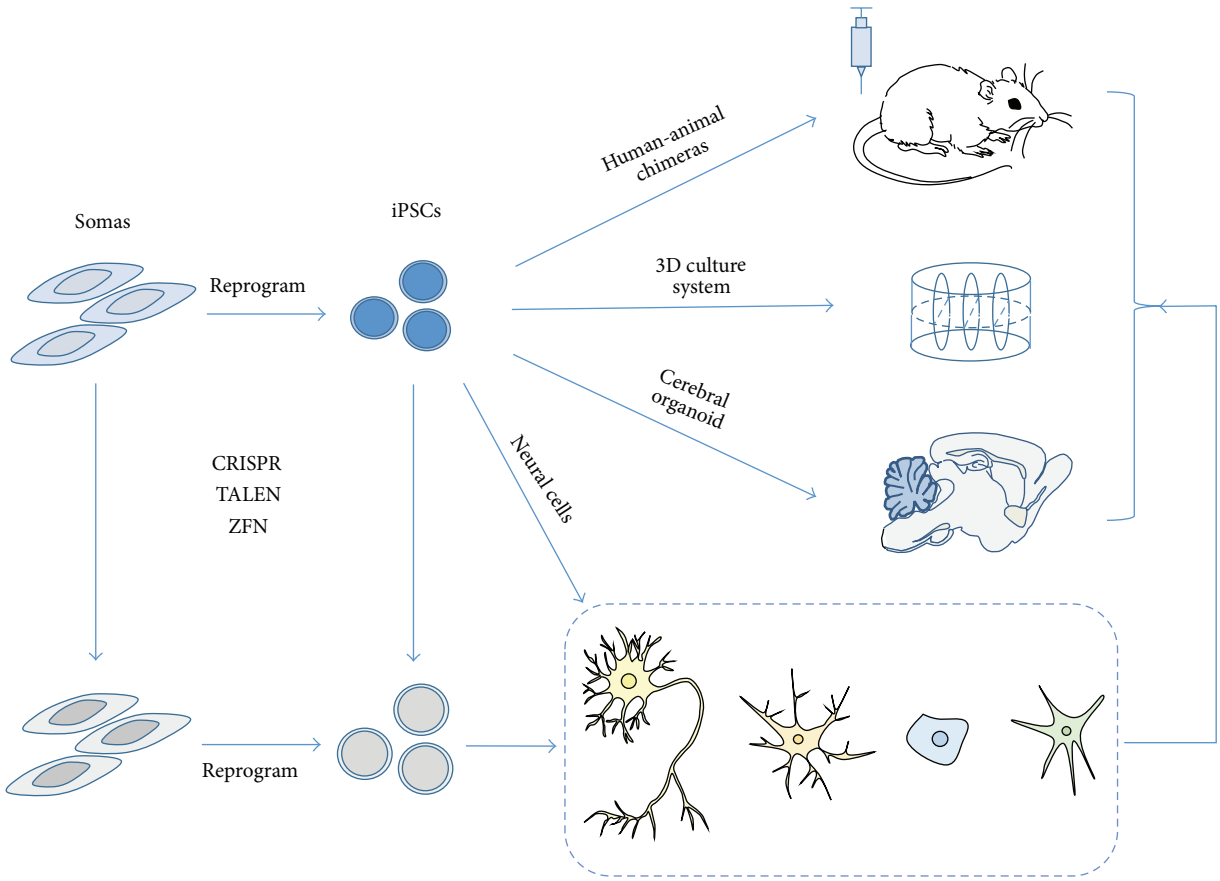

Figure 1: Applications of human iPSCs and iPSC derived neural cells in neurological diseases. In principle, induced pluripotent stem cells (iPSCs) are generated from somatic cells by reprogramming and gaining the capacity to self-renew indefinitely as well as the ability to differentiate into cells of different lineages. Up to date, the main applications of iPSC products in neurological diseases have included disease modeling, drug screening, and cell replacement therapy, for which the successful reprogramming of somatic cells to iPSCs is the most fundamental step. By generating human-animal chimeras, 3D culture systems, and cerebral organoids with disease specific iPSCs and iPSC derived neural cells, researchers can have various systems for disease modeling and drug screening. By inducing disease relevant cell types into wild type (WT) iPSCs, researchers and clinicians can have normal cells for cell replacement therapy. Recently, the rapid development of genome editing technology, including TALEN, ZFN, and CRISPR, makes it possible for diseases with defined genetic disorders to switch phenotype between WT and mutant at the single cell level, providing a new strategy to create syngeneic WT cells for cell replacement therapy [103]. Overall, human iPSCs have a great potential for clinical applications in neurological diseases.

deficits, which would provide insight into new therapy targets or drug screening strategies [19–21, 23, 28, 30–33]. However, as promising and straightforward as it is, this strategy is also facing lots of challenges. First, the iPSC model is not an equal substitute for the ESC model. For example, studies of FXS-ESCs showed that FMR1 is unmethylated and expressed at a level close to the normal [34]. By contrast, FMR1 in iPSCs reprogrammed from somatic cells of FXS patients remains silent [6]. To elucidate this contradiction, further biological studies focusing on the difference between ESC and iPSC are needed. It is very likely that some key pathogenic mechanisms are hiding behind the subtle difference between iPSCs and ESCs. Another challenge is the variability between different patients or different clones derived from the same patient. For some diseases like SCZH, it is hard to determine whether the observed phenotype is really disease relevant or merely due to specific genetic background. Therefore, instead of just comparing one single iPSC line from one patient versus one control, it is indispensable to generate a panel of disease relevant iPSC lines and controls from different patients and normal people to overcome the bias attributed to the individual iPSC line variation. Last but not least, there are still lots of technical limitations to model the late-onset disease like PD. The manifestation of PD associated phenotypes requires environmental stimuli, such as progerin, to accelerate the pathological process. Thus, it would be of great help to define age-related markers which could be used to monitor the induced aging process to mimic the decade long aging process in several months [35].

The second strategy would be generating humanized animals with human iPSCs via neonatal mouse brain injection [36] and human mouse chimera [37, 38], which is a technologically conventional but ethically controversial strategy. It has been reported that undifferentiated human ESCs directly injected into the brain ventricles of fetal mice can integrate into the mice brain and form human neurons and glia [39].

Similar studies further support that stem cell injection, either human ESCs or human ESC/iPSC derivatives, has a great potential in cell replacement therapy [40–42]. For example, It has been reported that locomotor recovery was associated with engraftments of human neural progenitor cells (NPC) in the spinal cord-injured immunodeficient mice [42]. Albeit the underlying mechanisms for these phenomena remain mysterious, this modeling strategy is still the basis for studies involving cell replacement therapy. Of note, much less evidence has shown to generate humanized animals with direct iPSCs injection[43], which is probably due to the difficulty controlling in vivo differentiation of iPSCs precisely, suggesting iPSC derived NPC would be a better choice for cell replacement therapy.

The 3rd strategy would be establishing three-dimensional (3D) structured in vitro models that can emulate human homeostasis vividly. At present two-dimensional (2D) culture systems are the platform most often being used. However, these 2D systems cannot mimic the delicately structured in vivo environment. Instead, 3D culture system could emulate the complex in vivo environment in vitro by mimicking the highly organized cytoarchitectural features. First, the 3D culture system can achieve compartmentalization of different cell types. Different cells will reside in organized chambers to mimic the architectural features in real tissue niches [44, 45]. In addition, based on compartmentalization of cells, using special biomaterial that can create concentration gradients, researchers can better control delivery of biochemical compounds towards specific cells types, which mimics the delivery of signal cues in vivo. For instance, by utilizing a 3D micropatterned culture system, researchers could regulate synapse distribution via concentration gradients of neurotrophic factors and cell layer positions. It is observed that synergistic NGF/B27 gradients could increase synaptic density by stimulating growth of cortical neurons, which would be disturbed by homogenous B27 distribution, while cell layer positions could impact the spatial distribution of synapses [46]. Lastly, it should not be ignored that the combination of iPSCs and microfabrication technologies also holds great potential for tissue engineering. Engraftment of human iPSC derived NPC into nanofibrous tubular scaffolds resulting in nerve regeneration is a typical example [47]. In a word, not only can the 3D culture system give us highly organized in vitro models to study but also it enables us to control the cellular microenvironment more precisely.

Last but not least would be the newly developed exciting technology, the mini brain. In 2013, Austrian scientists Lancaster et al. reported successful generation of cerebral organoids, known as the mini brains, in a dish by culturing human iPSC in a fine-tuned 3D culture system. Basically, it comprises multiple discrete, albeit interdependent embryonic brain like regions. Despite lacking delicate organizations and structures that develop in vivo, such as the complete cortical lamination, this model represents a great leap towards modeling the most complex tissue, human brain. On the one hand, the cerebral organoids can reach up to 4 mm in size and can grow in vitro as long as 10 months with a simplified protocol using bioreactor. On the other hand, they are much more closer to embryonic brains compared to those generated via conventional protocols [48]. More importantly, they can recapitulate human cortical development features that mouse models cannot achieve, which is termed "the characteristic progenitor zone organization with abundant outer radial glial stem cells." Researchers further utilize this method to model a developmental disorder, microcephaly. Interestingly, this human iPSC derived mini brain can mimic severe phenotypes which cannot be manifested in mouse models carrying the same mutation [49]. Overall, it is very promising that in the near future more and more neurological diseases will have corresponding mini brain models to study. And mechanisms remaining mysterious now, especially those involving neurodevelopmental diseases, can be unveiled at that time.

3. Drug Screening Strategies and Challenges

Generally, the prevailing pharmaceutical pipeline is inefficient and expensive, whereas more than 90% of the drugs tested in clinical trials fail to be approved [50]. Furthermore, among those hit compounds selected through non-human iPSC platforms, not every candidate is effective on humans, and one of the most important reasons is the lack of faithful disease models. Since patients derived iPSCs can better emulate the real disease mechanisms, it is reasonable to postulate that the patients derived iPSCs based high throughput screens would be a better strategy for drug screening. For example, in the study using human iPSC derived dopaminergic neurons to evaluate candidate PD therapeutic agents, of the 44 hit compounds selected by rodent systems, only 16 were demonstrated therapeutic effects in the human PD model [18], suggesting the superior specificity conferred by the human iPSCs based platform. To establish highly efficient drug screen platforms, two issues are essential: the large-scale production of iPSCs or iPSCs derived neural cells as well as the definition of in vitro readouts suitable for high throughput assays [43]. A good example is a recent drug screening for compounds that can restore FMRP expression in FXS NPC. To adapt related experiments to be done in large scale, researchers first induced FXS iPSC to NPC which is easy manipulating and can rapidly grow in monolayer. Next, FMRP translation level was defined as the readout and a sensitive and quantitative TR-FRET-based FMRP assay was developed, in which FMRP would be detected by a pair of FMRP antibodies, one labeled with a donor dye and the other labeled with an acceptor dye. Briefly, NPC was first seeded into a 1536-well plate, followed by stimulation of chemical libraries comprising 1280 drugs. After that cells were lysed and incubated with the pair of FMRP antibodies. Fluorescence signals given by positive wells with FMRP restoration would be detected and analyzed by the plate reader. In this way, 6 hits were identified and 4 of them were confirmed by secondary qPCR assays [51]. Similar screening platforms have also been applied in other neurological diseases, such as familial dysautonomia [52] and spinal muscular atrophy (SMA) type I [53]. Taken together, this screening strategy is more time saving and less expensive as compared to those traditional ways.

Aside from evaluation of therapeutic effects of a large number of chemical compounds, human iPSC based

platforms can also be used to assess cell type specific off-target effects and toxicities. For instance, inducing human iPSCs to hepatocytes and cardiac myocytes provides the opportunity to assess whether compounds of interest have true hepatotoxicity and cardiotoxicity [50]. Moreover, by screening compounds on a cell panel comprising iPSC lines or iPSC derived affected cell types from different patients, compounds that are only effective on certain patients, either with specific genetic profiles or with disease subtypes, can be identified [43]. In this sense, the iPSC drug screening strategy will help narrow target patient populations, contributing to the individualized medicine in the future.

4. Cell Replacement Strategies and Challenges

The most exciting potential of human iPSCs would be the cell replacement therapy. The hematopoietic stem cells have already been used in clinic to treat disease such as multiple myeloma and leukemia [54]. Recently, Japanese scientists applied human iPSCs in curing ophthalmological diseases in real patients [55]. It has also been well envisioned that in the near future we can have human iPSC derived organs for transplantation surgery [56]. Human iPSCs and iPSC derivatives have been proven to have therapeutic potentials in neurological diseases including spinal cord injury [42, 57], Huntington disease [58], and PD [59]. However, exciting as these results were, safety issues such as tumor formation and inappropriate localization of transplanted cells were also reported. Therefore, to achieve cell replacement therapy in real clinical settings, strict standards should be set to ensure quality control of clinic grade human iPSCs. Below we will discuss each of them briefly.

4.1. Integration-Free Reprogramming Methods. There has been the concern that virus vector mediated reprogramming may introduce unwanted insertion of vector fragments into the iPSC genome, which may affect the biological properties of the derived iPSCs and even induce malignant transformation, rendering the iPSC unsafe for clinic use. To solve this, researchers have been working on integration-free reprogramming methods to get safer iPSCs. A good example is the lentiviral vector plus Cre strategy, in this way, the reprogramming vectors flanking by loxP sites will be excised by Cre-recombinase transfected transiently [4]. But this is labor and time consuming and vector DNA external to the loxP sites may still remain integrated. Other approaches include piggyBac transposon [60, 61], episomal vectors [62], adenoviral vectors [63], sendai vectors [64], protein transfection [65, 66], nucleofection [67], and small molecules delivery [68, 69]. Although they can achieve the integration-free purpose, they share the same limitation which is the extremely low reprogramming efficiency compared to lentiviral vectors. Progress has been made in the significant enhancement of iPSC generation efficiency by Chen and Jin group, in which the transactivation domain of the Yes-associated protein is fused to the defined transcription factors, Oct4, Sox2, Nanog, and Klf4 (OSNK), to establish a new reprogramming system OySyNyK which can initiate rapidly within 24 h with up to 100-fold higher efficiency compared to the conventional OSNK system [70]. Hopefully progress in such studies can finally pave the way for producing the real clean and safe iPSCs efficiently.

4.2. Tumorigenicity. Tumorigenicity refers to the ability of iPSCs or iPSC derived cells to form tumors after being transplanted into hosts, which is often evaluated by the teratoma formation propensity assay. Up to date it remains mysterious how teratoma forms in transplantation settings using syngeneic iPSCs or iPSC derivatives. The first reason is that the cell source giving rise to the teratoma is unclear. One possible hypothesis is that undifferentiated iPSCs continually present within the iPSC derived progenitor cells or terminally differentiated tissues are the source of teratoma initiation [71, 72]. Another important reason is that the risk factors affecting teratoma formation are versatile. The first risk factor is the retroviral transgene integration. A major concern is that off-target integration of transgene elements and dysregulation of stem cell programs may cause aberrant tumor related genes expression [73, 74]. Moreover, reactivation or incomplete suppression of retroviral transgenes has been thought to be correlated with increased tumorigenicity. In support, transplantation of secondary neurospheres obtained from a partially reprogrammed iPSC line into the brains of immunodeficient mutant mice results in robust teratoma formation due to the incomplete suppression of transgenes encoding Oct4, Sox2, Klf4, and c-Myc [71]. Optimistically, this concern may be eliminated by using integration-free methods for reprogramming as mentioned above. Another factor that may play a role is the somatic tissue origin. Miura group reported that secondary neurospheres from murine iPSCs reprogrammed from different adult mouse tissues varied substantially in their teratoma-forming propensity. For example, secondary neurospheres derived from iPSCs generated from adult tail tip fibroblasts showed significantly higher tumorigenicity compared to those generated from mouse embryonic fibroblasts [75]. However, this could also be caused by the fact that different tissues have different mutation accumulation, epigenetic memory, and age. More evidence is needed to support this view. If somatic tissue origin really contributes to tumorigenicity, careful selection of original somatic tissue for reprogramming should be considered as a major standard in clinical application, because this will affect what the patient will suffer from at the very beginning, an invasive surgery or just pulling out a single hair. Overall, all evidence shown above is from mice. Since tumor formation mechanism is complex and could be species specific, more evidence from primates or human patients is needed.

