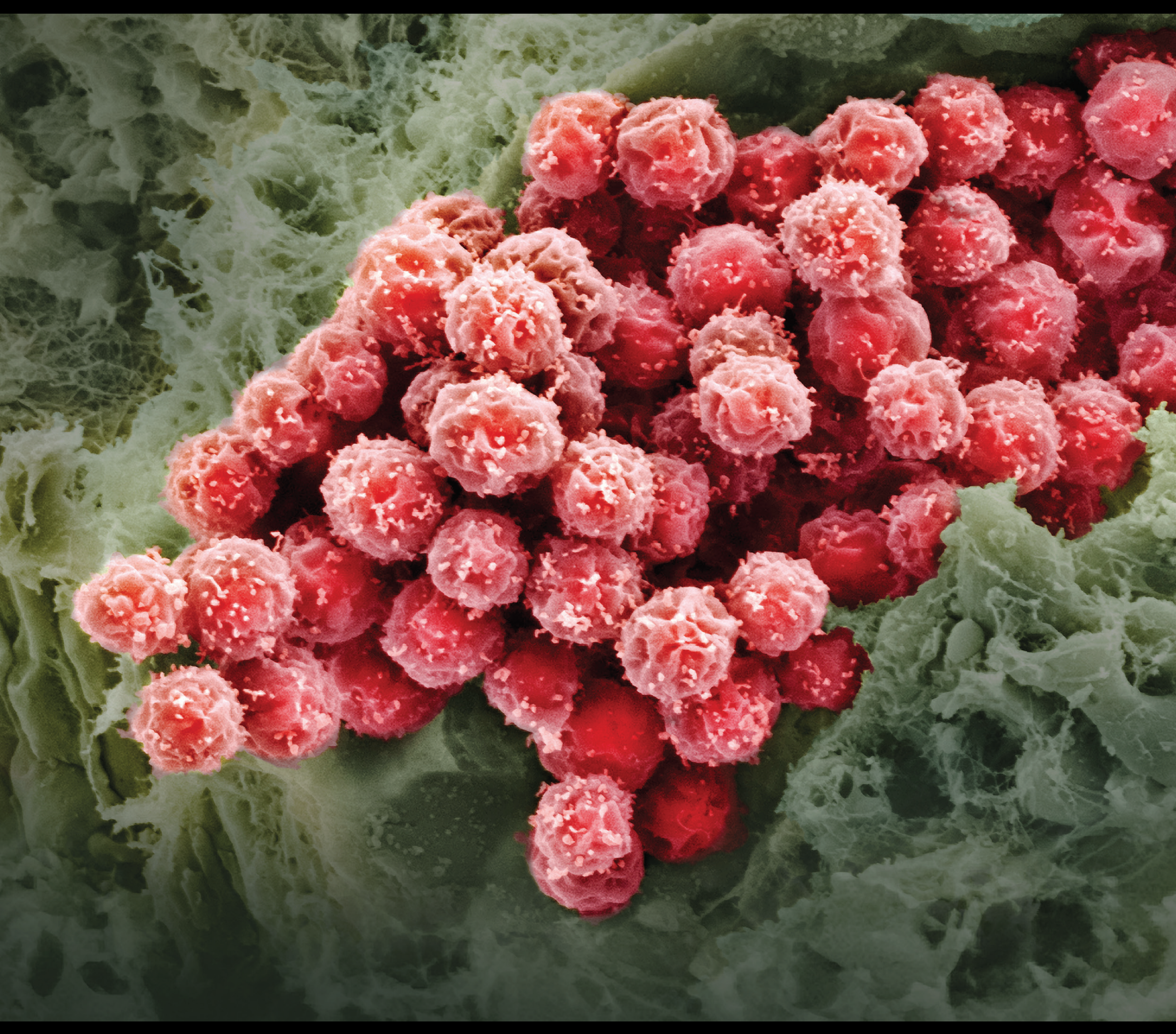
Stem Cells International

Developing Clinical Applications of ESCs and iPSCs

Lead Guest Editor: Yun-Wen Zheng
Guest Editors: Yuyou Duan and Li-Ping Liu



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Review Article

Gene Editing in Pluripotent Stem Cells and Their Derived Organoids

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With the rapid rise in gene-editing technology, pluripotent stem cells (PSCs) and their derived organoids have increasingly broader and practical applications in regenerative medicine. Gene-editing technologies, from large-scale nucleic acid endonucleases to CRISPR, have ignited a global research and development boom with significant implications in regenerative medicine. The development of regenerative medicine technologies, regardless of whether it is PSCs or gene editing, is consistently met with controversy. Are the tools for rewriting the code of life a boon to humanity or a Pandora's box? These technologies raise concerns regarding ethical issues, unexpected mutations, viral infection, etc. These concerns remain even as new treatments emerge. However, the potential negatives cannot obscure the virtues of PSC gene editing, which have, and will continue to, benefit mankind at an unprecedented rate. Here, we briefly introduce current gene-editing technology and its application in PSCs and their derived organoids, while addressing ethical concerns and safety risks and discussing the latest progress in PSC gene editing. Gene editing in PSCs creates visualized *in vitro* models, providing opportunities for examining mechanisms of known and unknown mutations and offering new possibilities for the treatment of cancer, genetic diseases, and other serious or refractory disorders. From model construction to treatment exploration, the important role of PSCs combined with gene editing in basic and clinical medicine studies is illustrated. The applications, characteristics, and existing challenges are summarized in combination with our lab experiences in this field in an effort to help gene-editing technology better serve humans in a regulated manner. Current preclinical and clinical trials have demonstrated initial safety and efficacy of PSC gene editing; however, for better application in clinical settings, additional investigation is warranted.

1. Introduction

Pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are extensively used and considered to be viable cellular therapies against complicated and malignant diseases, like leukemia [1]. Additionally, autologous stem cells, when used as a gene therapy vehicle, can minimize host vs. graft responses and facilitate the correction of mutated genes, consequently correcting an enzyme/protein deficiency and treating a variety of diseases [2]. For instance, gene editing in hematopoietic stem cells (HSCs) has been shown to correct the genotype

of transfusion-dependent β-thalassemia in human cells [3, 4]. Furthermore, transplantation of gene-edited HSCs and progenitor cells (HSPCs) to a leukemia patient with a simultaneous HIV-1 infection was proven to be safe [5]. These outcomes encourage more work in the field of genetic therapy for inherited and currently incurable diseases.

Gene editing is broadly applied in disease modeling [6], exploring disease mechanisms [7] and disease targeting treatments [8]. Jennifer Doudna and Emmanuelle Charpentier, who pioneered gene-editing technology, were awarded the 2020 Nobel Prize in Chemistry, driving an unprecedented boom in the field [9]. Although gene editing is leading to breakthroughs in regenerative medicine and represents a major innovation in medical technology, several challenges remain, including ethical issues and off-target effects. In 2018, the controversial case of a Chinese team who modified the CCR5 gene in the embryonic cells of a pair of twin babies through clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 gene-editing technology sparked intense debate [10]. The scientists were attempting to provide the babies with partial immunity to HIV; however, the experiment raised serious ethical issues surrounding genetic manipulation, especially given that the genetically edited twins were exposed to potentially detrimental and fatal mutations. This case also serves as a warning that despite the continuous development of gene-editing technology, the challenges of targeted deletion, retention of foreign genetic material, and viral infection can result in unpredictable health hazards. This technology can aid the progression of medicine only when used under strictly controlled parameters.

The applications and potential expansion of gene editing of PSCs and their derived organoids are endless. Here, we systematically analyze and compare several gene-editing methodologies and provide examples of how gene editing has been used in the treatment of diseases, construction of disease models, and exploration of disease mechanisms. Combined with the experiences and ongoing work in our lab, we have expounded the perspectives as well as opportunities associated with gene editing in PSCs and their derived organoids.