Although mechanisms underlying tumorigenicity remain unknown, clinical trials using autologous patient derived iPSCs are already underway marked by the Takahashi group using patient specific iPSC derived retinal pigment epithelium (RPE) cells for the treatment of wet type age related macular degeneration. Notably, in the preclinical study they evaluated the tumorigenicity of patient iPSC derived RPE using immunodeficient rodents. By transplanting iPSC derived RPE cells into 65 nonobese diabetic mice subcutaneously and into the subretinal space of 26 nude rats they concluded that the tumorigenic potential of the patient iPSC derived RPE

cells is negligible, for no tumors were found in the following 6–15 months of monitoring [76]. However, it is still risky to state that the iPSC derived RPE cells which are safe for rodents are also safe for human. Thus, strict preclinical evaluation and posttransplantation monitoring should be mandatory to ensure clinical safety. For example, primate models would be a more reliable system compared to rodent models for tumorigenicity assessment [77]. Positive selection of differentiated cells without contamination of undifferentiated cells before the transplantation surgery may also help reduce the risk of tumorigenicity [78]. A more fundamental and ideal solution for the posttransplantation setting would be the prodrug strategy or suicide genes controlled by inducible promoters [79]. By integrating a gene coding for enzymes that can convert prodrug to toxins or a suicide gene into the cells to be transplanted, researchers and clinicians can eliminate the transplanted cells easily just by administrating corresponding drugs when there is tumorigenic event ongoing [80, 81]. However, given all the technical limitations in the way, applying these strategies in real clinical cases still has a long way to go.

4.3. Immunogenicity. Another major concern for clinical transplantation of iPSCs is the immunogenicity. Whether syngeneic iPSC is immunogenic remains controversial. The debate was stimulated by a study in 2011 reporting unexpected immune rejections to teratomas derived from syngeneic murine iPSCs. Before that people believed that syngeneic iPSCs or iPSC derived cells are self-tolerant, which is a perfect autologous transplantation source. However, in this study, it is indicated that iPSC derived teratomas have significant higher immune rejection responses than the ESC derived counterparts. The B6 mice recipients receiving syngeneic ESCs transplantation showed efficient teratomas formation with negligible rejection, while those receiving syngeneic iPSCs transplantation, whether reprogrammed from mouse embryonic fibroblasts using retroviral transduction or episomal method, showed robust immune rejection evidence including failure of teratomas formation, T cell infiltration, and tissue damage [73]. In contrast to this study, other two later studies exploring differentiated cells, more therapeutically relevant, showed the opposite results. The first study showed negligible immune rejection of terminally differentiated cells of skin grafts and bone marrow, which are derived from chimeric iPSC or ESC derived mice [82]. The other study observed no immune rejections of terminally differentiated cells from both iPSCs and ESCs representing three germ layers by assessing immunogenicity in vitro and after their transplantation into autologous recipients [83]. Both of the two studies optimistically came to the same conclusion that syngeneic iPSCs or iPSC derivatives are the safe source for cell replacement therapy. Whether such variability between studies is due to the difference between different ESCs and iPSCs lines is unknown yet. Interestingly, a recent study indicated that the differentiation of iPSCs may result in a loss of immunogenicity and the induction of self-tolerant immune response [84], which helped explain the discrepancy of the studies discussed above. Moreover, another recent study of Zhao group reported differential immunogenicity of differentiated cells from syngeneic iPSCs in a humanized mouse model with a functional human immune system, where the iPSC derived smooth muscle cell is highly immunogenic while the iPSC derived retinal epithelial cell is tolerant, though direct transplantation of iPSCs is still immunogenic as their previous results [85]. So far it is difficult to form a hypothesis to explain different phenomena observed by different study groups due to poor understanding of underlying mechanisms. However, the consensus is that improvement in reprogramming technology and insight into the underlying mechanisms is needed for generating clinical grade iPSCs. Similar to tumorigenicity, evaluation of immunogenicity prior to transplantation should also be mandatory.

4.4. Promising Preclinical Trials on Animal Models. The first in human clinical trials using stem cell products for neurological diseases is the one launched by Geron in 2009, aiming at remyelinating denuded axons in patients of thoracic level spinal injury with human ESC derived oligodendrocytes which have been preclinically proven to be safe and effective [86]. However, due to financial concerns, this trial was terminated 2 years later [87]. Up to date, no such clinical trials using iPSC products have been reported; however, types of neurological diseases with preclinical data supporting clinical trials using iPSC products are increasing, including PD [88], Huntington's disease [89], amyotrophic lateral sclerosis [90], and SMA [91]. Among them, PD is probably the most promising neurodegenerative disease for cell replacement therapy trial, because it is mainly caused by the loss of midbrain dopaminergic neurons. As a proof of principle, transplantation of midbrain dopamine neurons could be an effective treatment by covering the shortage. Although dopaminergic reagents have been proven to be an effective treatment strategy, it is already recognized that the curative effect of the medications would be compromised due to adverse effects over time. As the logical next step, cell replacement therapy is viewed as a better alternative by scientists, with the potentials of overcoming limitations of medications, such as off-target effect and nonphysiological delivery of dopamine [92]. Clinical trials using fetal brain tissue transplantation and ESC derived neurons for PD have come into reality, highlighted by the "TRANSEURO" and "GForce-PD initiative" programs [92, 93]. However, given the safety issues discussed above, iPSCs and iPSC derivatives based cell therapy are still at the preclinical stage. Cell replacement therapy using iPSC derived neural cells has been trialed on rodent and non-human primate PD models with encouraging outcomes. For example, after transplantation rodent PD models all showed alleviated behavioral phenotypes, such as reduced amphetamine-induced rotations with no obvious side effects. Of note, in the non-human primate, a cynomolgus macaque PD model, autologous iPSC derived neural cells not only elicited motor improvement but also survived 2 years after the transplantation without any immunosuppression therapy [59, 88, 94–97].

As impressive as the preclinical data is, it is still too hasty to state that clinical trials using iPSC products are ready to go. A mature transplantation protocol for PD treatment should not only address the safety issues discussed above but also

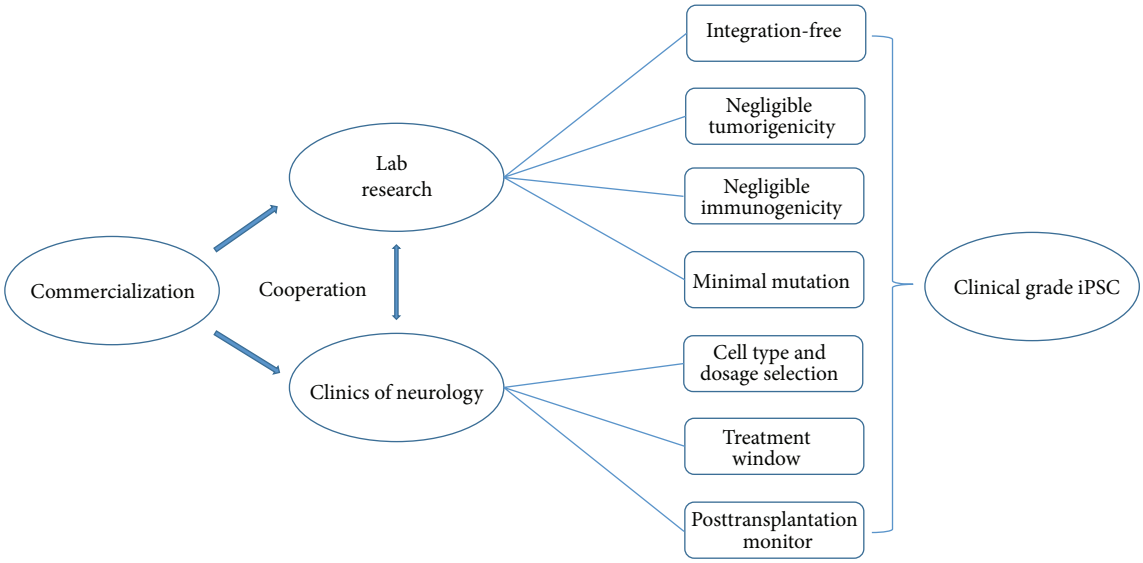

Figure 2: A strategy map towards clinical grade iPSCs. Strict standards should be set to ensure the safety of cell replacement therapy using iPSC products. These standards for clinical grade iPSCs must cover but are not limited to generation of iPSCs with minimal mutation, assessment for tumorigenicity and immunogenicity, cell type and dosage selection, length of treatment window, and posttranslation monitoring, which requires the cooperation of lab researchers, clinicians, and business industry.

refine an optimal differentiation protocol to ensure proper cell identities and long-term functions [98–100]. Hopefully, lessons and progress learned from clinical trials using iPSC products in other diseases can lend some useful experience [55, 86].

5. Concluding Remarks

To make human iPSCs applicable in drug screening and cell replacement therapy, aside from issues discussed above, other factors should also be taken into consideration to establish a comprehensive and multifaceted clinical standards. In some cases, therapeutists may require the use of genetically corrected syngeneic cells for transplantation; therefore, other than tumorigenicity and immunogenicity assessment related to the reprogramming process, clonal iPSC lines or iPSC derived cell lines after genome modifications should be further assayed carefully to isolate a pure line with minimal mutation to ensure safety. In addition, more efficient methods for reprogramming, colony isolation, and validation should be exploited to achieve scalable and robust biomanufacturing of iPSCs, which will not only promote the development of the drug screening industry but also facilitate collaborations across nations and laboratories. When iPSCs of consistent high quality and comparability can be generated by different institutes at desired scales flexibly, it is the time to establish a worldwide repository, where every individual could find the human leukocyte antigen (HLA) matched cells for transplantation [101]. Finally, the importance of cooperation between clinicians and researchers should not be overlooked. For the monitor markers, cell dosage for transplantation, specific surgery site, and treatment windows should be determined based on both the data from bench and the bedside medical records (Figure 2). Although lots of hurdles remain to be addressed, the good news is that researchers are not held back by these difficulties; their bold ambitions and endeavor recently yielded a guidance published by US food and drug administration, entitled "Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products" [102].

As a summary, undoubtedly the ultimate goal for human iPSCs studies is to provide new insight into diagnosis and therapeutics in real patients. As more knowledge is being accumulated, it is believable that commercialization of clinical grade iPSCs will come into reality in the future.

Conflict of Interests

The authors have declared that there is no conflict of interests.

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Review Article

Induced Pluripotent Stem Cells: Generation Strategy and Epigenetic Mystery behind Reprogramming

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Possessing the ability of self-renewal with immortalization and potential for differentiation into different cell types, stem cells, particularly embryonic stem cells (ESC), have attracted significant attention since their discovery. As ESC research has played an essential role in developing our understanding of the mechanisms underlying reproduction, development, and cell (de)differentiation, significant efforts have been made in the biomedical study of ESC in recent decades. However, such studies of ESC have been hampered by the ethical issues and technological challenges surrounding them, therefore dramatically inhibiting the potential applications of ESC in basic biomedical studies and clinical medicine. Induced pluripotent stem cells (iPSCs), generated from the reprogrammed somatic cells, share similar characteristics including but not limited to the morphology and growth of ESC, self-renewal, and potential differentiation into various cell types. The discovery of the iPSC, unhindered by the aforementioned limitations of ESC, introduces a viable alternative to ESC. More importantly, the applications of iPSC in the development of disease models such as neurodegenerative disorders greatly enhance our understanding of the pathogenesis of such diseases and also facilitate the development of clinical therapeutic strategies using iPSC generated from patient somatic cells to avoid an immune rejection. In this review, we highlight the advances in iPSCs generation methods as well as the mechanisms behind their reprogramming. We also discuss future perspectives for the development of iPSC generation methods with higher efficiency and safety.

1. Introduction

Due to their characteristic pluripotency, stem cells have the capacity to unveil the mystery behind reproduction, regeneration, and (de)differentiation, rendering stem cell—in particular, embryonic stem cell (ESC)—research essential for the development of a fundamental understanding of biomedical mechanisms and the discovery of clinical therapeutic strategies [1]. However, stem cell research has suffered setbacks due to ethical controversy, resource limitation, and technological barriers, hindering its biomedical research and clinical applications for regeneration medicine and therapy. To overcome these limitations, biologically similar alternatives that can bypass the ethical issues surrounding stem cells are essential. Significant efforts in this regard have led to the generation of induced pluripotent stem cells, an important advancement in biomedical research. Specifically, iPSC has been applied for development of disease models for neurodegenerative disorders amongst others, greatly enhancing our understanding of the pathogenesis of such diseases, as well as allowing for the development of clinical therapeutic strategies using iPSC from patient somatic cells. As such, the research advances in neurodegenerative disease models have been well reviewed [2–5].

iPSC was initially generated by reactivating nuclear reprogramming factors to reverse differentiated cells into a reprogramming state [6–8], maintaining the abilities of self-renewal and potential differentiation into various cell types. iPSC, like ESCs, can differentiate into nearly all the cell types in the organism from which they originated, shedding light on cell-based therapies and regenerative medicine to which patient-specific iPSC could be applied in order to regenerate

tissues or organs destroyed by injury, degenerative diseases, aging, or cancer while avoiding rejection by the host's immune system. This method is undoubtedly a milestone for stem cell research, as iPSC has been and will continue to be the primary substitute for or perhaps even surpass ESCs in their ability to serve as a tool to uncover the mystery behind differentiation.

Although an increasing number of groups thereafter have made significant efforts in the generation of iPSC from a variety of somatic cell populations, available information about the genome-wide epigenetic alterations that somatic cells must undergo to become fully reprogrammed remains limited. In addition, some concerns about the current procedures, particularly the insufficient efficiency and specificity required for clinical application, remain. Thus, a better understanding of the downstream events following the activation of silenced master reprogramming factors could provide essential information to aid in the development of patient-specific iPSC lines in a faster and safer way. In this review, recent advances in iPSC generation strategies and the detailed mechanisms that underlie reprogramming are highlighted, and future perspectives are discussed.

2. Technological Advances in iPSC Generation

In addition to efficiency and specificity concerns with regard to iPSC generation methods, there has been a concern over the virus based reprogramming as it may integrate unwanted vector fragments into iPSC genome, given that the Yamanaka factors such as Oct4, Sox2, Myc, and Klf4 (OSMK) are introduced into the fibroblast cells with the help of a virus. This would affect the clinical application of derived iPSC as it introduces the possibility of negative effects on the biological properties of iPSC and increases the likelihood of malignant transformation. Indeed, recent study showed that reactivation of viral genes integrated in host genome during differentiation of the reprogrammed iPSC leads to tumorigenesis [9]. To overcome the shortcomings conferred by the traditional methods, efforts have been made to address the efficiency and safety issues as described below.

2.1. Epigenetic Operation. To tackle the problem of low efficiency, chemical as well as epigenetic approaches have been adopted with the aim of enhancing iPSC generation efficiency [10–12]. Epigenetic regulations drive the reprogramming of histone methylation and acetylation levels. As some histone methyltransferases have been acknowledged to play significant roles in the inhibition of reprogramming efficiency via methylation, it is logical to speculate that repression of histone methyltransferase expression or inhibition of its activities would enhance reprogramming efficiency. Indeed, shRNA-based knockdown of H3K9 histone methyltransferase DOT1L, leading to significant elevation of NANOG and LIN28 levels at an early stage of reprogramming, dramatically promotes the generation of iPSC colonies even without the overexpression of KLF4 and c-Myc [13]. A related mechanism is DOT1L repression-mediated loss of H3K9me2 in genes involved in mesenchymal to epithelial transition (MET). Accordingly, independent studies show that the overexpression of demethylase Kdm4b, along with a deficiency in H3K9 methyltransferases Ehmt1, Ehmt2, and Setdb1, or heterochromatin protein-1γ (Cbx3), a protein known to recognize H3K9 methylation, could significantly promote reprogramming [14, 15].

Paradoxically, JMJD3, a histone H3K27 demethylase [16] expected to enhance reprogramming efficiency, instead represses reprogramming [17] through two potential pathways. The first is demethylase dependent; by increasing the demethylation of H3K27me3 at Ink4a/Arf loci, JMJD3 elevates expression levels [18]. The importance of this is well evidenced by the fact that knockdown or deletion of Ink4a/Arf drastically increases reprogramming efficiency. The second potential pathway is demethylase-independent degradation and ubiquitination of PHF20, which is required for reprogramming [17, 19].

Changes affecting the dynamic balance between acetylation and deacetylation may also affect reprogramming, as is evidenced by the effects of several core members of nucleosome remodeling and deacetylation (NuRD) repressor complexes on reprogramming efficiency. Serving as a core component in Methyl-CpG Binding Domain Protein 3 (Mbd3) NuRD repressor complexes, Mbd3 can interact with core reprogramming factors (OSKM) and assemble directly with Mbd3/NuRD to recruit the repressor complex to downstream OSKM target genes. As would be expected, Mbd3 depletion was capable of significantly enhancing the reprogramming efficiency of human and mouse fibroblast cells to near 100% within seven days [20], with the only concern being the quality of the induced iPSCs in the absence of Mbd3. It has been acknowledged that protein kinases make significant contributions to signal transduction in eukaryotic cells [21], suggesting their potential role in regulating somatic reprogramming. To this end, kinome-wide RNAi-based screening has been performed to identify the specific protein kinases that regulate reprogramming efficiency [22]. Among the 59 of kinases serving as potential barriers to reprogramming, serine/threonine kinases TESK1 and LIMK2 have been further tested to confirm their roles in MET during mouse embryonic fibroblast (MEF) reprogramming. Furthermore, TESK1 deficiency in human fibroblasts could significantly enhance reprogramming efficiency [22].

2.2. MicroRNA Manipulation. MicroRNA has been acknowledged to function as essential regulators for gene expression in almost all metabolic pathways, suggesting their potential involvement in the regulation of the nuclear reprogramming (Figure 3), and providing insight into means of enhancing reprogramming efficiency by alteration of miRNA expression levels. Certain microRNA, such as miR29b, directly target mRNA coding for several enzymes responsible for the methylation of cytosine (C) and demethylation of 5-methylcytosine (5-mC), mediated by 5-hydroxymethylcytosine (5-hmC) [23]. As a balance of 5-mC and 5-hmC has been essentially linked to somatic reprogramming [24], this suggests the regulatory functions of miR-29b in this regard. The miR-290–295 clusters, 2.2-kb region on chromosome 7 [25], constituting over 70% of the entire miRNA population in mouse ESCs and the most abundant miRNA family in ESCs,

have been believed to be important regulators for the ESC-specific cell cycle. The miRNA members in the cluster such as miR-291-3p, miR-294, and miR-295 had the capacity to enhance Klf4-, Oct4-, and Sox2-mediated reprogramming efficiency, although they were unable to further promote pluripotency efficiency in the presence of cMyc. Further study shows that the miRNA is downstream effector of cMyc [26]. More excitingly, overexpression of the miR302/367 cluster alone could enhance the reprogramming of mouse and human somatic cells to an iPSC state much more rapidly and efficiently than endogenous overexpression of the master transcription factors Oct4/Sox2/Klf4/Myc. iPSCs generated from mouse and human somatic cells via the overexpression of miR302/367 display similar characteristics to the ones from the conventional reprogramming factors, from pluripotency marking to teratoma formation [27]. More and more miRNA which regulated reprogramming had been identified, for example, three miRNA clusters, miR-17~92, miR-106b~25, and miR-106a~363, which have the ability to significantly enhance the induction efficiency at early reprogramming stages. Furthermore, miR-93 and miR-106b share very similar seed regions and dramatically promote iPSC induction, resulting in mesenchymal to epithelial transition (MET) at the initiation stage of reprogramming. More interesting is the capability of these miRNA-mediated iPSC clones to reach a fully reprogrammed state. Further study shows that the miRNA functions as reprogramming enhancer by targeting p21 and TGF-β receptor II, as is evidenced by the fact that siRNA based knockdown of both targets significantly increases iPSC induction efficiency. Another mechanism for the enhancement of miRNA based reprogramming efficiency is the regulation of cell cycle-related genes [28, 29].

2.3. Activation of Core Factors for Reprogramming. Although the native forms of core factors have been widely employed in iPSC generation, their relatively low transactivation activity remains a barrier for somatic cell reprogramming [30]. Recent studies have shown that the modification of OCT4, SOX2, and NANOG provides a new approach to overcoming these barriers [30, 31]. The yes-associated protein (YAP) has been demonstrated to be a transcriptional coactivator with a potent transactivation domain (TAD) in the C-terminal region; ectopic expression of YAP promotes cell growth and induces tumor formation [19, 32]. In addition, YAP also plays a critical role in the maintenance of stem cell pluripotency [33].

To enhance iPSC generation efficiency, the Oct4, Sox2, Nanog, and Klf4 (OSNK) reprogramming factors were engineered such that the transactivation domain of YAP is fused to defined factors labeled as OySyNyK. The efficiency of OySyNyK-induced iPSC generation is dramatically enhanced due to these modifications (about 100-fold greater efficiency relative to that of the wild-type OSNK-induced iPSCs). Furthermore, the initiation of reprogramming by OySyNyK is much faster, usually occurring within 24 hours. To understand the mechanism underlying this enhanced reprogramming, an epigenetic study was performed, the results of which indicated that the engineered reprogramming factors significantly increase the expression level of one member, namely, Tet1, of the ten-eleven translocation proteins (TETs, Tet1, Tet2, and Tet3 in the genome of mammalian cells) at the early reprogramming stage and also produce a marked increase in 5-hydroxymethylcytosine (5-hmC) levels, collectively suggesting that the engineered reprogramming factors collaborate with TETs to regulate 5-hmC mediated epigenetic control of somatic reprogramming [12].

2.4. Elimination of the Unwanted Virus Vector Parts in iPSCs. The integration of unwanted vector fragments into the iPSC genome can adversely affect the clinical applications of iPSCs in therapy. Thus, the improvement of nonviral and integration-free alternative methods to eradicate the safety issues currently associated with iPSCs has been a goal since the early stages of iPSC development.

2.4.1. Complete Removal of the Viral Vector in Cell Reprogramming. The initial approach for the removal of the unwanted viral vector was to combine a lentiviral vector with Cre to excise the reprogramming vectors flanked by loxP sites using transiently expressed Cre-recombinase. Although a large part of the lentiviral vector flanked by loxP sites can be removed, a small part of the vector DNA external to the loxP sites most probably still remains integrated. In addition, this strategy is difficult to operate and time consuming.

In another attempt to rectify this issue of viral vector integration, the PiggyBac (PB) transposon system has been used to efficiently integrate the construct harboring core factor genes into TTAA sites in the target genome. The inserted PiggyBac vector can be excised by PB transposase in a footprint-free removal [34–36].

2.4.2. Nonviral Methods. Nonviral methods have also been developed, such as the utilization of episomal and minicircle vectors [37, 38], adenoviral vectors [39], and Sendai vectors [40–42], and these special nonviral vectors have been demonstrated to enhance the reprogramming efficiency without introduction of viral components into the iPSC genome. As episomal DNA vectors with smaller molecular size free of bacterial plasmid DNA backbone, minicircles are designed for circular expression cassettes by significantly enhancing the transfection efficiency and offering over a period of weeks expression instead of only for several days conferred by standard plasmid vectors. Since the sequences within the bacterial plasmid backbone harbor the signals for methylation and transgene silencing, the minicircles based transfections can overcome the short period expression conferred by traditional transient transfections of plasmids.

Compared to other virus vectors such as lentivirus vectors, adenovirus vectors possess many advantages such as conferring the very efficient nuclear entry and low pathogenicity for humans, transducing large genes of more than 30 kb, avoiding integration into the host cell genome, targeting cell specificity, and maintaining long-term expression of transgenes. As such, the adenovirus vectors have been developed as popular gene delivery vehicles in a wide range of transduction for different cell types, particularly for quiescent and differentiated cells in basic biomedical research, clinical

applications such as gene therapy, and industrial applications such as vaccine development.

Different from all the conventional DNA vectors that so far have been extensively applied, Sendai virus (SeV) vector is a cytoplasmic RNA vector with RNA genome, a material chemically different from the patient's genome DNA. Thus, since the SeV vector replicates its genome exclusively in the cytoplasm instead of entering cell nucleus, it overcomes the fundamental risks in host cell chromosomal alteration caused by integration of DNA vectors into chromosomes or genetic recombination. Additionally, the SeV vector could produce protein in large quantity in the host cells. Given so many advantages over the conventional DNA vectors, SeV has been successfully applied in clinical therapy as well as basic biological research including iPSC generation.

To absolutely exclude virus vectors, electroporation of the constructs with nucleofection into somatic cells has become an alternative also [43].

2.4.3. Integration-Free Method: Modified mRNA Strategy. This strategy is based on the administration of engineered mRNA coding for the core reprogramming factors (ONSMK) to avoid innate antiviral responses and has been proven to be significantly more efficient compared to the previously established protocols in reprogramming human fibroblast cells to generate RNA-induced pluripotent stem cells (RiPSCs) [44]. In addition to fibroblast cells, this technique has also been applied to iPSC generation from bone marrow-derived mesenchymal stromal cells (BM-MSCs). However, although this strategy bears an advantage in that it avoids involving transgenes, its low efficiency remains a big concern.

2.4.4. DNA/RNA-Free Strategy. Besides the methods utilizing engineered mRNA (Section 2.4.3) and small molecules as described in Section 2.4, integration-free strategies, such as the treatment of somatic cells with purified core reprogramming factor proteins, significantly enhance reprogramming efficiency [45, 46].

2.5. Chemical Approach to Improving Reprogramming Efficiency. Theoretically, any molecules that target core epigenetic enzymes to alter the dynamic balance of methylation/demethylation could be potential candidates for enhancing or inhibiting somatic reprogramming efficiency (Figure 3). As expected, several small molecules have been identified to function as inhibitors of histone demethylases, such as BIX-01294, RG108, parnate, 5-azacytidine, or 3 histone deacetylase inhibitors (suberoylanilide hydroxamic acid, trichostatin A, and valproic acid). These compounds enhance reprogramming efficiency either individually or in collaboration with the transduction of certain core reprogramming factors by reducing the methylation level of H3K9mono-Me, H3K9di-Me, or the L-calcium channel agonist Bayk8644 [47–54]. In particular, the incomplete epigenetic reprogramming attributed to the epigenetic memory of original cell-type-specific genes may contribute to the subtle difference between iPSCs and ESCs and even among iPSC clones [55–61], leading to low quality iPSCs with limited clinical applications. In order to elevate iPSC quality, epigenetic memory must be largely erased, where 5-azacytidine and trichostatin A could efficiently function as erasers [58]. However, possible off-target effects may lower the viability of iPSC treatment with epigenetic memory erasers.

Altogether, recent studies suggest that small molecules which function to alter the dynamic balance between methylation and demethylation—either individually or collaboratively—could significantly enhance reprogramming and largely erase epigenetic memory in cell-specific genes retained in iPSCs, leading to a substantial improvement in the quality of reprogrammed iPSCs [58, 60, 61]. The advantage of the small molecule-based reprogramming is that no genetic engineering is necessary, avoiding the integration of the unwanted virus vector sequences as well as the side effects caused by transgenes.

However, its shortcomings cannot be ignored, one of which is that the off-target effects may adversely affect quality of the iPSCs generated.

In summary, methods have been developed to remove the unwanted virus vector sequences or exclude use of viruses altogether in virus-free and integration- or transgene-free methods. However, although integration-free methods are within the realm of possibility, these strategies still share the same shortcomings, one of which is extremely low reprogramming efficiency relative to lentiviral vector-mediated strategies. One more issue needs attention for comparison of the reprogramming efficiency mediated by modified mRNA and viral vector strategies such as lentiviral vector. Although the modified mRNA confers the lower induction efficiency relative to the viral vector, the percentage of the normal iPSC generated by this strategy is higher than that by the lentiviral vector due to immune response as well as other adverse factors such as genome instability and chromosomal variation caused by viral vector integration into the chromosome of the iPSC.

2.6. Automated, High-Throughput Derivation, Characterization, and Differentiation of iPSCs. For large scale generation and further differentiation of iPSCs into special tissues to be used in regeneration medicine for therapeutic purposes, an automatic platform was established starting from fibroblast cell preparation [62]. Since this strategy is a robot-based platform combining many related protocols for cell isolation, culture, distribution, induction of reprogramming with modified mRNA delivery, and differentiation of iPSCs, very limited manual intervention was employed. It was proven that using this platform high-quality and stable iPSCs could be induced with less line-to-line variation than is found in those generated via conventional strategies. Although this combined automatic platform would significantly contribute to iPSC-based regeneration medicine in the long run, high demands with regard to the equipment necessary hamper its application at present.

3. Mechanisms behind Somatic Reprogramming

3.1. Epigenetic Regulation at the Chromatin Level. Not all cells that gain expression of the core reprogramming factors

are pluripotent, though they may be self-renewing, because some of them are trapped in a state of partial reprogramming [47, 48, 63]. To understand the barriers to reprogramming, epigenetic identification was carried out to determine levels of histone and DNA modification in partially reprogrammed cells, fully reprogrammed cells, ESCs, and starting somatic cells. Epigenetic marks were found to be altered genome-wide, leading to reactivation of the core pluripotency genes and large scale 5-mC demethylation [49]. When the somatic cells are treated with inhibitors of HDACs, DNMTs, and the G9a methyltransferase, reprogramming efficiency is significantly enhanced [47, 64–66], suggesting the regulatory role of epigenetic modifiers in reprogramming. Indeed, many of the epigenetic regulators are directly recruited by the reprogramming factors to stimulate the expression of downstream pluripotency genes during iPSC generation, and chromatin remodelers serve as the key components of the interactome between the epigenetic regulators, core factors, and pluripotency genes. Some modulators, such as Smarca4/Brg1 and Smarcc1/BAF155 AS, the ESC-specific BAF (esBAF) components, INO80, Wdr5, and Mbd3, can physically interact with or be recruited by some or all of the OSKM master factors, enhancing the binding of these master reprogramming factors to promoters, leading to either an increase of activation-associated markers H3K4me3 and H3K9 acetylation (H3K9ac) at target genes, or the recruitment of RNA polymerase II to promote the expression of their target genes [67–75].