2. Superiority of PSCs and Gene Editing for Precision Medicine and Therapy

2.1. PSCs and Their Organoids. PSCs are self-renewing with infinite proliferation and multipotency. In 2006, Shinya Yamanaka was the first Japanese scholar to use a viral vector to introduce four transcription factors (Oct4, Sox2, Klf4, and c-Myc) into somatic cells to obtain iPSCs, which revolutionized the field of regenerative medicine [11]. Like ESCs, iPSCs are pluripotent and can proliferate indefinitely. However, unlike ESCs, iPSCs are generated from somatic cells and do not have ethical implications; more importantly, they allow for the isolation of patient-derived cells that carry all of the genetic alterations that cause a specific disease. Patient-derived cells provide an experimental system for the construction of patient-derived disease models for pathogenesis investigation and drug screening, as well as cell-based transplantation therapies [12]. Organoids derived from PSCs are three-dimensional cell masses that contain multiple differentiated cells that are highly similar to the respective organ or tissue; thus, they have an advantage in imitating the developmental process of human organs. Such organoids reflect the human environment more comprehensively than conventional stem cells, enabling the identification of pathological mechanisms that more accurately resemble physiological conditions, owing to their consanguinity advantage over animal models. Therefore, PSCs and their derived organoids, which possess irreplaceable advantages over other models, have already contributed much to this field, including in the treatment of heart valve disease [13] and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [14]. Hence, PSCs and their derived organoids have established their position in the field of precision medicine.

2.2. Current Status of Gene Editing. Genome editing tools can be divided into four types that are described here according to the timeline of their discovery from the earliest to the most recent: meganucleases (MegNs, also termed homing endonucleases), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR RNA-guided nucleases. The characteristics of each of these four editing tools relative to existing genetic technologies, as well as their advantages and disadvantages, are summarized in Table 1.

Meganucleases (MegNs) rely on the length of the target sequence and the structure of the DNA contact surface to specifically, accurately, and effectively identify the target. The mechanism of DNA recognition by MegNs involves binding patterns of protein side chains and nucleotide bases [15], deformation of groove dimensions, electrostatic distribution of the molecular surface, and additional contacts within and near the minor groove [16]. Binding affinity and cleavage activity sometimes have different efficiencies. Identifying a relatively good performing MegN can consume enormous time and cost [17]. Different substrates change the activity and/or specificity of the inherent function of MegNs [18], illustrating the importance of the context dependent protein–DNA interactions.

Zinc finger nucleases are constructed by fusing a DNA cleavage domain, like the Type II restriction enzyme FokI, to a zinc finger protein (ZFP) [19], enabling it to cleave the target DNA recognized by the ZFP. Four key amino acid residues of the α-helix specifically contact each base of the DNA target site; altering these residues allows for the targeting of any desired sequence. However, the intermolecular interaction among individual zinc fingers alters the binding force with the DNA, making the optimization of assembling and testing multiple pairs of ZFN engineering extraordinarily complex.

Transcription activator-like effector nucleases (TALENs) evolved from transcription activator-like (TAL) effectors, which are transcription activators that have peculiar properties of DNA recognition. The monomeric protein chains of TALENs bind DNA in a right-handed spiral manner, without inducing any bend or other substantial structural

Table 1: Characteristics of current gene editing technologies and their advantages and limitation.

	Identifying patterns	Cleavage domain	Recognition length	Identification conditions	Minimum identification unit	Accuracy	Molecular weight size of editing tools	Operational difficulty	Off-target level	Cytotoxicity	Advantages	Limitations
MegNs	Binds specific DNA through protein-DNA interactions	4 bp	Double-stranded DNA sequences of 12 to 40 base pairs	Monomer, target DNA	Indeterminate	+++	200-400 aa	+++	+	+	Higher specificity	Limited variety; difficult to retrofit
ZFN	Binds specific DNA through protein-DNA interactions	5-7 bp	9-18 bp per ZFN	Dimers, 3 bp units of target DNA	3 bp	++	500-1300 aa	++	++	++	Mature platform; more efficient than homologous recombination	High off-target rate; low specificity; design dependent on upstream and downstream sequences; only for in vitro operations
TALEN	Binds specific DNA through protein-DNA interactions	5-7 bp	14-20 bp per TALEN	Dimer, transcription activator-like effector or transcription activating effector nuclease 5′ preceded by a central structural domain of T	1 bp	++	900-1100 aa	++	++	+++	Unrestricted target sites; easier design than ZFN; higher specificity	Cumbersome module assembly; requires large sequencing effort; high cost
CRISPR	Binding of specific DNA through base complementary pairing and protein-DNA interactions	0 bp	20 bp	Monomer, 3′ sequence for NGG's guide RNA	1 bp	++	1300-1500 aa	+	+	+	High rate of gene modification; diverse gene regulation; allows simultaneous knockout of multiple target loci; precise targeting inexpensive	No PAM in the pretarget region cannot be cut; transfection difficulties

PAM: protospacer adjacent motif.

distortion. Each base is recognized by a highly conserved sequence of typically 33–35 amino acids. Based on the one-to-one corresponding relationship [20], it is relatively easy to assemble a specific identification domain. TALENs exhibit relatively high precision and flexibility.

CRISPR, clustered regularly interspaced short palindromic repeats, is named for the conserved primitive sequence structure of the bacteria and archaea immune defense system [21]. CRISPR-associated protein 9 (Cas9) is an enzyme with cutting and nucleotide-binding protein domains. Cas9 binds to a single guide RNA (sgRNA), which is engineered by fusing CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) into a single RNA molecule. If the CRISPR RNA (crRNA) is followed by a protospacer adjacent motif (PAM), the complementary target DNA sequence is precisely sheared. In the editing process, the RNA-DNA interaction is the cornerstone of DNA recognition, which differs from MegNs, ZFNs, and TALENs. The superiority of this approach in gene editing is that synthesis of a sgRNA is the only component researchers need to construct; thus, complicated protein domain manipulation is no longer needed.

Among gene-targeting nucleases, MegNs are the most difficult to synthesize. However, they exhibit small sizes, single-chain structures, and high specificity. TALENs are good at targeting specific individual DNA base pairs without affecting the activity or binding force of the nucleases. Only a pair of TALENs can accurately bind to a double-strand break, which may result in a low probability of off-target effects. Engineering and redesigning specific recognition of DNA-binding proteins are a challenging area of research and development. Proteins and DNA have different molecular interface compositions, and their complex relationships include directional hydrogen bonds, electrostatic contacts, ordered solvent molecules, and bound counterions, making protein–DNA interactions elusive and unpredictable. The CRISPR/Cas9 system is the most operable tool because of its RNA-DNA recognition characteristics, which avoids complex protein engineering.

3. Applications of Gene Editing in PSCs and Their Organoids

3.1. Ex Vivo Organoid Models and beyond. The combination of stem cell and gene-editing technologies has led to new innovations in the field of medicine, opening up a new wave of personalized and precision medicine. The creation of organoid disease models through genetic engineering and gene-editing technologies has led to the elucidation of underlying mechanisms of major diseases, with clinically translatable applications. Table 2 summarizes the more mature research applications of current gene-editing technologies in basic medicine. Both gain- and loss-of-function phenotype disease models can be created by CRISPR/Cas9 in human iPSCs, serving as an efficient tool for human genetic functional studies and drug screening [22].

3.1.1. Visualization of Cell Fate. PSC-derived organoid models can be used to visually trace the fate of cells through development or growth, by constructing knockin reporter genes for specific target genes. To study vocal dysphonia, caused by vocal fold (VF) disorder, a hiPSC-derived VF model with a GFP reporter was transfected via TALEN to simulate the development of VF epithelial cells in utero. This system consisted of a 3D *in vitro* visualized system for VF mucosal disease modeling [23]. To trace the process of melanocyte development and reconstitution into structured tissue, visualizing melanocytic stem cells is key; this is an active area of research in our lab.

3.1.2. Functional Evaluation. To elucidate physiological mechanisms, gene editing and PSC differentiation models may be a perfect combination. Through CRISPR/Cas9-based genome editing technology, key segmentation-clock gene expression showed phase changes in the hPSC-derived presomitic mesoderm. This provided insights into the human segmentation clock related to diseases associated with human axial skeletogenesis [24]. Targeting endogenous genes in hPSCs with small molecule-assisted shut-off helped reveal how FOXG1 syndrome gene dosage affects the generation of neurotransmitter [25]. CRISPR/Cas9 gene-editing produced 11 variants of the HCM-causing mutation in genome-edited human pluripotent stem cell-cardiomyocytes (hPSC-CMs). The main hallmarks of HCM were exhibited through phenotypic rescue and functional evaluation, providing novel putative diagnostic biomarkers and gene-based therapeutic targets for HCM [26].