Modifications of both histones and genomic DNA have been essentially linked to the regulation of master transcription factor-mediated reprogramming. At the histone level, a balance between the enzymes responsible for methylation and those responsible for demethylation, such as methyltransferases and demethylases, determines the global H3K9me3 levels during reprogramming. Genome regions occupied by H3K9me3, a heterochromatic histone mark, have been identified to efficiently prevent the binding of master transcription factors in both human and mouse fibroblasts, serving as major roadblocks during reprogramming [76–78].

Accordingly, Cbx3, a reader of H3K9me3, and H3K9 methyltransferases (Ehmt1 and Ehmt2) have been detected in the Nanog protein complexes in mouse ESCs, suggesting that the Nanog autorepression mechanism is mediated by the recruitment of these readers and methyltransferases by Nanog in the cells remaining in a nonreprogramming state [78–80].

3.2. Epigenetic Regulation at the Genomic DNA Level. At the genomic DNA level is a situation similar to that at the chromatin level—a balance between DNA methylation and demethylation determines the dynamic status of the somatic cell, leading it towards either reprogramming or remaining differentiated. Again, this balance is also regulated by DNA methyltransferases and demethylases. The demethylation of the promoter regions of the genes conferring pluripotency has become a prerequisite for epigenetic somatic reprogramming [81] and most probably occurs during posthistone modification [82].

More and more evidence is available to contribute to an understanding of the mechanisms of passive and active DNA demethylation. However, these two mechanisms do not contribute equally to the dynamic regulation of demethylation. The passive mechanism renders the automatic and gradual loss of methylation during cell cycles possible. In contrast, several enzymes are believed to be involved in the active mechanisms for demethylation, including activation-induced cytidine deaminase (AID) [83], TET [84], and thymine DNA glycosylase (TDG) [12, 24, 85–87]. AID is responsible for the deamination of 5-mC to thymine, leading to cytosine exchange and demethylation and thus subjecting it to DNA repair pathways. However, the contribution of AID-mediated active demethylation to nuclear reprogramming remains controversial due to inconsistent experimental results [83, 88, 89].

Probably the most important mechanism for active DNA demethylation during induced pluripotency is the conversion of methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) catalyzed by TET family (TET1, TET2, and TET3 in mammals). Once generated, the 5-hmC has to face several fates, either being directly converted into cytosine (C) through mechanisms involving the base excision repair pathway or sequentially becoming 5-fC and 5-caC to be finally converted into regular cytosine [90] (Figure 1).

Tet1 and Tet2 have been proven to facilitate cell reprogramming [84, 91]. To understand the roles that TET1 and TET2 play during somatic reprogramming, coimmunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP) using antibodies raised against the TETs and master reprogramming factors were conducted. The results showed that TET1 and TET2 physically interact with NANOG, SOX2, and OCT4 at the protein level and that the master reprogramming factors such as NANOG, SOX2, and OCT4 recruit TET1 and TET2 to the key target genes to oxidize 5-mC to 5-hmC and then to cytosine either directly or indirectly [12, 84, 85].

Although knockdown of all three Tet genes in ESCs seems to confer ESCs normal self-renewal and pluripotency, deletion of Tet1 slightly enhances the reprogramming efficiency [92] and Tet3 deficiency had little effect, in contrast to the fact that inactivation of Tet2 reduced the reprogramming by 70%. While the double knockout of both Tet1 and Tet2 or Tet1 and Tet3 still does not significantly affect the reprogramming efficiency marked by the amount of the colonies with positive AP and SSEA1, deficiency of all three Tet genes completely abolished the reprogramming potential of MEFs. Further studies show that deletion of Tet2 from the Tet1 and Tet3 double knockout (DKO) or deficiency of Tet3 from Tet1 and Tet2 double KO MEFs completely inhibits the reprogramming, suggesting that the reprogramming deficiency of TKO MEFs could not be ascribed to inherent genomic or epigenomic alterations potentially arisen from the constitutive Tet deletion. Altogether this indicated that the Tet enzymes play essential roles in the key factors-driven reprogramming of somatic cells.

The complete abolition of the reprogramming of the Tet TKO MEFs has been linked to failing to undergo mesenchymal to epithelial Transition during MET [92]. Since the multistep process of the factor-driven reprogramming

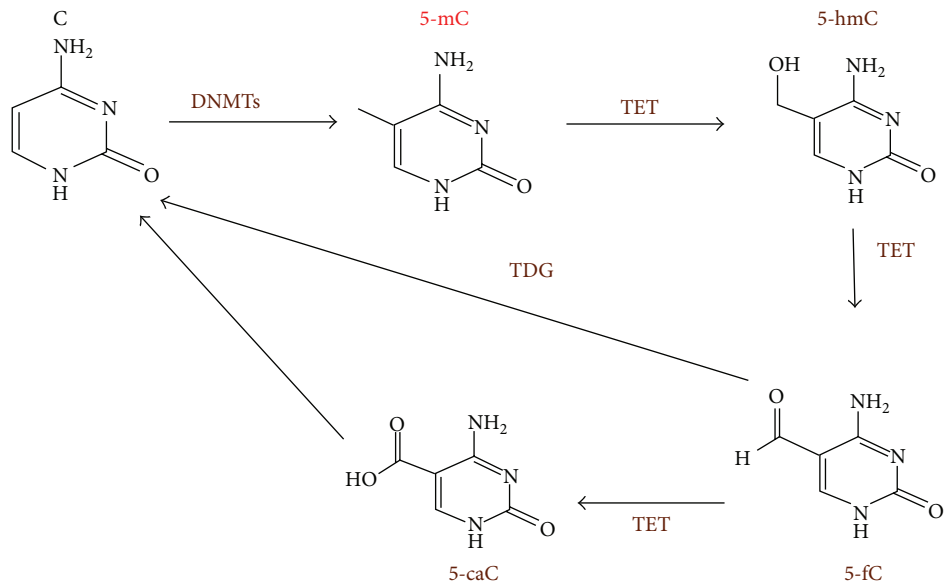

Figure 1: Base methylation at DNA levels. Cytosine (C) is methylated to methylcytosine (5-mC) by methyltransferases (DNMTs), and the 5-mC is oxidized or hydroxylated by ten-eleven translocation proteins (TETs) to 5-hydroxymethylcytosine (5-hmC). And the 5-hmC is believed to be mediator for demethylation 5-mC to C through serial steps: 5-mC is further oxidized by TETs to generate 5-formal C (5-fC) and 5-carboxy-C (5-caC), and finally 5-fC and 5-caC are converted to regular C catalyzed by thymidine DNA glycosylase (TDG). Methylation of adenosine at 6 position (6mA) has been also detected in the genomes of mammalian, *Chlamydomonas*, *Drosophila*, and *C. elegance* cells. TETs or TET analogues are responsible for generation of the 6mA.

is initiated by MET [93, 94], any event affecting the MET process will be vital to the reprogramming. Indeed, Tet TKO MEFs showed no sign of epithelium-like morphological shift, in contrast to wild-type, individual, or double knockout of Tet. MEFs displayed a substantial MET, and the TKO MEFs significantly exhibited MET defect. The MET process in TKO MEFs could be rescued by ectopic expression of the active catalytic domains of the TETs, but not the inactive form of the full TETs, suggesting the TET might epigenetically function as 5-hmC mediated demethylation in MET process.

Applying the Tet-assisted bisulfite sequencing strategy by which the 5-mC and 5-hmC could be distinguished from each other [95], it was found that at early stage of the reprogramming the 5-hmC levels in the 5′ region of the promoters of miRNA such as miR-200s reached high percentage in WT MEFs, and strikingly the 5-hmC levels dramatically decreased in the Tet KO particularly in Tet2 KO and even completely abolished in the TKO MEFs [89]. Taken together, these indicated that the impaired oxidative demethylation of miRNA genes in Tet-deficient MEFs leads to inactivation of the miRNA such as miR-200s, miR-200a, miR-200b, miR-200c, miR-141, and miR-429 critically involved in both cancer metastasis and experimental cell reprogramming. And further evidences showed the inverse correlation between 5-hmC and 5-mC among promoters of the genes associated with cell adhesion, suggesting that the genes involved in MET serve as targets for Tet-catalyzed hydroxylation during the early phase of reprogramming [92].

More interestingly, while both TET1 and TET2 are responsible for the conversion of 5-mC into 5-hmC, their roles in reprogramming are very different, especially in the presence of vitamin C [86]. While TET2 constitutively enhances the reprogramming regardless of vitamin C level, TET1 serves as a barrier to reprogramming by interfering with MET in the presence of vitamin C. In addition, TET2 but not TET1 can work together with Parp1 to balance the levels of 5-mC and 5-hmC. Besides Parp1, the DNA repair proteins that have been identified and linked to reprogramming include members in the XPC nucleotide excision repair complex. Unlike Parp1, the XPC family members are recruited by Oct4 and Sox2 to the Nanog and Oct4 promoters, working with TET2 to regulate reprogramming [96]. These findings suggest that various modulators required for reprogramming, such as TET1, TET2, and TET3, are recruited to specific DNA targets by core transcriptional factors to regulate the reprogramming state (Figure 2).

Besides the methylation of cytosine to form 5-mC, deoxyadenosine methylation has also been detected in the genomes of some eukaryotes, such as *Chlamydomonas, Drosophila,* and *C. elegans*, generating N6-methyldeoxyadenosine (6mA or m6A) [97–99]. Although the importance of the dynamic balance between the methylation and demethylation of adenosine in eukaryotes remains elusive, mammalian TET analogues in *Drosophila* (DMAD) and *C. elegans* (NMAD-1) function as erasers of 6mA methylation and play essential roles in reproduction and neuronal activities, further suggesting a TET- or TET analogue-mediated epigenetic regulation spectrum in eukaryotes.

3.3. Epigenetic Regulation at the Histone MacroH2A Level. Unlike TETs, which are guided by the core reprogramming factors and specifically localize to regions of the target genes

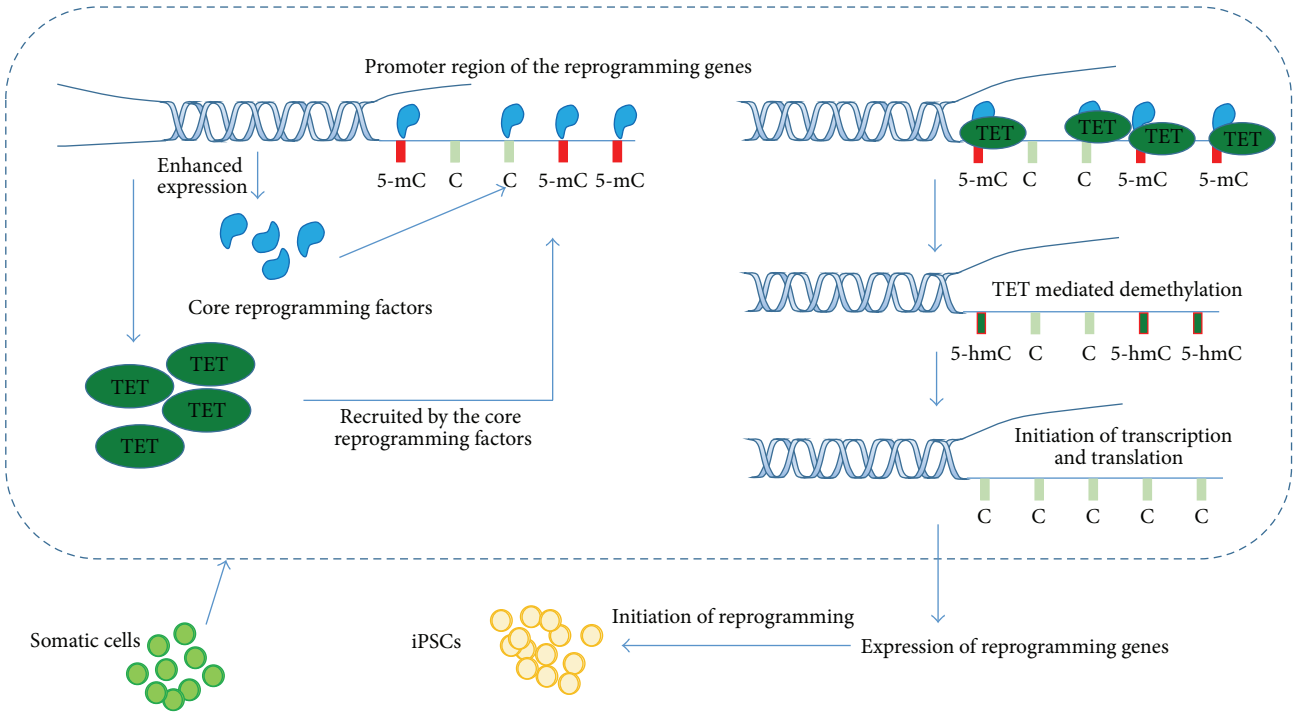

FIGURE 2: Enhanced expression of the core reprogramming factors and TETs switches the cell fate towards nucleic reprogramming to generate iPSCs. The TET enzymes are recruited by core transcription factors and localized to the methylated promoter regions of the downstream genes involved in the reprogramming. Then the TET oxidizes the 5-mC into 5-hydroxymethylcytosine (5-hmC) that serves as mediator of demethylation of the 5-mC, leading to demethylation of the promoter regions and expression of these genes and thereby initiation of the reprogramming.