3.1.3. Role of Pathogenic Genes. The ability to selectively modify genes is important to identify the role of genes in specific pathological changes. In one study, genetically modified hPSCs were generated by CRISPR/Cas9 editing revealing that noncoding gene variants have undeniable effects on *GATA6* gene expression and penetrance during pancreatic agenesis [27]. Using CRISPR/Cas9, the *DISC1* gene in iPSCs was modified, altering the relationship among molecular function, risk factors, and the particular cellular context in psychiatric diseases [28]. Increased cardiac microtissue contraction was caused by CM-associated TNNT2 variants, thus, revealing the gene variants associated with hypertrophic and dilated CMs [29]. Using CRISPR/Cas9, QKI-deficient hESCs (hESCs-QKI [del]) were generated. The analyses of the physiological role of QKI in CM differentiation, maturation, and contractile function demonstrated that QKI was a critical alternative splicing regulator in human cardiogenesis and heart function [30]. Neurooncological ventral antigen 1 (NOVA1) plays a critical role in neural development. The reintroduction of the archaic allele into hiPSCs using CRISPR/Cas9 technology revealed a discrepancy between controls and edited hiPSC-derived cortical organoids. This suggested that NOVA1 may have functional consequences for human neural phenotype evolution [31]. Susceptibility to herpes simplex virus-1 (HSV-1) of hPSC-derived cortical neurons with *SNORA31* mutations is increased, revealing the neuron-intrinsic immunity mechanism of HSV-1 infection [32].

Table 2: Landmarks and trends of gene editing in life and medical sciences.

	Editing methods	Target cells	Targeted genes	Virus transfection	Animal models	Points	Year
MegN	KI	mESCs	Villin locus	Yes	/	Induction of gene-targeting and homologous recombination events	1998 [45]
	M	293T	RAG1 locus	No	/	Targeting endogenous genes; low targeting efficiency; with cytotoxicity	2009 [46]
	KI	293T	π10 locus	Yes	/	Delivering meganucleases into cells in a transient and dose-controlled manner; low targeting efficiency; with cytotoxicity	2011 [47]
ZFN	KI/GFP	hESCs	OCT4 locus、AAVS1 locus	Yes	m	Gene targeting in hESCs	2009 [48]
	M	hESCs	Genomic α-synuclein locus (SNCA)	No	/	Genome editing in hESCs; off-target detection needs to be improved; targeting efficiency needs to be enhanced	2011 [49]
	KO	hiPSCs	LRRK2 (sigma)	No	m	Parkinson's pathogenesis; patient-derived iPSCs; low targeting efficiency; with cytotoxicity	2013 [50]
	M	hiPSCs	MAPT	Yes	/	Designed mutation iPSCs; FTD pathogenesis; targeting efficiency needs to be enhanced	2018 [51]
	KO	FRT cells	CFTR	No	r	Disease targets; designed KO model	2020 [52]
TALEN	KI/GFP	hPSCs	OCT4 locus	No	/	Genetic engineering for hPSCs; targeting efficiency like ZFN	2011 [53]
	KO	hiPSCs	TNNT2, LMNA/C, TBX5, MYH7, ANKRD1NKX2.5	Yes	/	Human-based KO cell model *in vitro*; greater freedom and flexibility in target site selection than CRISPR	2017 [36]
CRISPR	KI/GFP	hiPSCs	AAVS1 locus	No	/	3D organoid models; GFP; mechanistic studies	2019 [23]
	KO	hiPSCs	AAVS1 safe harbor locus	No	/	TetO inducible system; feasibility and reversibility of CRISPRi; high off-target efficiency	2016 [54]
	M	hiPSC	FBN1	No	/	Vascular models, human iPSCs; pathogenesis of MFS	2017 [55]
	KI/GFP	hESCs	gRNAs made from the lentiGuide-puro construct	Yes	/	A genome-scale screening; hESCs; impaired differentiation	2019 [56]
	KO	hPSCs	NRL	No	/	A 3D organoid model; disease pathogenesis; high targeting efficiency	2021 [43]

ESCs: embryonic stem cells; FTD: frontotemporal dementia; FRT: Fischer rat thyroid; h: human; iPSCs: induced pluripotent stem cells; KI: knockin; KO: knockout; m: mouse; M: mutation; MFS: Marfan syndrome; PSCs: ESCs and iPSCs; r: rat.

3.1.4. Mechanism Exploration of Known Mutations. To clarify the mechanism of action of known mutations, gene-editing interventions were carried out on pathogenic genes in patient-derived iPSCs or organoids. Mutations in *TSC1* or *TSC2* are known to disturb multisystem development in tuberous sclerosis complex (TSC) [33]. Blair et al. established TSC models using CRISPR-Cas9, and second-hit somatic mutations were found to have an essential effect on the large heterogeneity of tuber number and size among TSC patients [33]. Similarly, the pathogenesis of Cockayne syndrome was revealed using the gene-corrected CS-iPSC (GC-iPSC) model [34]. Knocking out different regions of the TTN gene, mutations in which are known to cause familial dilated cardiomyopathy, revealed that Cronos is crucial to sarcomere formation in human CMs [35]. iPSC-derived cardiac myocytes with KO mutations, mediated by TALENs, could also provide a platform for studying biological function and the pathology of genetic variants in cardiovascular diseases [36].

3.1.5. Functional Exploration of Unknown Mutations. To explore unknown mutations and their effects, comparing PSC models with and without mutations may be effective. For instance, comparing CRISPR/Cas9-based gene editing in hPSC-derived neurons and isogenic controls, it was determined that the internal mechanism of neuronal network dysregulation was due to the *V337M* tau mutation impairing the cytoskeleton in the axon initial segment [37]. Amyotrophic lateral sclerosis (ALS) has long been seen as an energy metabolism-related disease. In another study, using iPSC-derived motor neuron (MN) as a disease model and CRISPR/Cas9 as a tool to correct FUS mutations, surprisingly, metabolic dysfunction was found to not be the underlying cause of the ALS-related phenotypes [38]. Establishing

a stepwise model of congenital neutropenia to acute myeloid leukemia (AML), derived from congenital neutropenia patient-derived iPSCs by CRISPR/Cas9, revealed that BAALC and MK2a phosphorylation may be excellent targets for preventing leukemogenic transformation or eliminate AML blasts [39].

3.1.6. Screening for Pathogenic Genes. To screen for unknown genes responsible for diseases, creating mutations and overexpressing or inhibiting gene expression in PSCs and organoid models could help clarify and define key genes of interest. Using CRISPR/Cas9 in hPSCs with an E50K mutation in the optineurin (*OPTN*) gene resulted in differentiation of the cells into retinal ganglion cells. This method establishes an *in vitro* model of neurodegeneration and provides the opportunity to develop novel therapeutic approaches for glaucoma [40]. Generating TREM2 mutation hPSCs using CRISPR/Cas9 in human microglia-like cells, demonstrated TREM2 expression related to amyloid plaque metabolism, which might advance the current understanding regarding Alzheimer's disease (AD) [41]. Moreover, CRISPR/Cas9-mediated FOXO3-enhanced or FOXO3-related protein ablated hESC differentiation into hVSMCs. Vascular protection function was demonstrated for FOXO3, and novel mechanistic insights could be investigated [42]. By knocking out individual 15q13.3 microdeletion genes using CRISPR/Cas9, downstream effects in pathways in neuropsychiatric disorders and interactions between genes were revealed [7]. CRISPR/Cas9 engineered NRL-deficient hESCs- (*NRL* [-/-]) derived retinal organoids demonstrated that NRL is required to define rod identity. Otherwise, S-cone-like cells would develop by default into photoreceptor cell types [43]. *RAP1*-deficient hESCs, also generated with CRISPR/Cas9, revealed that RAP1 may play an important role in aging-associated disorders by telomeric and nontelomeric regulation of cell homeostasis [44].

3.2. Mutation Correction and Potential Treatment. Gene editing in PSCs through knockout (KO) or knockin (KI) genes enables observation of phenotypic changes and, potentially, the identification of disease targets for clinical research and therapy. Table 3 summarizes the uses and existing challenges of gene-editing technologies in the clinical treatment of different diseases.

3.2.1. Immunogenicity Reduction. Immunological rejection is common following organ transplantation. A study based on hiPSC gene editing found that the *ETV2* mutation generates exogenous organs with reduced immunogenicity [82]. In addition, HLA-C-retained immunocompatible donor iPSCs edited by disrupting both HLA-A and -B alleles that evade T cells and natural killer cells *in vitro* and *in vivo* [83].

3.2.2. Patient PSC-Derived Disease Models. Disease models were constructed by PSC differentiation and gene editing. For fragile X syndrome (FXS), an inherited intellectual disability in males, FMR1 was reactivated after the heterochromatin status switched, by targeting demethylation of the CGG expansion using dCas9-Tet1/single guide RNA (sgRNA) in FXS iPSCs. This suggested potential therapeutic strategies for FXS [77]. iPSC-derived cerebral organoids with Alzheimer's disease features and CRISPR/Cas9-edited isogenic lines were used to screen and test blood-brain barrier-permeable drugs; this system may illuminate strategies for precision medicine therapy [8]. Patient iPSC-derived CMs were disputed with *RAF1* mutations by CRISPR/Cas9, and mitogen-activated protein kinase 1/2 (MEK1/2) and extracellular regulated kinase 5 pathways were found to serve as new therapeutic targets to treat HCM [62]. Fibrin-based engineered heart tissue was generated from *DNMT3A* knockout hiPSC-derived CMs. DNA methylation plays an important role in CM development, which suggests that it could be a potential target for cardiac therapy [84]. Generation and subsequent conversion of *CTNS*-KO lines into iPSCs or kidney organoids helped establish disease models. Cystine-depleting drugs were tested in the model [85].

3.2.3. Refractory Disease Models. There are many refractory diseases without effective treatment, some of which are fatal. Although the best therapy cannot be confirmed at once, potential targets can be identified through gene editing carried out on hiPSC models. Both long-QT syndrome and short-QT syndrome are fatal inherited arrhythmogenic syndromes, which can cause apopsychia and death. A human ether-a-go-go-related gene-deficient CM model [6] with a pathogenic mutation, or mutation-corrected hiPSC-CMs [86], was established separately using CRISPR/Cas9, providing clues for malignant hereditary arrhythmia [6]. Moreover, the underlying molecular mechanism of congenital hepatic fibrosis (CHF) remains unclear. PKHD1-KO and heterozygous mutated PKHD1 iPS clones were established. Following analysis of the composition of serum, interleukin-8 (IL-8) and connective tissue growth factor (CTGF) were found to be essential in CHF pathogenesis. Thus, IL-8 and CTGF could be seen as new therapeutic candidate targets for CHF [87]. Based on a CRISPR/Cas9 KO strategy, a study found that adhesion, metastasis, and propagation of somatic cancer cells were closely related to OCT4A, indicating that targeting OCT4A may be a promising combination therapy for human cancers [88]. A significant decrease or increase in the expression of knockin and knockout *PARK2*, respectively, in iPSCs by CRISPR/Cas9 technology revealed that the *PARK2* mutation, related to catechol-O-methyltransferase (COMT), may make a difference in the initial process of Parkinson's disease; treatment with central COMT inhibitors may thus be useful [89]. In another study, researchers combined iPSCs and CRISPR/Cas9 technologies to develop a clonal evolution model of AML. Cell-autonomous dysregulation of inflammatory signaling was identified as an early and persistent event in leukemogenesis, which suggested a promising early therapeutic target [90].

3.2.4. Rescue Models of Definite Etiology. Sometimes, as the basis of a definitive etiology, gene-editing treatment methods can be manipulated in PSC-derived models to identify rescue treatments. PSC-derived alveolar epithelial type 2 cells (AEC2s) provide a platform for disease modeling, exhibit self-renewal capacity, and display additional AEC2

Table 3: Current challenges of gene editing in different diseases.

	Disease names	Related genes	Editing technologies	Model types	Clinical trials	Challenge points and limitation	Years
Respiratory disorders	CF	CFTR	CRISPR	Organoids	\	Proof of concept only, gene editing off-target effects; needs further evaluation for safety	2013 [57]
			TALEN	Cells	\	Delivery efficiency needs to be improved; targeting accuracy needs to be improved	2019 [58]
			CRISPR	Patient-derived cells	\	Difficulty of in vivo delivery, genetically corrected airway stem cell transplantation and recovery of in vivo mucus cilia transport	2021 [59]
	NSCLC	PD-1	CRISPR	\	Phase I (first)	Underexpansion and low response rate of T cells after gene editing; small study sample	2020 [60]
Circulatory disorders	HC	Protein PCSK9	MegNs	Macaques	\	Off-target effects, with cytotoxicity, immunogenicity to be overcome	2018 [61]
	NS-associated HCM	RAF1	CRISPR	Patient-derived cells	\	RAF1 lacks a nuclear localization sequence (NLS), its translocation mechanism is unknown, and the molecular mechanism of the disease needs to be further explored	2019 [62]
	HC	Ldlr	CRISPR	Mouse	\	Genome editing efficiency to be improved and off-target effects to be overcome	2020 [63]
	NS-associated HCM	LZTR1	CRISPR	Patient-derived cells	\	Proof of concept only, needs in vivo evaluation, patient-specific iPSC-CM model is still immature and needs to be improved	2020 [64]
	LDS	TGFBR1	CRISPR	Patient-derived cells	\	Needs further proof from in vivo experiments, off-target effects	2021 [65]
Infectious diseases	HIV	CCR5	ZFN	\	Yes	A serious adverse event was associated with the infusion of ZFN-modified autologous CD4 T cells, with off-target safety issues to be overcome	2014 [66]
			ZFN	Mouse	\	Reduced proliferation of editorial cells transplanted in vivo, delivery efficiency and targeting accuracy need to be improved	2013 [67]
			TALEN	Cells	\	Delivery efficiency and targeting accuracy need to be improved	2015 [68]
			CRISPR	Mouse	\	Safety issues to be further assessed	2017 [69]
			CRISPR	\	Yes	Off-target efficiency needs to be improved, targeting accuracy needs to be improved, and generalizability needs to be further assessed	2019 [5]
			CRISPR	Patient-derived cells	\	Off-target efficiency needs to be improved, and targeting accuracy needs to be improved	2020 [70]
Hematologic disorders	TDT & SCD	BCL11A	CRISPR	\	Yes	No comprehensive genomic analysis of clinical samples and the generalizability of the results needs to be further determined	2021 [71]
	TDT & SCD	HPFH5	CRISPR	Cells	\	Off-target effects to be overcome and safety to be improved	2016 [72]
	SCD	HBB	CRISPR	Mouse	\	The off-target efficiency needs to be reduced, and more sensitive off-target analysis methods are needed	2019 [73]
	SCD	HBB	CRISPR	Humanized mouse	\	Delivery methods to be optimized and delivery efficiency to be improved	2021 [74]
	ALL	CD52	TALEN	\	Yes	Immunogenicity needs to be further reduced; safety needs to be further tested; small sample size	2017 [75]
	MM	TRAC、CD52	TALEN	Mouse	\	Delivery efficiency needs to be improved, and long-term safety issues need to be further studied	2019 [76]

Table 3: Continued.

	Disease names	Related genes	Editing technologies	Model types	Clinical trials	Challenge points and limitation	Years
Neurological disorders	FXS	FMR1	CRISPR	Mouse	\	The off-target efficiency needs to be reduced, more sensitive off-target analysis methods are needed, and safety issues need to be further tested	2018 [77]
	AD	TREM2	CRISPR	Humanized SCD mouse	\	Further analysis of the mechanism of action is needed to find effective therapeutic targets for disease treatment	2020 [78]
	CD	ASPA	TALEN	Mouse	\	Proof of concept only, how to achieve sustained efficacy remains to be addressed, and the issue of safety still needs to be improved	2020 [79]
Ophthalmology	XLRP	RP2	CRISPR	Organoids	\	Retinal-like organs are still immature and need further improvement	2020 [80]
	LCA10	CEP290	CRISPR	Mouse	Yes	Impact of individual differences on safety of off-target effect delivery, durability of efficacy to be further assessed	2019 [81]

AD: Alzheimer's disease; ALL: acute lymphocytic leukemia; CD: Canavan disease; CF: cystic fibrosis; FXS: fragile X syndrome; HC: hypercholesterolemia; HCM: hypertrophic cardiomyopathy; iPSC-CMs: iPSC-derived cardiomyocytes; LDS: Loeys-Dietz syndrome; LCA10: Leber congenital amaurosis type 10; MM: multiple myeloma; NS: Noonan syndrome; NSCLC: non-small-cell lung cancer; SCD: sickle cell disease; TDT: transfusion-dependent β-thalassemia; XLRP: X-linked retinitis pigmentosa.