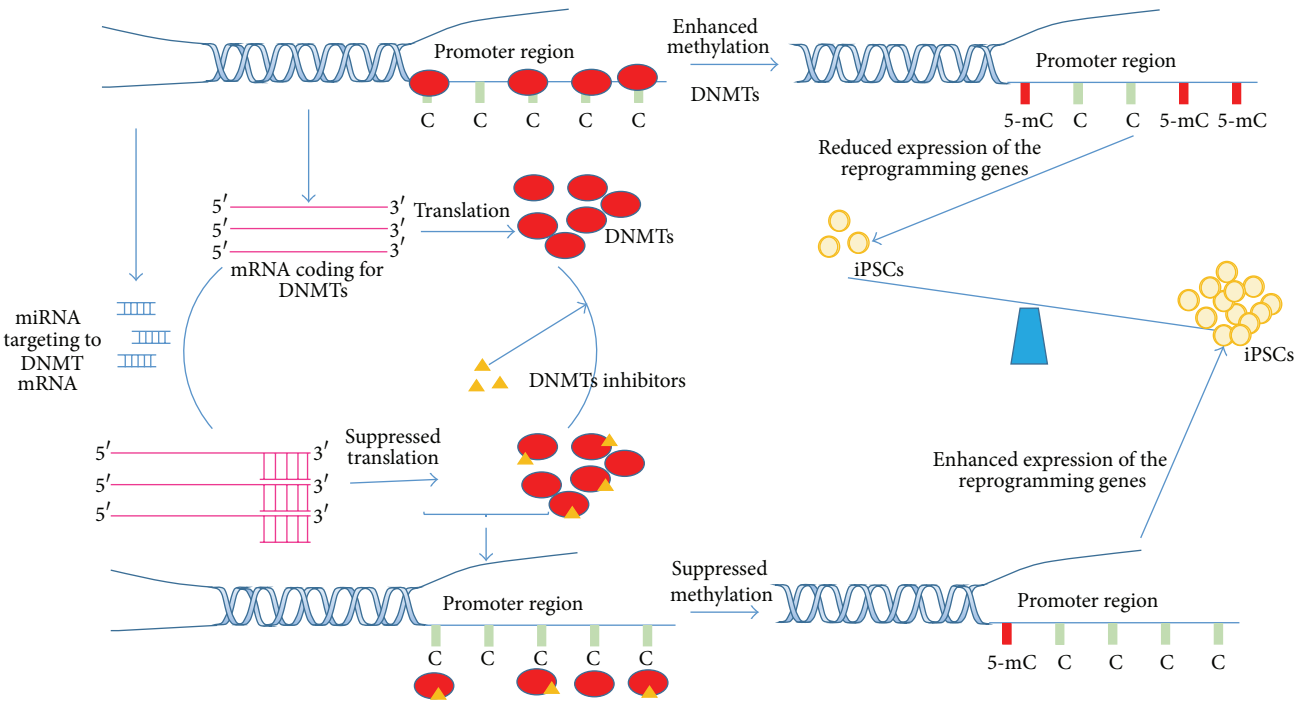

FIGURE 3: Regulation of the dynamic balance between methylation and demethylation by miRNA and small molecules functions as DNMTs inhibitors. Some miRNA targeted the mRNA coding for DNMTs and some small molecules function as inhibitors of DNMTs. Due to overexpression of some miRNA specifically targeting to DNMTs mRNA as well as treatment with DNMTs inhibitors, methylation is repressed, leading to dynamic alteration of methylation and demethylation balance and enhancing the reprogramming efficiency.

to enhance active demethylation during reprogramming, some epigenetic regulators, such as macroH2A and peptidylarginine deiminase Padi4, globally rather than specifically affect the pluripotent state either negatively or positively [100–102]. MacroH2A, a histone variant, has a global adverse effect on reprogramming, evidenced by the fact that MacroH2A is depleted in pluripotent cells and enriched in differentiated cells, possibly to repress pluripotency factors, as well as that the removal of MacroH2A enhances the efficiency of reprogramming [98, 99]. In contrast, Padi4 plays positive roles in reprogramming by interfering with the binding of the histone H1 to nucleosomal DNA [99].

3.4. Nonepigenetic Regulation of Reprogramming Efficiency. In addition to the genome-wide epigenetic regulation of reprogramming, alterations of key components in some metabolic pathways such as p21 and p53, as well as some general transcriptional and translational apparatuses, also affect reprogramming efficiency. It has been shown that p21 can negatively affect reprogramming efficiency; consequently any alterations which enhance p21 transcription and translation would repress reprogramming [101]. Thus, it is logical to speculate that p53, which stimulates the transcription of p21, and eIF4E binding protein (4E-BPs), which enhances the translation of p21, could significantly inhibit reprogramming efficiency. This speculation has been confirmed by the fact that depletion of 4E-BPs in p53 deletion fibroblasts results in increased reprogramming efficiency compared to that in wild-type fibroblasts due to the reduced transcription of p21 and higher levels of Sox2 and c-Myc under the condition of 4E-BPs. Accordingly, the expression of exogenous Oct4 alone was sufficient to induce pluripotency in p53 and 4E-BP1/2 double deletion mutant fibroblasts.

4. Concluding Remarks

The establishment of iPSC methodology and dissection of the mechanisms regarding reprogramming are a milestone in the long journey of stem cell research both theoretically and practically, providing a sufficient tool with which to tackle fundamental biomedical questions regarding epigenetics-mediated (de)differentiation, as well as providing a valuable cell source for tissue regeneration, human disease modeling, and drug discovery. Due to the efforts made to improve the protocols for iPSC generation, particularly with regard to patient-specific somatic cells, with a focus on increased efficiency and safety, significant progress has been made by employing a variety of (epi)genetic and biochemical approaches. In addition, the molecular mechanisms behind reprogramming have been extensively studied at biochemical, genetic, and epigenetic levels. However, technical challenges in the generation of iPSCs and safety concerns for the use of iPSCs in clinical applications remain big issues which require solving.

Although various strategies have been invented to enhance reprogramming efficiency and to improve the issue of safety, regrettably, none of these strategies could ensure both the high generation efficiency and safety of iPSCs. For example, while virus-mediated ectopic expression of the core reprogramming factors or knockdown of key epigenetic factors led to high generation efficiency, the integration of virus vectors could lead to tumorigenesis. Likewise, some strategies without virus vector meditation such as virus vector-free or transgene-free methods, such as the modified mRNA strategy and small molecule treatments to inhibit some barriers or activate enhancers of the reprogramming process, can improve the safety of iPSCs but their efficiency is much lower than that conferred by the virus- or other transgene-mediated methods. In addition, some small molecules may have multiple targets and thus come with the possibility of off-target effects which could lead to the unpredictable quality and safety concerns for the generated iPSCs. Thus, it is of great importance to carry out high-throughput chemical screens, transcriptomic, and proteomic studies so that more small molecules, chromatin remodelers, and other epigenetic modifiers can be identified and employed to enhance iPSC generation efficiency without raising safety and quality issues.

On the other hand, mechanism studies may facilitate the discovery of new strategies focusing on different targets to significantly enhance reprogramming efficiency. Previous studies have made this approach applicable, such as those leading to the discovery of epigenetic barriers to the reprogramming. By removing these barriers, reprogramming efficiency could be dramatically promoted. Although several barriers have been identified, more efforts are required to unveil new barriers and activators.

Alternatively, microRNA approaches could be also applied for the dissection of pathways implicated in iPSC reprogramming to further understand the crosstalk among metabolic pathways and the molecular agents known to serve as (epi)genetic modifiers or drivers. Further understanding of reprogramming mechanisms and development of safer and more efficient reprogramming strategies will benefit biomedical studies as well as iPSC-mediated regeneration medicine and transplant therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Human Umbilical Cord Blood-Derived Serum for Culturing the Supportive Feeder Cells of Human Pluripotent Stem Cell Lines

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Although human pluripotent stem cells (hPSCs) can proliferate robustly on the feeder-free culture system, genetic instability of hPSCs has been reported in such environment. Alternatively, feeder cells enable hPSCs to maintain their pluripotency. The feeder cells are usually grown in a culture medium containing fetal bovine serum (FBS) prior to coculture with hPSCs. The use of FBS might limit the clinical application of hPSCs. Recently, human cord blood-derived serum (hUCS) showed a positive effect on culture of mesenchymal stem cells. It is interesting to test whether hUCS can be used for culture of feeder cells of hPSCs. This study was aimed to replace FBS with hUCS for culturing the human foreskin fibroblasts (HFFs) prior to feeder cell preparation. The results showed that HFFs cultured in hUCS-containing medium (HFF-hUCS) displayed fibroblastic features, high proliferation rates, short population doubling times, and normal karyotypes after prolonged culture. Inactivated HFF-hUCS expressed important genes, including Activin A, FGF2, and TGFβ1, which have been implicated in the maintenance of hPSC pluripotency. Moreover, hPSC lines maintained pluripotency, differentiation capacities, and karyotypic stability after being cocultured for extended period with inactivated HFF-hUCS. Therefore, the results demonstrated the benefit of hUCS for hPSCs culture system.

1. Introduction

Human pluripotent stem cells (hPSCs) can be generated from isolation of the pluripotent cells using preimplantation embryos, called "human embryonic stem cells (hESCs)" [1] or the reprogramming of somatic cells using exogenous genes, resulting in "human induced pluripotent stem cells (hiPSCs)" [2]. Both hESCs and hiPSCs share phenotypic and molecular genetic similarities [3]. hPSCs can be used as a model for the study of developmental biology, toxicity, and cell-based therapy [4]. Conventionally, hPSCs are derived and propagated through coculture with supportive feeder cells isolated from mice [1, 2]. Feeder cells play an important role in supporting the pluripotency of hPSCs by producing a complex microenvironment for the growth of hPSCs. Feeder cells secrete growth factors, such as FGF2, TGF-β1, or Activin A, involving the specific pathway controlling the pluripotency of hPSCs. Moreover, extracellular matrices, such as laminin, fibronectin, or collagen, produced from the feeders, are necessary for cell interaction, cell migration, or cell proliferation [5].

For therapeutic purposes, avoiding the cross-contamination of animal pathogens to hPSCs through the replacement of mouse feeder cells with the feeder cells derived from human tissue should be considered. Human feeder cells, such as human foreskin fibroblasts (HFFs), human mesenchymal stem cells (hMSCs), human amniotic epithelial cells (hAECs), or human fallopian tube-derived cells, were used as supportive feeder cells for the culture of hPSCs [6]. Moreover, previous studies have demonstrated the possibility

to generate genetically modified human feeder cells for the long-term support of hPSCs [7, 8]. For routine culture, human cells were cultured in medium supplemented with fetal bovine serum (FBS). Although FBS promotes human cell proliferation, human cells bare the risk of contamination of bovine viruses or other pathogens. For clinical purposes, the use of animal products in human cell culture should be avoided. Since human serum (HS) shows a positive effect on culture of mesenchymal stem cells, it appears to be a promising candidate for FBS replacement. The previous studies demonstrated that HS can be used for culturing the supportive feeder cells of hESCs [9, 10] and hMSCs showed greater proliferation in the HS-containing medium compared with FBS-containing medium [11, 12]. Nonetheless, HS not only promoted hMSC cell proliferation but also enhanced osteogenic differentiation [13]. Due to the progressive research for the therapeutic application of hPSCs, it is necessary to develop xeno-free culture conditions for the maintenance of hPSCs. Recent studies have shown that both feeder cells and hPSCS can be generated and cultured under the good manufacturing practice (GMP) [14, 15] as a useful step forward for the application of hPSCs in the field of regenerative medicine. Recently, human cord blood-derived serum (hUCS) showed a positive effect on culture of mesenchymal stem cells [16, 17]. Therefore, it is interesting to test whether hUCS can be used for culture of feeder cells of hPSCs.

The objective of the present study was to compare (i) growth, proliferation, and karyotypic stability of HFFs when cultured in a medium containing hUCS (HFF-hUCS) versus FBS (HFF-FBS), (ii) the characteristics of inactivated HFF-hUCS and inactivated HFF-FBS, and (iii) the pluripotent characteristics of hPSCs after having been cocultured for a long period with inactivated HFF-hUCS and inactivated HFF-FBS.

2. Results

2.1. Effect of Serum Supplementation on the Morphology and Proliferation of Human Foreskin Fibroblasts. The HFFs were cultured in medium containing either hUCS or FBS, and the morphology and growth were subsequently observed. We first observed the difference of cell attachment between the two culture media. At 5 hours after dissociation, nearly all dissociated HFF-FBS were attached to the surface of the culture dish. HFFs were transformed from a round to a fibroblastic-like shape. In contrast, most HFF-hUCS remained suspended in the culture medium. However, at 24 hours after dissociation, there were no notable differences in cell attachment and morphology between HFF-FBS and HFF-hUCS and most of the cells were attached to the culture dishes and displayed typical fibroblast cell morphology (Figure 1(a)).

After seeding the cells, the growth of HFF-hUCS and HFF-FBS was continuously observed until reaching confluency. We observed that HFFs cultured in both media progressively proliferated and reached confluency between 3 and 4 days after seeding the cells. The growth behaviors were similar at both early (p4 + 1) and intermediate passage (p4 + 10) (Figure 1(b)). In contrast, HFFs cultured in the medium without serum supplementation and in the medium supplemented with 10% KSR attached to the culture dishes but the cells could not reach confluency even being cultured for 7 days (Figure 1(c)).

Although the growth behavior of HFF-hUCS and HFF-FBS was not notably different, we observed some differences in the proliferation kinetics. The proliferation kinetics of HFF-hUCS and HFF-FBS were determined as the population doubling time (PDT) during continuous culture for up to 13 passages (p4 + 13). At passage numbers 4 + 3 to 4 + 13, HFF-hUCS displayed a significant shorter ($P < 0.05$) PDT compared with HFF-FBS (Figure 2(a)). The PDT of HFFs cultured in the medium without serum supplementation and supplemented with 10% KSR were not able to determine due to their proliferation insufficiency.

2.2. Karyotype Analysis of Human Foreskin Fibroblasts after Long-Term Culture in the Culture Medium Containing Human Umbilical Cord Blood-Derived Serum. The major effect of serum supplementation was observed in the proliferation of HFFs, showing that hUCS promotes better HFF proliferation than FBS. However, prior to using HFFs as feeder cells for culturing human pluripotent stem cells (hPSCs), we examined the genetic stability of HFFs through karyotype analysis of HFF-hUCS and HFF-FBS at p4 + 13 using the G-banding method. The results showed that culturing HFFs in hUCS-containing medium did not alter the karyotype of these cells. After culturing in either hUCS- or FBS-containing medium HFFs maintained a normal karyotype of 46,XY (Figure 2(b)).

2.3. Effect of Serum Supplementation on the Morphology and Gene Expression of Inactivated Human Foreskin Fibroblast Feeder Cells. In the present study, we used HFF-hUCS and HFF-FBS between p4 + 5 and p4 + 10 to prepare feeder layer. After mitomycin C-inactivation, HFF-hUCS and HFF-FBS displayed typical fibroblast features (Figure 3(a)). Inactivated HFF-hUCS and inactivated HFF-FBS were cultured in hPSC culture media for 24 hours, and total RNA were collected and subjected to gene expression analysis using RT-PCR. As shown in Figure 3(b), inactivated HFF-hUCS and inactivated HFF-FBS expressed Activin A, FGF2, TGF-β1, and BMP-4, which have been implicated in the maintenance of hPSC pluripotency.

2.4. Human Pluripotent Stem Cells Maintained Pluripotency after Coculture with Inactivated HFF-hUCS Feeder Cells. In the present study, karyotypic normal hPSC lines, including Chula2.hES and HFSK#11.hiPS, were used. Prior to examining the pluripotency of hPSCs, the cells were continuously grown on inactivated HFF-FBS or HFF-hUSC feeder cells for at least 10 passages. The morphology of hPSCs, when cultured on inactivated HFF-hUCS feeder cells, was not notably different from that of cells cultured on inactivated HFF-FBS feeder cells. Both hPSC lines showed undifferentiated colonies with defined boundaries and distinct prominent nuclei (Figure 4(a)).