functional capacities. In iAEC2s generated from a child with severe lung disease carrying an *SFTPB* mutation, the mutation was corrected by CRISPR-based gene editing rescued surfactant processing in AEC2s [91]. Calcium ion plays a central role in heart failure development; CRISPR/Cas9-mediated CRISPLD1-KO led to dysregulated Ca^{2+} handling in hPSC-CM. This study provided new evidence on the critical role of Ca^{2+} in heart failure pathophysiology; simultaneously, novel candidate genes were found for therapeutic interventions [92]. Excision of the FXN intron by CRISPR/Cas9 in iPSC-derived dorsal root ganglia organoids rescued molecular and cellular deficits of the disease. This system revealed several pathological mechanisms for repairing complex neuronal circuits [93]. iPSCs carrying a heterozygous K219T mutation in *LMNA* generated an iPSC-based model of LMNA-cardiomyopathy (CMP). When corrected by CRISPR/Cas9, the functional and molecular defects of the disease model were rescued, describing a new pathogenic mechanism for the conduction defects associated with LMNA-CMP [94]. Another study using a similar strategy identified the underlying mechanism of LMNA-CMP conduction abnormalities [95]. Using patient-derived iPSCs and CRISPR/Cas9 engineering to develop a Leigh syndrome (LS) model, mechanistic insights and potential interventional strategies were indicated for a rare mitochondrial disease [96]. Marfan syndrome (MFS) is a genetically inherited connective tissue disorder; a vascular model derived from MFS patient-iPSCs was used to assess the molecular mechanisms. A *FBN1* mutation, a critical pathogenic factor of MFS, was corrected by CRISPR-based editing, and abnormalities of the model were subsequently rescued, thus identifying novel targets for treatment [55]. Splicing defects in cystic fibrosis were corrected by allele-specific genome editing with AsCas12a-crRNA nuclease system, paving the way for a permanent splicing correction in genetic diseases [97].

3.2.5. In Vivo Transplantation to Validation. Some mutations can be rescued by gene editing, and gene-rescued PSCs can differentiate into mature cells and be transplanted into animal models, improving and possibly curing the animal. TWIK-related spinal cord K^+ channel (TRESK) is implicated in nociception and pain disorders; a CRISPR/Cas9-corrected TRESK function-related mutation, F139WfsX2, showed a reversal in neuronal excitability. This suggests TRESK activators may be a promising therapeutic approach to pain and migraine [98]. iPSC-based cell therapy was developed for Canavan disease by introducing the aspartoacylase (*ASPA*) gene into patient iPSC-derived neural progenitor cells or oligodendrocyte progenitor cells using TALEN-mediated genetic engineering [79]. The approach established in this study provides a robust proof of principle for cell therapy strategies. *BEST1* mutant iPSC-derived retinal pigment epithelium models in the study showed that gene augmentation or gene editing had equal efficacy, which guides some genotypically diverse disorders [99]. In the late stage of diabetes, patients must regularly inject exogenous insulin. In this study, researchers used CRISPR/Cas9 to correct a diabetes-causing pathogenic variant in iPSCs derived from a patient with Wolfram syndrome. After transplantation, the diabetes phenotype was rescued in mice [100]. The iPSC-derived Duchenne muscular dystrophy disruption model using CRISPR/Cas9 offers new options for restoring muscle function, potentially treating patients in the future [101].

3.3. Risk Control for Ethics and Off-Target Effects. Ethical issues have always been unavoidable in the context of gene editing [102]. CRISPR/Cas9-mediated adenine base editors can correct STAT3 p.R382W in patient-derived iPSCs, providing a potential treatment for STAT3-hyper IgE

syndrome; however, for clinical translation, safety and ethical implications still need to be resolved [103]. For human medical development, ethics should be a priority and appliance stringently monitored, but also not be a stumbling block. The International Society for Stem Cell Research recognized this and permitted heritable changes to the human genome under the premise of safety [104]. Safety must always be a crucial prerequisite for clinical applications. Indeed, various promising stem cell treatments were stopped owing to the carcinogenic potential of the cells. Meanwhile, research using genome-engineering strategies has demonstrated the protective effect of a suicide system for inactivating dividing cells. In this study, human ESCs with homozygous modifications of *CDK1* exhibited normal morphology, self-renewing capacity, and differentiation capacity compared with control hESCs. Researchers also established a system to assess and quantify the safety of cell-based therapies [105]. Although a second-generation PCSK9-specific MegN showed reduced off-target cleavage, it still appeared at ~30 off-target cleavage sites. Cells derived from human iPSCs may provide a perfect *in vitro* model for observing the propensity to cleave at off-target sites [61]. Safeguard mechanisms ameliorate the potential cell therapy risks; for example, one metabolic engineering study using genome editing methods to disrupt uridine monophosphate synthetase generated a transgene-free safety switch for cell therapy [106].

3.4. Future Perspectives. Although viral vectors are known to have high delivery efficiency, they can be double-edged swords, with continuous expression of CRISPR/Cas9 nuclease and gRNA causing off-target mutagenesis and immunogenicity. Off-target risk has always been a major concern for genetic treatment; however, through the use of PSC culture and differentiation technology, cells that are deemed to be safe can potentially be used for clinical applications. At the same time, more studies that are committed to safe and efficient gene-editing strategies are needed, similar to those described below.

3.4.1. Transfection. A nanovesicle-based delivery system, NanoMEDIC, delivers large molecules, such as ribonucleoprotein; the nanovesicles are cleared within 3 days [107]. CRISPR/Cas9 RNA-guided endonucleases (RNP) can be transported to certain cells by modifying the surface affinity of the extracellular vesicles for certain cells [108], both of which potentially reduce off-target risks and improve targeting efficiency. Moreover, a technically simple system has been described that employs electroporation to significantly enhance genome targeting capabilities in primary human hematopoietic cells [109].

3.4.2. Base Editing. Cytosine and adenine base editors (CBEs and ABEs) are powerful tools for single-base modification. However, editor components, DNA repair proteins, and local sequence context interact, resulting in unpredictable editing outcomes. Researchers who focused on illuminating base editing have provided refined and novel insights, which may improve the precision of base editing [110]. By applying SpCas9-ABE (PAM recognition sequence: NGG) and xCas9-ABE (PAM recognition sequence: NGN) to cystic fibrosis intestinal organoids, genetic and functional repair was obtained. Furthermore, no off-target mutations were detected, indicating that ABE may be safely applied in human cells [111].

3.4.3. Homology Repair. Nonhomologous end-joining (NHEJ), microhomology-mediated end-joining (MMEJ), and homology-directed repair (HDR) are the three main types of cellular DNA repair machinery. To determine the most efficient HDR strategy, researchers introduced different forms of donor DNA and observed that editing with a 400 bp dsDNA repair template increased the efficiency of repair [112]. Combining the small molecular compounds M3814 and trichostatin A inhibited NHEJ repairs predominantly and increased HDR efficiency, which potentially improves the efficiency of knockins [113]. MMEJ-based therapeutic strategies could be used in diseases that are associated with microduplications [114].

3.4.4. Newly Developed Editing Tools. Prime editing is a genome editing technology combining Cas9-nickase and reverse transcriptase with greater precision than Cas9-mediated HDR. When performed, nearly no off-target effects are observed; thus, it has potential in future clinical applications to safely repair human monogenic diseases [115]. Furthermore, the *Natronobacterium gregoryi*-derived Ago protein demonstrated nickase activity at 37°C [116] five years after the technology had been thrown out. It will be interesting to observe the applications of this technology going forward.

Although various gene-editing methods have emerged, their broad and direct use in clinical settings remains a long road ahead.

4. Conclusions

The rapid advancement of genome editing technologies, from MegNs to CRISPR, has improved the operability, efficiency, and safety of gene editing. The combination of gene editing and stem cell technologies has advanced the research and development of the life and medical sciences. Through knockin and knockout technologies, human genetic and pathogenic mechanisms of disease can be better explored, and gene expression and disease progression can be traced. Drug development can also be accelerated, contributing to the advancement of personalized precision gene therapy for inherited diseases.

The existing gene-editing technologies each have their particular characteristics and advantages, but all have some corresponding challenges. Although MegNs have high specificity and low cytotoxicity, they are difficult to manipulate, limited in variety, and time-consuming, and it is expensive to design sequence-specific enzymes. ZFNs, although a relatively mature platform and more efficient than homologous recombination, are highly off-target and cytotoxic, have low specificity, are sequence-dependent upstream and downstream, and are only suitable for *in vitro* manipulation.