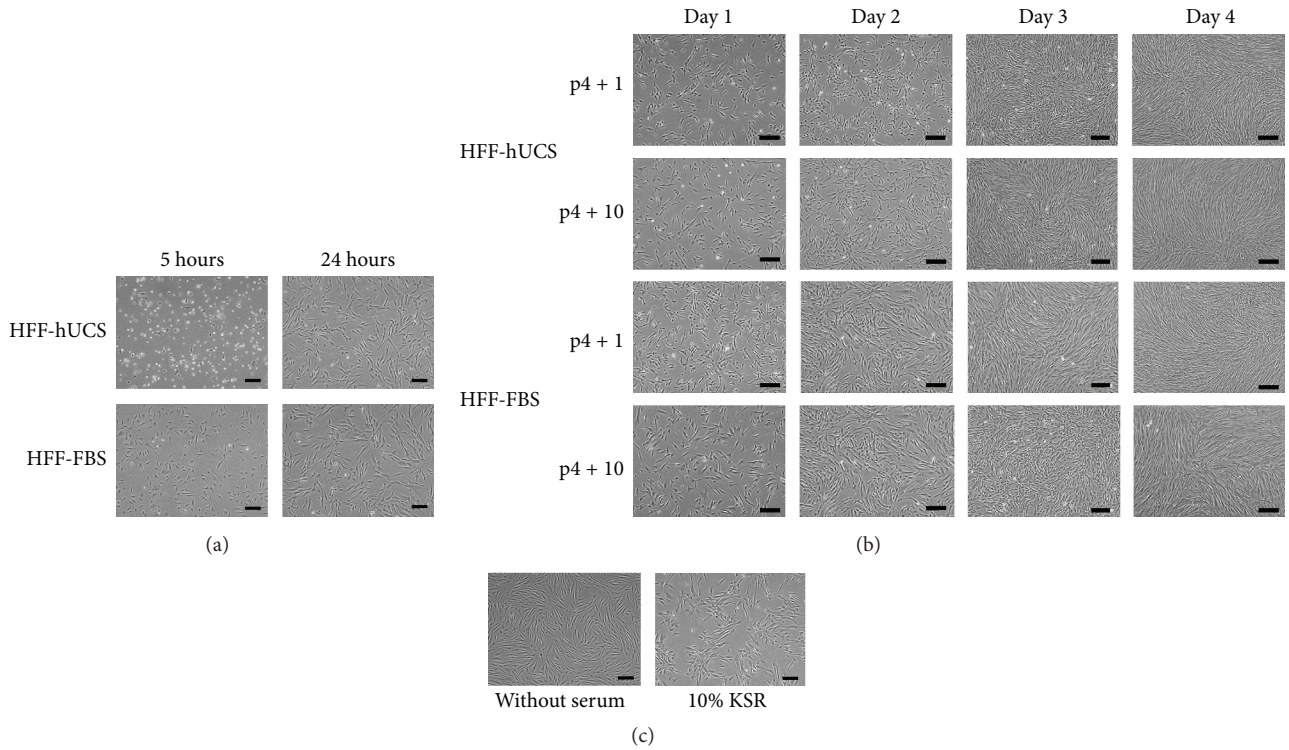

Figure 1: Effect of serum supplementation on the growth kinetics of human foreskin fibroblasts. Human foreskin fibroblasts were enzymatically dissociated and plated onto culture dishes. HFFs were cultured in the media containing human cord blood-derived serum (hUCS; HFF-hUCS), fetal bovine serum (FBS; HFF-FBS), knockout serum replacement (KSR) or without serum supplementation. Dissociated cells were observed for attachment behavior and growth kinetics. Differences in cell attachment between HFF-hUCS and HFF-FBS were observed. At 5 hours after plating, nearly all of the dissociated HFF-FBS were attached to the surface of the culture dish and exhibited a fibroblastic-like morphology, while most of HFF-hUCS remained suspended in the culture medium. At 24 hours after plating, all HFF-FBS and HFF-hUCS were attached to the culture dishes and displayed typical fibroblast cell morphology (a). Similar proliferation patterns between HFF-hUCS and HFF-FBS were observed. HFF-hUCS and HFF-FBS progressively proliferated and reached confluency at 3 to 4 days after seeding. The proliferation behavior at early passage (p4 + 1) was similar to that at intermediate passage (p4 + 10) (b). In contrast to HFF-hUCS and HFF-FBS, HFFs cultured in the medium containing 10% KSR and without serum supplementation could not reach the confluency even being cultured for 7 days (c). HFF = human foreskin fibroblasts, FBS = fetal bovine serum, hUCS = human cord blood-derived serum, KSR = knockout serum replacement, and p = passage number. Scale bars = 200 μm.

hPSCs cultured on inactivated HFF-hUCS and inactivated HFF-FBS feeder cells expressed pluripotent genes, including OCT-4, NANOG, REX1, and UTF (Figure 4(b)). Immunostaining demonstrated that hPSCs cultured on inactivated HFF-hUCS and inactivated HFF-FBS feeder cells expressed pluripotent markers, including SSEA-3, TRA-1-60, TRA-1-81, and OCT-4 (Figure 4(c)). In addition, the quantitative analysis of SSEA-4 expression using flow cytometry demonstrated that the percentage of SSEA-4 positive cells between hESCs cocultured with inactivated HFF-FBS and inactivated HFF-hUCS was not significantly different (92.8 ± 0.6 versus 92.7 ± 0.8, resp.). Interestingly, the percentage of SSEA-4-positive hiPSCs cocultured with inactivated HFF-hUCS (96.5 ± 0.3) was significantly higher than that of hiPSCs cocultured with inactivated HFF-FBS (93.2 ± 0.1) ($P < 0.05$) (Figure 4(d)).

2.5. Differentiation Ability and Karyotypic Stability of Human Pluripotent Stem Cells. The differentiation of hPSCs cultured on inactivated HFF-hUCS and inactivated HFF-FBS was confirmed based on embryoid body (EB) formation subsequent to differentiation in vitro. The results showed that hPSCs cultured on inactivated HFF-hUCS and inactivated HFF-FBS feeder cells formed three-dimensional EBs in suspension culture (Figure 5(a)). The in vitro differentiation of EBs toward three embryonic germ layers was confirmed by using immunostaining for ectoderm (NESTIN, PAX6), mesoderm (BRACHYURY, SMA), and endoderm (AFP). The RT-PCR results also confirmed the differentiation abilities of EBs toward ectoderm, (NESTIN), mesoderm (BRACHYURY), and endoderm (AFP). Moreover CDX2, a trophoblast marker, was also detected in EBs (Figures 5(b) and 5(c)).

In order to evaluate the in vivo differentiation of hPSCs, the cells were allowed to grow and differentiate after injection into immunodeficient mice. The teratoma formation assay was performed for in vivo differentiation test. The presence of structures that resemble the ectodermal, endodermal, and mesodermal tissues in the teratoma confirmed the differentiation capacities of hPSCs after being cocultured with inactivated HFF-hUCS and inactivated HFF-FBS (Figure 5(d)).

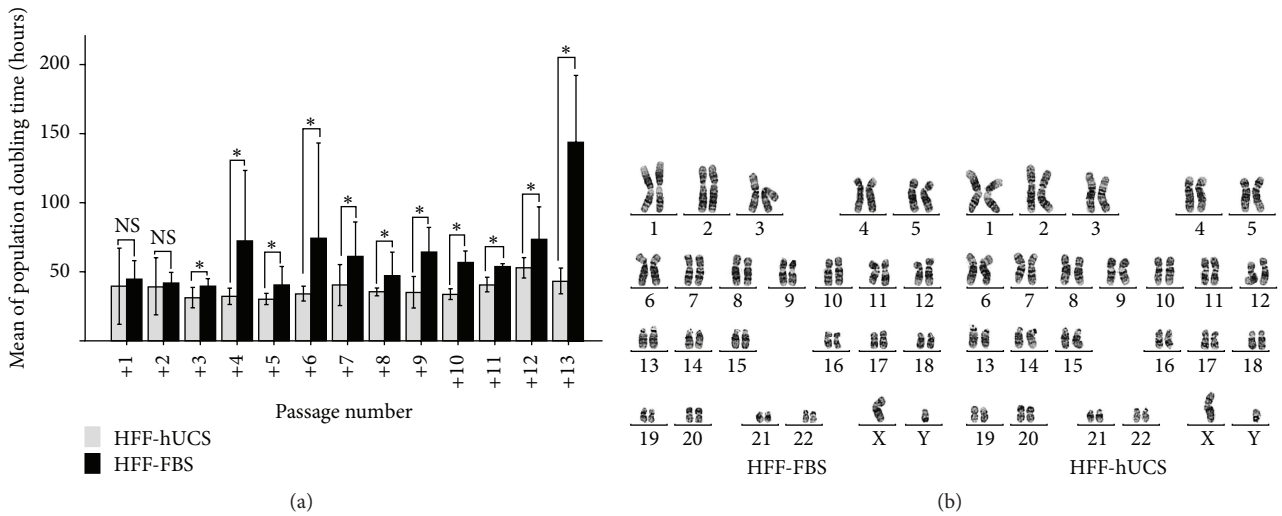

Figure 2: Effect of serum supplementation on cell proliferation and karyotype stability of human foreskin fibroblasts. The cell proliferation of human foreskin fibroblasts (HFFs) was evaluated by determination of their population doubling time (PDT). The ability of HFFs to maintain their normal karyotype was assessed by the G-banding method. The PDT of HFFs cultured in the media containing human cord blood-derived serum (hUCS; HFF-hUCS) and fetal bovine serum (FBS; HFF-FBS) from p4 + 1 to p4 + 13 were determined. The HFF-hUCS from p4 + 3 to p4 + 13 displayed a significantly shorter ($P < 0.05$) PDT than HFF-FBS (a). The karyotype analysis of HFF-FBS and HFF-hUCS demonstrated that both HFFs cultured in hUCS- or FBS-containing media maintained a normal 46,XY karyotype after prolonged culture (b). Error bars represent the standard error of mean (SEM). HFF = human foreskin fibroblasts, FBS = fetal bovine serum, hUCS = human cord blood-derived serum, and NS = not significant. Scale bars = 200 μm, $^{*}P < 0.05$.

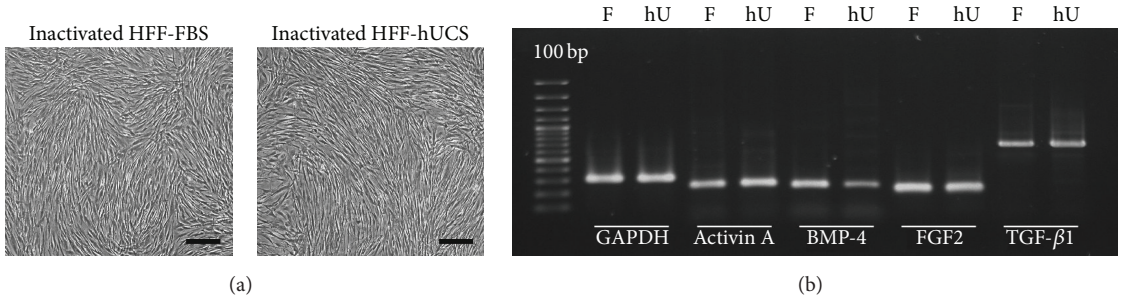

Figure 3: Morphology and gene expression of inactivated human foreskin fibroblasts prior to use as the feeder layer. HFF-hUCS and HFF-FBS survived inactivation through mitomycin-C treatment and maintained typical fibroblast-like features (a). RT-PCR was performed for the detection of supportive feeder cell-related genes. Inactivated HFF-hUCS and inactivated HFF-FBS expressed Activin A, FGF2, TGF-β1, and BMP-4. GAPDH was used as the house keeping gene (b). BMP = bone morphogenetic protein, FGF = fibroblast growth factors, TGF = transforming growth factor, HFF = human foreskin fibroblasts, F and FBS = fetal bovine serum, hU and hUCS = human cord blood-derived serum, RT-PCR = reverse transcription polymerase chain reaction, and GAPDH = glyceraldehyde 3-phosphate dehydrogenase. Scale bars = 200 μm.

After coculturing hPSCs with inactivated HFF-hUCS and inactivated HFF-FBS for more than 10 passages, hPSCs were subjected to karyotype analysis. The results demonstrated that the coculture of diploid hPSC lines with inactivated HFF-hUCS feeder cells did not change the karyotype stability of these cells. Chula2.hES maintained the 46,XY karyotype and HFSK#11.hiPS maintained the 46,XY karyotype on both types of feeder cells (Figure 6).

3. Discussion

The successful derivation of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2], is promising not only for use in the treatment of patients suffering from cell or tissue damage but also for drug discovery and the exploration of human developmental biology. hESCs can be derived from the preimplantation embryos, while hiPSCs can be generated through the reprogramming of somatic cells to the embryonic stem cell-like stage. Both hESCs and hiPSCs were conventionally derived and cocultured with mouse embryonic fibroblasts (MEFs) [1, 2]. Due to the progress of using hPSCs derivatives for therapeutic development program, the production of clinical grade hPSCs is needed. Therefore, it is important to develop a culture system being practical, simple, and clinically relevant for the generation

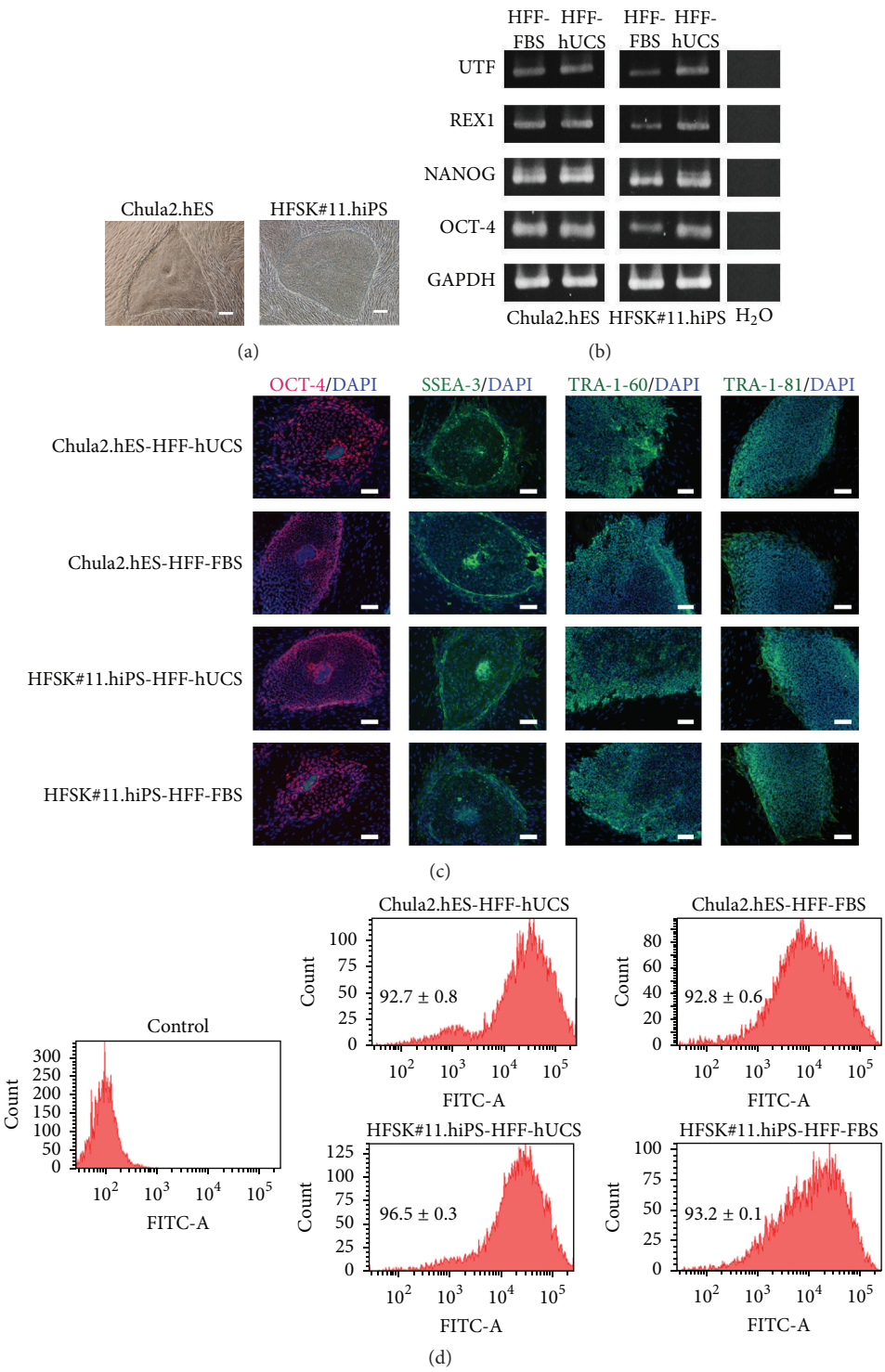

Figure 4: Characterization of human pluripotent stem cells after coculture with HFF-hUCS feeder cells. Two karyotypic normal hPSC lines, hESC (Chula2.hES) and hiPSC (HFSK#11.hiPS), were cocultured with inactivated HFF-hUCS or inactivated HFF-FBS, and subsequently the morphological appearance, pluripotent gene expression, and pluripotent marker expression were examined. hPSC lines showed undifferentiated colonies with defined boundaries and distinct prominent nuclei (a). hPSCs cocultured with inactivated HFF-hUCS expressed pluripotent genes, including OCT-4, NANOG, REX1, and UTF, similar to hPSCs cocultured with inactivated HFF-FBS (b). The pluripotent markers, including SSEA-3, TRA-1-60, TRA-1-81, and OCT-4, were detected in hPSCs cocultured with inactivated HFF-hUCS and inactivated HFF-FBS (c). The quantitative analysis of SSEA-4 expression through flow cytometry demonstrated that the percentage of SSEA-4-positive cells was not significantly different between hESCs cocultured with inactivated HFF-FBS and inactivated HFF-hUCS (92.8 ± 0.6 versus 92.7 ± 0.8, resp.). The percentage of SSEA-4-positive hiPSCs cocultured with inactivated HFF-hUCS (96.5 ± 0.3) was significantly higher than that for hiPSCs cocultured with inactivated HFF-FBS (93.2 ± 0.1) ($P < 0.05$; (d)). HFF = human foreskin fibroblasts, FBS = fetal bovine serum, hUCS = human cord blood-derived serum, and DAPI = 4′,6-diamidino-2-phenylindole. Scale bars = 200 μm.

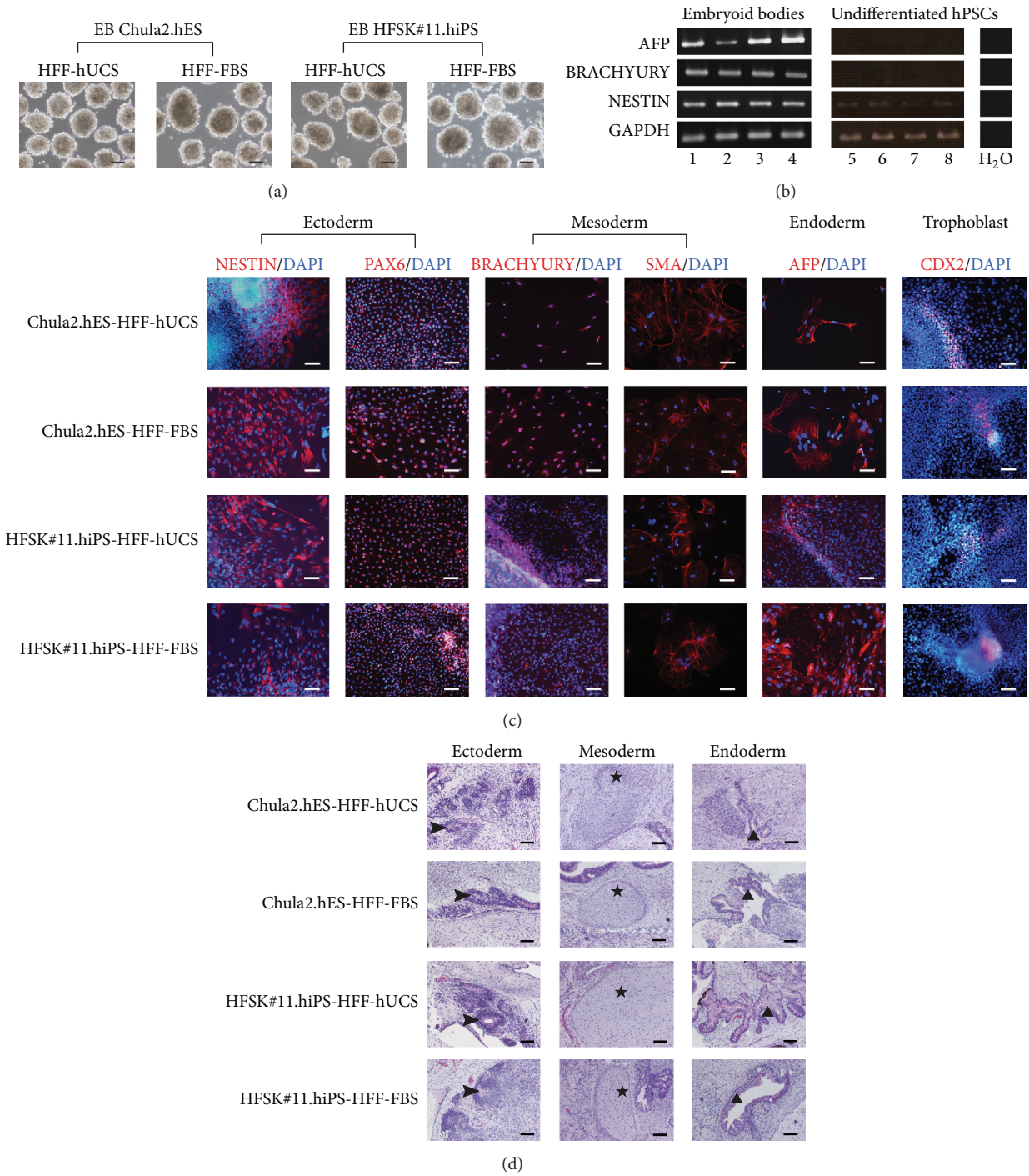

Figure 5: In vitro and in vivo differentiation of human pluripotent stem cell lines cocultured with inactivated HFF-hUCS. The differentiation capacities of hPSC lines cocultured with inactivated HFF-hUCS and inactivated HFF-FBS were determined through the formation of embryoid bodies (EBs) and teratoma formation. hPSC lines cocultured with inactivated HFF-hUCS formed EBs in suspension culture similar to hPSC lines cocultured with inactivated HFF-FBS (a). After the EBs were plated into culture dishes and continuously cultured for 21 days, the EBs differentiated to embryonic germ layers, including the ectoderm (NESTIN, PAX6), mesoderm (BRACHYURY, SMA), endoderm (AFP), and the trophoblast markers (CDX2) were detected through immunostaining (b) and RT-PCR (c). hPSCs lines cocultured with inactivated HFF-hUCS formed teratoma tissue and differentiated into ectoderm (neural rosette-like structure; arrowhead), mesoderm (cartilage; star), and endoderm (gut-like structure; triangle) similar to those cocultured with inactivated HFF-FBS (d). AFP = alpha-fetoprotein, smooth muscle actin (SMA), HFF = human foreskin fibroblasts, FBS = fetal bovine serum, hUCS = human cord blood-derived serum, hPSCs = human pluripotent stem cells, DAPI = 4′,6-diamidino-2-phenylindole, and RT-PCR = reverse transcription polymerase chain reaction. Lane 1 = EB Chula2.hES-HFF-hUCS, Lane 2 = EB Chula2.hES-HFF-FBS, Lane 3 = EB HFSK#11.hiPS-HFF-hUCS, Lane 4 = EB HFSK#11.hiPS-HFF-FBS, Lane 5 = undifferentiated Chula2.hES-HFF-hUCS, Lane 6 = undifferentiated Chula2.hES-HFF-FBS, Lane 7 = undifferentiated HFSK#11.hiPS-HFF-hUCS, and Lane 8 = undifferentiated HFSK#11.hiPS-HFF-FBS. Scale bars (a) and (d) = 200 μm and (b) = 40 μm.

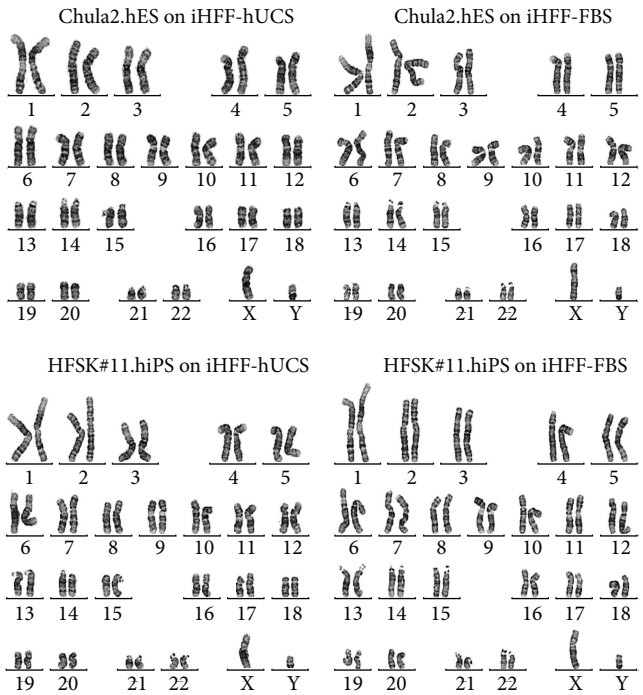

Figure 6: Karyotypic analysis of human pluripotent stem cell lines cocultured with inactivated HFF-hUCS. The karyotypic stability of hPSCs after prolonged coculture with inactivated HFF-hUCS was detected using the G-banding method. hPSC lines cocultured with inactivated HFF-hUCS maintained a normal karyotype similar to hPSCs lines cocultured with inactivated HFF-FBS. Chula2.hES maintained the 46,XY karyotype and HFSK#11.hiPS maintained the 46,XY karyotype on both types of feeder cells. iHFF-hUCS = inactivated HFF-hUCS and iHFF-FBS = inactivated HFF-FBS.

and propagation of hPSCs. Although the feeder-free culture system is the ideal culture condition for derivation and culture of clinical grade hPSCs, this culture system is still facing some limitations. Firstly, many studies reported the successful derivation and culture of hPSCs under feeder-free culture system using Matrigel instead of feeder layer, resulting in robust proliferation of hPSCs. However, Matrigel is extracellular matrices isolated from mice, which makes them unsuitable for use as extracellular matrices for culturing clinical grade hPSCs. Secondly, the more relevant extracellular matrices originated from human recombinant proteins, including vitronectin, fibronectin, and laminin and their combinations have been shown to support the pluripotency of hPSCs. However, large-scale purification or production of biologically functional human proteins by recombination technologies is laborious and expensive [18]. Thirdly, the ability of feeder-free culture system to maintain the genetic stabilities of hPSCs remains controversial [19]. hPSC lines derived and/or cultured under the feeder-free conditions acquired karyotypic abnormalities during subsequent culture [19, 20].

On the other hand, the conventional culture system, using mouse embryonic fibroblasts (MEF) as the feeder cells, is still widely applied to ensure self-renewal and the pluripotency of hPSCs. MEFs play important roles in the regulation of hPSC pluripotency through the secretion of essential cytokines or molecules and extracellular matrices [21, 22]. Due to concerns about animal pathogens and immunogens using MEFs, several reports recommended the use of human fibroblasts, including human neonatal foreskin fibroblasts (HFFs) instead of MEFs [23–25]. The results demonstrated that HFFs showed the characteristics of supportive feeder cells for hPSCs. Interestingly, the current clinical trials using hPSCs to treat patients suffering from macular degeneration adopted the feeder culture system for propagation of hPSCs prior to differentiation of cells into the retinal-pigment epithelial (RPE) cells [26, 27]. The feeder cells appear thus to remain a good choice for coculture with hPSCs prior to use hPSCs for clinical treatment.

However, HFFs were routinely cultured and propagated in fetal bovine serum- (FBS-) containing medium prior to inactivation and used as the feeder cells for culturing hPSCs. FBS contains several unknown factors and is widely used for supplementation in animal and human cell culture media. The FBS supplementation in the culture medium influences the proliferation, maintenance, and differentiation of human stem cells [28, 29]. However, using FBS for culture of the HFFs might cause the contamination of bovine pathogen(s) to hPSCs, through the feeder cells. To reduce the cross contamination, FBS can be replaced with the human serum.

In the present study, we attempted to improve the culture conditions for hPSCs using human serum and human feeder cells. The human serum used in the present study was the human cord blood-derived serum (hUCS). hUCS has previously been demonstrated to have beneficial effects on the isolation, proliferation, and differentiation of human amniotic fluid stem cells (hAFS) and Wharton's jelly mesenchymal stromal cells by our collaborators [16, 17]. The commercial human foreskin fibroblasts (HFFs) used in the present study have been demonstrated as supportive feeder cells for the derivation of hESCs and hiPSCs [23–25]. Therefore, the aim of the present study was to examine the feasibility of using hUCS for culturing HFFs prior to the preparation of feeder cells and the subsequent use of inactivated HFFs for coculture with hPSCs. We first demonstrated that hUCS can efficiently replace FBS for the routine culture of HFFs. hUCS improved the proliferation of HFFs and maintained the karyotype and normal features of these cells. Secondly, mitomycin-C-inactivated HFFs, previously cultured with hUCS-containing media, expressed candidate genes, including Activin A, FGF2, and TGF-β, which are normally expressed by hPSC supportive feeder cells [21]. Thirdly, hPSC lines cocultured with inactivated HFFs, previously grown in hUCS-containing media, maintained pluripotency, differentiation, and a normal karyotype.

Serum contains factors that supply nutrients to the cells, protect cells from stress, and influence the attachment of almost all cell types. A major advantage of using human serum for culturing human cells, particularly human stem cells, is avoiding the contamination of animal pathogens to human cells. However, only the beneficial effects of using human serum in human stem cell culture have been demonstrated [11–13]. Under the culture conditions used in the present study, we observed that the type of serum

presented in the HFF culture medium affected the attachment of HFFs after enzymatic dissociation. Shortly after replating, HFFs in FBS-containing medium attached to the surface of the culture dishes, while HFFs in hUCS-containing medium remained afloat. However, after 24 hours, the HFFs in the hUCS-containing medium were attached to the surface and displayed a morphology similar to the HFFs in FBS-containing medium. The delayed attachment of HFF-hUCS to the surface of the culture dish likely reflects differences in the cytokines, growth factors, or extracellular matrices contained in hUCS and FBS [30, 31].