Although TALENs are easier to design than ZFNs and their targets are not restricted, their modules are cumbersome to assemble, require extensive sequencing work, and are costly and cytotoxic. CRISPR has a high rate of gene modification and diverse gene regulation, enables simultaneous knockdown of multiple targets, is precise in its targeting, has a low off-target rate, is inexpensive, and is easy to operate. However, it still suffers from the inability to cut the pretarget region without PAM, off-target effects, and transfection difficulties.

Although current preclinical trials have demonstrated initial safety and efficacy of gene editing, existing studies have also shown that the immunogenicity and cytotoxicity of these vectors are of concern. Improving the accuracy of detecting and then reducing off-target effects remain a challenge. Only when these problems are solved can gene-editing technology be better applied in the clinical setting.

Conflicts of Interest

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

Authors' Contributions

LPL and YWZ conceived and designed the study. HZ and YW drafted and revised the manuscript. YWZ, LPL, and HZ contributed to reviewing and discussing the manuscript. All authors approved the final manuscript. YML supplied resources and materials. HZ and YW contributed equally to this work as co-first authors. YML and YWZ are senior authors and contributed equally to this work as cocorresponding authors. Hang Zhou and Yun Wang contributed equally to this work.

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References

[1] E. Kimbrel and R. Lanza, "Next-generation stem cells – ushering in a new era of cell-based therapies," *Nature Reviews. Drug Discovery*, vol. 19, no. 7, pp. 463–479, 2020.

[2] G. Ferrari, A. J. Thrasher, and A. Aiuti, "Gene therapy using haematopoietic stem and progenitor cells," *Nature Reviews. Genetics*, vol. 22, no. 4, pp. 216–234, 2021.

[3] S. Xu, K. Luk, Q. Yao et al., "Editing aberrant splice sites efficiently restores β-globin expression in β-thalassemia," *Blood*, vol. 133, no. 21, pp. 2255–2262, 2019.

[4] Y. Wu, J. Zeng, B. P. Roscoe et al., "Highly efficient therapeutic gene editing of human hematopoietic stem cells," *Nature Medicine*, vol. 25, no. 5, pp. 776–783, 2019.

[5] L. Xu, J. Wang, Y. Liu et al., "CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia," *The New England Journal of Medicine*, vol. 381, no. 13, pp. 1240–1247, 2019.

[6] Y. Chang, Y. N. Li, R. Bai et al., "hERG-deficient human embryonic stem cell-derived cardiomyocytes for modelling QT prolongation," *Stem Cell Research & Therapy*, vol. 12, no. 1, p. 278, 2021.

[7] S. Zhang, X. Zhang, C. Purmann et al., "Network effects of the 15q13.3 microdeletion on the transcriptome and epigenome in human-induced neurons," *Biological Psychiatry*, vol. 89, no. 5, pp. 497–509, 2021.

[8] J. C. Park, S. Y. Jang, D. Lee et al., "A logical network-based drug-screening platform for Alzheimer's disease representing pathological features of human brain organoids," *Nature Communications*, vol. 12, no. 1, p. 280, 2021.

[9] H. Ledford and E. Callaway, "Pioneers of revolutionary CRISPR gene editing win chemistry nobel," *Nature*, vol. 586, no. 7829, pp. 346-347, 2020.

[10] H. Wang and H. Yang, "Gene-edited babies: what went wrong and what could go wrong," *PLoS Biology*, vol. 17, no. 4, article e3000224, 2019.

[11] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[12] G. D. Vatine, R. Barrile, M. J. Workman et al., "Human ipsc-derived blood-brain barrier chips enable disease modeling and personalized medicine applications," *Cell Stem Cell*, vol. 24, no. 6, pp. 995–1005.e6, 2019.

[13] C. Theodoris, P. Zhou, L. Liu et al., "Network-based screen in iPSC-derived cells reveals therapeutic candidate for heart valve disease," *Science (New York, N.Y.)*, vol. 371, no. 6530, 2021.

[14] Y. Han, X. Duan, L. Yang et al., "Identification of SARS-CoV-2 inhibitors using lung and colonic organoids," *Nature*, vol. 589, no. 7841, pp. 270–275, 2021.

[15] J. D. Blanco, L. Radusky, H. Climente-Gonzalez, and L. Serrano, "FoldX accurate structural protein-DNA binding prediction using pada1 (protein assisted DNA assembly 1)," *Nucleic Acids Research*, vol. 46, no. 8, pp. 3852–3863, 2018.

[16] M. Laforet, T. A. McMurrough, M. Vu et al., "Modifying a covarying protein-DNA interaction changes substrate preference of a site-specific endonuclease," *Nucleic Acids Research*, vol. 47, no. 20, pp. 10830–10841, 2019.

[17] R. Werther, J. P. Hallinan, A. R. Lambert et al., "Crystallographic analyses illustrate significant plasticity and efficient recoding of meganuclease target specificity," *Nucleic Acids Research*, vol. 45, no. 14, pp. 8621–8634, 2017.

[18] T. A. McMurrough, C. M. Brown, K. Zhang et al., "Active site residue identity regulates cleavage preference of LAGLIDADG homing endonucleases," *Nucleic Acids Research*, vol. 46, no. 22, pp. 11990–12007, 2018.

[19] Y. G. Kim, J. Cha, and S. Chandrasegaran, "Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 3, pp. 1156–1160, 1996.

[20] G. Hensel and J. Kumlehn, "Genome engineering using TALENs," *Methods in Molecular Biology*, vol. 1900, pp. 195–215, 2019.

[21] A. M. Khalil, "The genome editing revolution: review," *Journal, Genetic Engineering & Biotechnology*, vol. 18, no. 1, p. 68, 2020.

[22] J. Grajcarek, J. Monlong, Y. Nishinaka-Arai et al., "Genome-wide microhomologies enable precise template-free editing of

biologically relevant deletion mutations," *Nature Communications*, vol. 10, no. 1, p. 4856, 2019.

[23] V. Lungova, X. Chen, Z. Wang, C. Kendziorski, and S. L. Thibeault, "Human induced pluripotent stem cell-derived vocal fold mucosa mimics development and responses to smoke exposure," *Nature Communications*, vol. 10, no. 1, p. 4161, 2019.

[24] M. Matsuda, Y. Yamanaka, M. Uemura et al., "Recapitulating the human segmentation clock with pluripotent stem cells," *Nature*, vol. 580, no. 7801, pp. 124–129, 2020.

[25] W. Zhu, B. Zhang, M. Li et al., "Precisely controlling endogenous protein dosage in hPSCs and derivatives to model FOXG1 syndrome," *Nature Communications*, vol. 10, no. 1, p. 928, 2019.

[26] D. Mosqueira, I. Mannhardt, J. Bhagwan et al., "CRISPR/Cas9 editing in human pluripotent stem cell-cardiomyocytes highlights arrhythmias, hypocontractility, and energy depletion as potential therapeutic targets for hypertrophic cardiomyopathy," *European Heart Journal*, vol. 39, no. 43, pp. 3879–3892, 2018.

[27] S. Kishore, E. de Franco, F. Cardenas-Diaz et al., "A non-coding disease modifier of pancreatic agenesis identified by genetic correction in a patient-derived iPSC line," *Cell Stem Cell*, vol. 27, no. 1, pp. 137–146.e6, 2020.

[28] B. Wilkinson, O. V. Evgrafov, D. Zheng et al., "Endogenous cell type-specific disrupted in schizophrenia 1 interactomes reveal protein networks associated with neurodevelopmental disorders," *Biological Psychiatry*, vol. 85, no. 4, pp. 305–316, 2019.

[29] A. M. Pettinato, F. A. Ladha, D. J. Mellert et al., "Development of a cardiac sarcomere functional genomics platform to enable scalable interrogation of HumanTNNT2Variants," *Circulation*, vol. 142, no. 23, pp. 2262–2275, 2020.

[30] X. Chen, Y. Liu, C. Xu et al., "QKI is a critical pre-mRNA alternative splicing regulator of cardiac myofibrillogenesis and contractile function," *Nature Communications*, vol. 12, no. 1, p. 89, 2021.

[31] C. A. Trujillo, E. S. Rice, N. K. Schaefer et al., "Reintroduction of the archaic variant ofNOVA1in cortical organoids alters neurodevelopment," *Science*, vol. 371, no. 6530, 2021.

[32] F. G. Lafaille, O. Harschnitz, Y. S. Lee et al., "Human *SNORA31* variations impair cortical neuron-intrinsic immunity to HSV-1 and underlie herpes simplex encephalitis," *Nature Medicine*, vol. 25, no. 12, pp. 1873–1884, 2019.

[33] J. Blair, D. Hockemeyer, and H. Bateup, "Genetically engineered human cortical spheroid models of tuberous sclerosis," *Nature Medicine*, vol. 24, no. 10, pp. 1568–1578, 2018.

[34] S. Wang, Z. Min, Q. Ji et al., "Rescue of premature aging defects in Cockayne syndrome stem cells by CRISPR/Cas9-mediated gene correction," *Protein & Cell*, vol. 11, no. 1, pp. 1–22, 2020.

[35] R. J. Zaunbrecher, A. N. Abel, K. Beussman et al., "Cronos titin is expressed in human cardiomyocytes and necessary for normal sarcomere function," *Circulation*, vol. 140, no. 20, pp. 1647–1660, 2019.

[36] I. Karakikes, V. Termglinchan, D. A. Cepeda et al., "A comprehensive TALEN-based knockout library for generating human-induced pluripotent stem cell-based models for cardiovascular diseases," *Circulation Research*, vol. 120, no. 10, pp. 1561–1571, 2017.

[37] P. D. Sohn, C. T. Huang, R. Yan et al., "Pathogenic tau impairs axon initial segment plasticity and excitability homeostasis," *Neuron*, vol. 104, no. 3, pp. 458–470.e5, 2019.

[38] T. Vandoorne, K. Veys, W. Guo et al., "Differentiation but not als mutations in fus rewires motor neuron metabolism," *Nature Communications*, vol. 10, no. 1, p. 4147, 2019.

[39] B. Dannenmann, M. Klimiankou, B. Oswald et al., "iPSC modeling of stage-specific leukemogenesis reveals BAALC as a key oncogene in severe congenital neutropenia," *Cell Stem Cell*, vol. 28, no. 5, pp. 906–922.e6, 2021.

[40] K. B. VanderWall, K. C. Huang, Y. Pan et al., "Retinal ganglion cells with a glaucoma OPTN(E50K) mutation exhibit neurodegenerative phenotypes when derived from three-dimensional retinal organoids," *Stem Cell Reports*, vol. 15, no. 1, pp. 52–66, 2020.

[41] C. Claes, J. van den Daele, R. Boon et al., "Human stem cell-derived monocytes and microglia-like cells reveal impaired amyloid plaque clearance upon heterozygous or homozygous loss of trem2," *Alzheimers Dement*, vol. 15, no. 3, pp. 453–464, 2019.

[42] P. Yan, Q. Li, L. Wang et al., "FOXO3-engineered human ESC-derived vascular cells promote vascular protection and regeneration," *Cell Stem Cell*, vol. 24, no. 3, pp. 447–461.e8, 2019.

[43] E. Cuevas, D. L. Holder, A. H. Alshehri, J. Tréguier, J. Lakowski, and J. C. Sowden, "NRL-/- gene edited human embryonic stem cells generate rod-deficient retinal organoids enriched in S-cone-like photoreceptors," *Stem Cells*, vol. 39, no. 4, pp. 414–428, 2021.

[44] X. Zhang, Z. Liu, X. Liu et al., "Telomere-dependent and telomere-independent roles of RAP1 in regulating human stem cell homeostasis," *Protein & Cell*, vol. 10, no. 9, pp. 649–667, 2019.

[45] M. Cohen-Tannoudji, S. Robine, A. Choulika et al., "I-scei-induced gene replacement at a natural locus in embryonic stem cells," *Molecular and Cellular Biology*, vol. 18, no. 3, pp. 1444–1448, 1998.

[46] S. Grizot, J. Smith, F. Daboussi et al., "Efficient targeting of a SCID gene by an engineered single-chain homing endonuclease," *Nucleic Acids Research*, vol. 37, no. 16, pp. 5405–5419, 2009.

[47] A. Izmiryan, S. Basmaciogullari, A. Henry, F. Paques, and O. Danos, "Efficient gene targeting mediated by a lentiviral vector-associated meganuclease," *Nucleic Acids Research*, vol. 39, no. 17, pp. 7610–7619, 2011.

[48] D. Hockemeyer, F. Soldner, C. Beard et al., "Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases," *Nature Biotechnology*, vol. 27, no. 9, pp. 851–857, 2009.

[49] F. Soldner, J. Laganière, A. W. Cheng et al., "Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations," *Cell*, vol. 146, no. 2, pp. 318–331, 2011.

[50] P. Reinhardt, B. Schmid, L. F. Burbulla et al., "Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression," *Cell Stem Cell*, vol. 12, no. 3, pp. 354–367, 2013.

[51] A. Verheyen, A. Diels, J. Reumers et al., "Genetically Engineered iPSC-Derived FTDP-17 *MAPT* Neurons Display Mutation- Specific Neurodegenerative and

Neurodevelopmental Phenotypes," *Stem Cell Reports*, vol. 11, no. 2, pp. 363–379, 2018.

[52] S. E. Birket, J. M. Davis, C. M. Fernandez-Petty et al., "Ivacaftor reverses airway mucus abnormalities in a rat model harboring a humanized G551D-CFTR," *American Journal of Respiratory and Critical Care Medicine*, vol. 202, no. 9, pp. 1271–1282, 2020.

[53] D. Hockemeyer, H. Wang, S. Kiani et al., "Genetic engineering of human pluripotent cells using tale nucleases," *Nature Biotechnology*, vol. 29, no. 8, pp. 731–734, 2011.

[54] M. A. Mandegar, N. Huebsch, E. B. Frolov et al., "Crispr interference efficiently induces specific and reversible gene silencing in human ipscs," *Cell Stem Cell*, vol. 18, no. 4, pp. 541–553, 2016.

[55] A. Granata, F. Serrano, W. G. Bernard et al., "An iPSC-derived vascular model of Marfan syndrome identifies key mediators of smooth muscle cell death," *Nature genetics*, vol. 49, no. 1, pp. 97–109, 2017.

[56] Q. V. Li, G. Dixon, N. Verma et al., "Genome-scale screens identify JNK-JUN signaling as a barrier for pluripotency exit and endoderm differentiation," *Nature genetics*, vol. 51, no. 6, pp. 999–1010, 2019.

[57] G. Schwank, B. K. Koo, V. Sasselli et al., "Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients," *Cell Stem Cell*, vol. 13, no. 6, pp. 653–658, 2013.

[58] E. Xia, Y. Zhang, H. Cao, J. Li, R. Duan, and J. Hu, "TALEN-mediated gene targeting for cystic fibrosis-gene therapy," *Genes*, vol. 10, no. 1, p. 39, 2019.

[59] S. Vaidyanathan, R. Baik, L. Chen et al., "Targeted replacement of full-length CFTR in human airway stem cells by CRISPR- Cas9 for pan-mutation correction in the endogenous locus," *Molecular therapy : the journal of the American Society of Gene Therapy*, 2021.

[60] Y. Lu, J. Xue, T. Deng et al., "Safety and feasibility of CRISPR-edited t cells in patients with refractory non-small-cell lung cancer," *Nature Medicine*, vol. 26, no. 5, pp. 732–740, 2020.

[61] L. Wang, J. Smith, C. Breton et al., "Meganuclease targeting of *PCSK9* in macaque liver leads to stable reduction in serum cholesterol," *Nature Biotechnology*, vol. 36, no. 8, pp. 717–725, 2018.

[62] F. Jaffré, C. L. Miller, A. Schänzer et al., "Inducible pluripotent stem cell-derived cardiomyocytes reveal aberrant extracellular regulated kinase 5 and mitogen-activated protein kinase kinase 1/2 signaling concomitantly promote hypertrophic cardiomyopathy in RAF1-associated Noonan syndrome," *Circulation*, vol. 140, no. 3, pp. 207–224, 2019.

[63] H. Zhao, Y. Li, L. He et al., "In vivo AAV-CRISPR/Cas9-mediated gene editing ameliorates atherosclerosis in familial hypercholesterolemia," *Circulation*, vol. 141, no. 1, pp. 67–79, 2020.

[64] U. Hanses, M. Kleinsorge, L. Roos et al., "Intronic CRISPR repair in a preclinical model of Noonan syndrome-associated cardiomyopathy," *Circulation*, vol. 142, no. 11, pp. 1059–1076, 2020.