Interestingly, the source of the serum also affected the proliferation kinetic of HFFs. The population doubling time (PDT) of HFFs after culture in medium containing hUCS was shorter than that observed with FBS. Extending the PDT limits the life span and use of cells for these experiments. Thus, hUCS provided more suitable nutrients and a better physiological environment for HFF cell division compared with FBS. Previous studies have demonstrated that using human serum for culturing HFFs extends the passage number of cultured HFFs. Therefore, numerous HFF stocks can be obtained for feeder supply for hPSC culture [5, 9]. The chromosomal stability of the cells is one of the most important issues for the application of stem cells for cell therapy. During the routine culture of human stem cells, chromosome stability was regularly analyzed every 5–10 passages. Prolonged culture or changing the culture conditions might lead to the karyotype instability of the cultured cells [18, 30]. Interestingly, the results of the karyotype analysis in the present study showed that although the culture medium was supplemented with hUCS, this serum did not alter the karyotype of HFFs after prolonged culture. Taken together, hUCS not only enhances cell proliferation but also preserves the HFF karyotypic stability. Therefore, hUCS is suitable for HFF culture medium supplementation.

To use HFFs as the feeder cells for culturing hPSCs, the cell division of HFFs should be inactivated. The inactivation of HFFs can be performed through exposure to gamma rays or supplementation with mitomycin-C into the culture medium. Prolonged incubation with a high concentration of mitomycin-C might damage fibroblast cells [32]. In the present study, HFFs cultured in hUCS- and FBS-containing media were treated with a standard concentration of mitomycin-C for a standard incubation time [33]. The difference in the morphological appearance was not notably observed between inactivated HFFs cultured in hUCS- and FBS-containing medium. HFFs cultured in hUCS were resistant and survived mitomycin C treatment, similar to the HFFs cultured in FBS. It has been previously demonstrated that the differences in the secretion of growth factors, cytokines, and extracellular matrices (ECM) distinguish supportive from nonsupportive feeder cells [21]. A proteomics approach is typically used to characterize the composition and interaction networks between the ECMs that support the maintenance of hESCs [34, 35]. In addition, the detection of the expression of supportive feeder-related genes, such as FGF2, Activin A, or TGF-β1, could also be used to examine supportive feeders prior to use for culturing hPSCs. In the present study, inactivated HFFs-hUCS and HFFs-FBS similarly expressed the selected genes, including Activin A, FGF2, and TGF-β1, which is typically detected in supportive feeder cells. These results were consistent with those of Eiselleova and colleagues [21]. The genes expressed from inactivated HFFs provided a complex network for the maintenance of hPSCs in the pluripotent state, suggesting that the supplementation of hUCS in the culture media might not change the supportive feeder cell nature of HFFs.

We further tested the pluripotency of hPSC lines after prolonged coculture with inactivated HFF-hUCS or inactivated HFF-FBS. The hPSC lines were cocultured with inactivated HFF in the serum-free culture medium. The serum-free culture medium is considered as the suitable culture medium for maintenance of hPSCs in order to use hPSCs for cell-based therapy. The results demonstrated that hPSC lines cocultured with inactivated HFF-hUCS feeders maintained pluripotency with normal hPSC colony morphology and the expression of pluripotent transcriptional factors, including OCT-4, NANOG, REX1, and UTF, and pluripotent markers, including SSEA-3, TRA-1-60, TRA-1-81, and OCT-4. Moreover, the quantified results of SSEA-4 expression, determined through flow cytometry, demonstrated that inactivated HFF-hUCS effectively support the pluripotency of hPSCs in a manner similar to inactivated HFF-FBS. In addition, hPSC lines differentiate into three embryonic germ layers in vitro and in vivo and maintain a normal karyotype, confirming the pluripotency of these cells [1, 2, 25].

The present study demonstrated the beneficial effect of hUCS for culturing the supportive feeder cells of hPSCs. Notably, the important issues of using serum are pooling the serum from different donors and the lot-to-lot variability of hUCS. To control the quality of hUCS, several issues need to be considered such as the donors must not have any history of infectious diseases, the serum producing processes has to be carried following a strictly aseptic technique, or the serum has to be tested against contamination of endotoxins. Importantly, the ability of each batch of serum should be preliminary tested for culturing the fibroblast feeder cells prior to use the serum in a large scale. Alternatively, the HFF-hUCS conditions referred to in our study might be used for derivation of newly established hPSC lines prior to adaptation of such lines to the xeno-free/feeder-free culture conditions for the robust propagation of hPSC lines.

In conclusion, these results demonstrated that hUCS is an effective alternative source of serum for supplementation in the culture medium of HFFs. Not only does hUCS support the growth of HFFs, but inactivated HFF-hUCS also maintain the characteristics of supportive feeder cells for the culture of hPSC lines. These findings benefit the optimization of the xeno-free culture conditions of hPSCs.

4. Materials and Methods

4.1. Human Subjects. In the present study, the use of human subjects, human cell lines, and serum was conducted in accordance with the guidelines and regulations of the Medical Council of Thailand and Faculty of Medicine, Chulalongkorn University.

4.2. Serum. Two types of serum were used in the present study, human umbilical cord blood-derived serum (hUCS) and fetal bovine serum (FBS). The hUCS was collected by our collaborator and the serum collecting protocol was described previously [16, 17]. In brief, each mother who donated the cord blood was monitored to ensure no history of infectious diseases such as hepatitis and human immunodeficiency virus (HIV). Cord blood serum was obtained by following blood clotting at room temperature for 6–12 hours and centrifugation at 2,800 rpm for 5 minutes at 20°C. The hUCS was filtered through the 0.22 um pore size. Twenty serum samples were pooled in each batch, tested for mycoplasma contamination, and kept at −20°C before being used. This hUCS is sufficient for ensuring growth and for maintaining the multipotency of human mesenchymal stem cells as described previously [16, 17]. HyClone FBS (recommended by the provider of human foreskin fibroblasts) was purchased from Thermo Fisher Scientific (Logan, UT, USA).

4.3. Culture of Human Foreskin Fibroblasts. Frozen human foreskin fibroblasts (HFFs; catalog number CRL 2429) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). CRL-2429 HFFs demonstrated to be a supportive feeder cells for the generation and culture of hESC lines [23–25]. The CRL-2429 HFFs were initially isolated and cultured in FBS-containing medium. HFFs at passage number (p) p4 were purchased from the supplier. Upon receiving the frozen stock, the cells were thawed and expanded according to the manufacturer's instructions with slight modifications. Briefly, the cells were cultured using HFF culture medium comprising 88% Iscove's modified Dulbecco's medium (IMDM; Thermo Scientific), 1% Glutamax (Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Invitrogen), and 10% FBS in 10 cm tissue culture dishes at 37°C in 5% CO_2 atmosphere. At 80–90% confluency, the cells were dissociated with TrypLE Select (Invitrogen) and from p5 (p4 + 1) onward, HFFs were cultured in the medium containing either (i) 10% FBS or (ii) 10% hUCS. In addition, HFFs were cultured in the medium without serum supplementation and 10% knockout serum replacement (KSR; Invitrogen).

4.4. Proliferation of HFFs. The proliferation of HFFs was determined by calculating the population doubling time (PDT). The HFFs were cultured in hUCS-containing medium (HFF-hUCS) or FBS-containing medium (HFF-FBS), and the PDT was determined between p4 + 1 and p4 + 13. A total of 1×10^5 cells were plated per one well of a 6-well dish. The cells were dissociated, counted, and passaged at 80–90% confluency, which occurred at approximately 4 days after replating. At each passage, the PDT was determined using the formula PDT = [log10(NH) − log10(N1)]/log10(2), where N1 is the plated cell number and NH is the cell number at harvest, as previously described [12]. The cell proliferation test was performed in triplicate.

4.5. Preparation of Feeder Cells. HFF-hUCS and HFF-FBS from p4 + 5 to p4 + 10 were used to prepare the feeder cells. HFFs were treated with 10 μg/mL of mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours. Treated HFFs were washed 5 times with PBS, dissociated with TrypLE Select (Invitrogen), and plated on the culture dishes at a density of 5×10^4 cells/cm^2.

4.6. Culture of Human Pluripotent Stem Cells. The karyotypically normal hESC line (Chula2.hES) was derived from frozen embryos [25] between p15 and p30, and the non-integrating hiPSC line (HFSK#11.hiPS) was generated from human dermal fibroblasts using a Sendai virus carrying exogenous genes [36] between p15 and p30. Originally, both hPSC lines were derived and cultured on HFF feeder layer. The hPSC lines were continuously maintained on either mitomycin-C inactivated HFF-FBS or mitomycin-C inactivated HFF-hUCS in serum-free hPSC culture medium. The serum-free hPSC culture medium comprises 80% knockout Dulbecco's modified Eagles medium (KO-DMEM), 20% knockout serum replacement (KSR), 1% nonessential amino acid, 1% Glutamax, 1% penicillin-streptomycin, 0.1 mM β-mercaptoethanol (all from Invitrogen), and 8 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA).

The culture medium was changed daily, and the hPSC lines were mechanically passaged using a 23 G needle every 5–7 days. After continuous culturing on inactivated HFF-FBS or HFF-hUCS for at least 10 passages, the expression of pluripotent markers was determined through immunostaining, RT-PCR, and flow cytometry. In addition, karyotypic analysis and in vitro and in vivo differentiation were performed.

4.7. Immunostaining for Pluripotent Markers. After continuous culturing for at least 10 passages on either inactivated HFF-FBS or HFF-hUCS, the expression of surface and intracellular markers, including stage-specific embryonic antigens (SSEA)-3, tumor-rejection antigen (TRA)-1-60, TRA-1-81, and transcriptional OCT-4, was determined. To detect the surface markers, hPSC colonies were fixed using 4% PFA for 15 minutes, washed 3 times with PBS, and incubated with blocking solution, containing 5% goat serum or 5% rabbit serum (Sigma) in PBS, for 1 hour. The fixed cells were incubated with primary antibodies at 4°C overnight. All primary antibodies were diluted 1 : 100 with blocking solution. After overnight incubation with primary antibodies, the cells were washed 3 times with PBS, followed by incubation with secondary antibodies for 1 hour at room temperature. The secondary antibodies were diluted at 1 : 200 with PBS. Negative controls were performed without the addition of primary antibodies. After incubation with secondary antibodies, the cells were washed 3 times with PBS and incubated for 10 minutes with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for nuclear staining. Subsequently, the cells were washed again and examined under using fluorescence microscopy. The primary antibodies used in the present study were SSEA-3 (Abcam, Cambridge, MA, USA), TRA-1-60 (Chemicon Millipore, Billerica, MA, USA), TRA-1-81 (Chemicon Millipore), OCT-4 (Abcam), NESTIN (Chemicon), PAX6

(Abcam), BRACHYURY (Abcam), smooth muscle actin (SMA; Abcam), alpha-fetoprotein (AFP; Abcam), and CDX2 (Abcam). The secondary antibodies used in the present study were FITC-conjugated goat anti-rabbit IgM (Abcam), FITC-conjugated goat anti-mouse IgM (Abcam), or Cy3-conjugated goat anti-mouse IgG (Chemicon).

4.8. Gene Expression Analysis. Reverse transcription polymerase chain reaction (RT-PCR) was performed for analyzing the expression of supportive feeder cell-related genes (Activin A, FGF2, TGF-β1, and BMP4) in inactivated HFF-FBS and inactivated HFF-hUCS and the expression of pluripotent-specific genes (OCT-4, NANOG, REX1, and UTF) in hPSCs cocultured with inactivated HFF-FBS or inactivated HFF-hUCS as well as the differentiated genes (NESTIN, BRACHYURY, and AFP) in embryoid bodies (EBs). The total RNA of each condition from three independent experiments was extracted and the expression of genes was analyzed.

Total RNA from EBs, hPSCs, inactivated HFF-FBS, and inactivated HFF-hUCS was extracted using GeneJet (Fermentas, Thermo Fisher Scientific, Baltics, Vilnius, Lithuania) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to complementary DNA (cDNA) using the RevertAid H Minus First Strand cNDA Synthesis Kit (Fermentas, Thermo Fisher Scientific) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using the KAPA2G Fast HotStart ReadyMix (2x) (KAPABIOSYSTEMS, Cape Town, South Africa). PCR products were visualized in a 1% agarose gel. The PCR conditions and primers have been described elsewhere [2, 21].

4.9. Flow Cytometry. After coculture of hPSCs with inactivated HFF-FBS or inactivated HFF-hUCS feeder cells for at least 10 passages, the expression of SSEA-4 of hPSCs was evaluated using flow cytometry. At day 6 after passage, the hPSCs colonies were mechanically harvested and dissociated into single cells using TrypLE Select (Invitrogen), followed by centrifugation at 1500 rpm for 5 minutes. The cells were washed with PBS, centrifuged, and stained with FITC anti-human SSEA-4 (BioLeagent, San Diego, CA, USA) at 37°C for 1 hour. The cell nuclei were counterstained using propidium iodine (PI; Sigma). The cells were washed once in PBS, resuspended in 500 μL of PBS, and subjected to analysis using flow cytometry (BD Bioscience, San Jose, CA, USA). The experiment of flow cytometric analysis has been repeated three times.

4.10. In Vitro Differentiation. To evaluate the hPSC differentiation in vitro, the hPSC colonies were cut into small clumps and cultured in hESC culture medium lacking bFGF to induce EB formation. EBs were cultured in suspension medium for 7 days, and subsequently plated on Matrigel-coated dishes for an additional 14 days. After culturing for a total of 21 days, the cells were immunostained. The differentiated cells were fixed, permeabilized, and immunostained as described above. Differentiated cells were immunostained for ectoderm (NESTIN, PAX6), mesoderm (BRACHYURY, SMA), endoderm (AFP), and trophoblasts (CDX2) markers. In addition, RT-PCR was performed for detection of differentiated gene expression of 21-day-old EBs as described above.

4.11. In Vivo Differentiation. The in vivo differentiation of hPSCs was examined by teratoma formation assay. The protocol of teratoma formation assay was similar to our previous report [25]. In brief, a total of 1×10^6 cells of undifferentiated hPSCs were subcutaneously injected into the flank area of 6–8-week-old nude mice. Two mice were used for teratoma test of each group of hPSCs. The injected cells were allowed to differentiate for 8–10 weeks. After that, the mice were euthanized and the teratoma tissues were removed from the mice. The teratoma tissues were fixed with a 10% paraformaldehyde solution and embedded in paraffin block. The 4 μm sections were stained with hematoxylin and eosin (H&E) and examined for embryonic germ layers. The care of immune-deficient mice was conducted in accordance with the institute guideline of the Ethical Committee for Animal Laboratory Use (Approval Number 7/57).

4.12. Karyotypic Analysis. To analyze chromosomal stability, the karyotype analysis of HFF-FBS and HFF-hUCS at p4 + 13 and hPSCs after continuous culturing on two different types of feeder cells for 15 passages was performed. HFF-FBS and HFF-hUSC were incubated with 0.1 μg/mL of colcemid (Invitrogen) for 15 hours, while hPSCs were incubated for 3 hours. The cells were subsequently trypsinized and incubated in 0.075 M KCl for 20 minutes at 37°C in a 5% CO_2 atmosphere, followed by fixing with 3 : 1 methanol/acetic acid. The metaphases were spread onto microscope slides and stained using a standard G banding technique. The chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN).

4.13. Statistical Analysis. Statistical analyses were performed using SPSS software version 22.0. Student's t-test was used to evaluate differences in the rate of population doubling time and the percentage of SSEA-4 expression. The results were given as means ± standard error of mean (SEM), and values of $P < 0.05$ were considered statistically significant.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Ruttachuk Rungsiwiwut and Praewphan Ingrungruanglert contributed equally to this work.

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