[65] D. Zhou, H. Feng, Y. Yang et al., "hiPSC modeling of lineage-specific smooth muscle cell defects caused byTGFBR1A230T-Variant, and its therapeutic implications for Loeys-Dietz syndrome," *Circulation*, vol. 144, no. 14, pp. 1145–1159, 2021.

[66] P. Tebas, D. Stein, W. W. Tang et al., "Gene editing ofCCR5in autologous CD4 T cells of persons infected with HIV," *The New England Journal of Medicine*, vol. 370, no. 10, pp. 901–910, 2014.

[67] L. Li, L. Krymskaya, J. Wang et al., "Genomic editing of the HIV-1 coreceptor CCR5 in adult hematopoietic stem and progenitor cells using zinc finger nucleases," *Molecular therapy: the journal of the American Society of Gene Therapy*, vol. 21, no. 6, pp. 1259–1269, 2013.

[68] U. Mock, R. Machowicz, I. Hauber et al., "mRNA transfection of a novel TAL effector nuclease (TALEN) facilitates efficient knockout of HIV co-receptor CCR5," *Nucleic Acids Research*, vol. 43, no. 11, pp. 5560–5571, 2015.

[69] L. Xu, H. Yang, Y. Gao et al., "CRISPR/Cas9-mediated CCR5 ablation in human hematopoietic stem/progenitor cells confers HIV-1 resistance in vivo," *Molecular therapy: the journal of the American Society of Gene Therapy*, vol. 25, no. 8, pp. 1782–1789, 2017.

[70] L. Ye, J. Wang, F. Teque et al., "Generation of HIV-1-infected patients' gene-edited induced pluripotent stem cells using feeder-free culture conditions," *AIDS (London, England)*, vol. 34, no. 8, pp. 1127–1139, 2020.

[71] H. Frangoul, D. Altshuler, M. D. Cappellini et al., "CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia," *The New England Journal of Medicine*, vol. 384, no. 3, pp. 252–260, 2021.

[72] L. Ye, J. Wang, Y. Tan et al., "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: an approach for treating sickle cell disease and β-thalassemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 113, no. 38, pp. 10661–10665, 2016.

[73] S. H. Park, C. M. Lee, D. P. Dever et al., "Highly efficient editing of the β-globin gene in patient-derived hematopoietic stem and progenitor cells to treat sickle cell disease," *Nucleic Acids Research*, vol. 47, no. 15, pp. 7955–7972, 2019.

[74] G. A. Newby, J. S. Yen, K. J. Woodard et al., "Base editing of haematopoietic stem cells rescues sickle cell disease in mice," *Nature*, vol. 595, no. 7866, pp. 295–302, 2021.

[75] W. Qasim, H. Zhan, S. Samarasinghe et al., "Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells," *Science translational medicine*, vol. 9, no. 374, 2017.

[76] C. Sommer, B. Boldajipour, T. C. Kuo et al., "Preclinical evaluation of allogeneic CAR T cells targeting BCMA for the treatment of multiple myeloma," *Molecular therapy: the journal of the American Society of Gene Therapy*, vol. 27, no. 6, pp. 1126–1138, 2019.

[77] X. S. Liu, H. Wu, M. Krzisch et al., "Rescue of Fragile X Syndrome Neurons by DNA Methylation Editing of the *FMR1* Gene," *Cell*, vol. 172, no. 5, pp. 979–992.e6, 2018.

[78] A. McQuade, Y. J. Kang, J. Hasselmann et al., "Gene expression and functional deficits underlie TREM2-knockout microglia responses in human models of Alzheimer's disease," *Nature Communications*, vol. 11, no. 1, p. 5370, 2020.

[79] L. Feng, J. Chao, E. Tian et al., "Cell-based therapy for Canavan disease using human iPSC-derived NPCs and OPCs," *Advanced Science*, vol. 7, no. 23, article 2002155, 2020.

[80] A. Lane, K. Jovanovic, C. Shortall et al., "Modeling and rescue of RP2 retinitis pigmentosa using iPSC-derived retinal organoids," *Stem Cell Reports*, vol. 15, no. 1, pp. 67–79, 2020.

[81] M. L. Maeder, M. Stefanidakis, C. J. Wilson et al., "Development of a gene-editing approach to restore vision loss in

Leber congenital amaurosis type 10," *Nature Medicine*, vol. 25, no. 2, pp. 229–233, 2019.

[82] S. Das, N. Koyano-Nakagawa, O. Gafni et al., "Generation of human endothelium in pig embryos deficient in *ETV2*," *Nature Biotechnology*, vol. 38, no. 3, pp. 297–302, 2020.

[83] H. Xu, B. Wang, M. Ono et al., "Targeted disruption of HLA genes via CRISPR-Cas9 generates iPSCs with enhanced immune compatibility," *Cell Stem Cell*, vol. 24, no. 4, pp. 566–578.e7, 2019.

[84] A. Madsen, G. Höppner, J. Krause et al., "An important role for DNMT3A-mediated DNA methylation in cardiomyocyte metabolism and contractility," *Circulation*, vol. 142, no. 16, pp. 1562–1578, 2020.

[85] J. A. Hollywood, A. Przepiorski, R. F. D'Souza et al., "Use of human induced pluripotent stem cells and kidney organoids to develop a cysteamine/mTOR inhibition combination therapy for cystinosis," *Journal of the american society of nephrology*, vol. 31, no. 5, pp. 962–982, 2020.

[86] R. Shinnawi, N. Shaheen, I. Huber et al., "Modeling reentry in the short QT syndrome with human-induced pluripotent stem cell-derived cardiac cell sheets," *Journal of the American College of Cardiology*, vol. 73, no. 18, pp. 2310–2324, 2019.

[87] T. Tsunoda, S. Kakinuma, M. Miyoshi et al., "Loss of fibrocystin promotes interleukin-8-dependent proliferation and CTGF production of biliary epithelium," *Journal of Hepatology*, vol. 71, no. 1, pp. 143–152, 2019.

[88] Y. Zhou, X. Chen, B. Kang et al., "Endogenous authentic OCT4A proteins directly regulate FOS/AP-1 transcription in somatic cancer cells," *Cell Death & Disease*, vol. 9, no. 6, p. 585, 2018.

[89] N. Kuzumaki, Y. Suda, C. Iwasawa et al., "Cell-specific overexpression of COMT in dopaminergic neurons of Parkinson's disease," *Brain*, vol. 142, no. 6, pp. 1675–1689, 2019.

[90] T. Wang, A. Pine, A. Kotini et al., "Sequential CRISPR gene editing in human iPSCs charts the clonal evolution of myeloid leukemia and identifies early disease targets," *Cell Stem Cell*, vol. 28, no. 6, pp. 1074–1089.e7, 2021.

[91] A. Jacob, M. Morley, F. Hawkins et al., "Differentiation of human pluripotent stem cells into functional lung alveolar epithelial cells," *Cell Stem Cell*, vol. 21, no. 4, pp. 472–488.e10, 2017.

[92] S. Khadjeh, V. Hindmarsh, F. Weber et al., "CRISPLD1: a novel conserved target in the transition to human heart failure," *Basic Research in Cardiology*, vol. 115, no. 3, p. 27, 2020.

[93] P. G. Mazzara, S. Muggeo, M. Luoni et al., "Frataxin gene editing rescues Friedreich's ataxia pathology in dorsal root ganglia organoid-derived sensory neurons," *Nature Communications*, vol. 11, no. 1, p. 4178, 2020.

[94] N. Salvarani, S. Crasto, M. Miragoli et al., "The K219T-Lamin mutation induces conduction defects through epigenetic inhibition of _SCN5A_ in human cardiac laminopathy," *Nature Communications*, vol. 10, no. 1, p. 2267, 2019.

[95] X. Y. Tang, L. Xu, J. Wang et al., "DSCAM/PAK1 pathway suppression reverses neurogenesis deficits in iPSC-derived cerebral organoids from patients with Down syndrome," *The Journal of Clinical Investigation*, vol. 131, no. 12, 2021.

[96] G. Inak, A. Rybak-Wolf, P. Lisowski et al., "Defective metabolic programming impairs early neuronal morphogenesis in neural cultures and an organoid model of Leigh syndrome," *Nature Communications*, vol. 12, no. 1, p. 1929, 2021.

[97] G. Maule, A. Casini, C. Montagna et al., "Allele specific repair of splicing mutations in cystic fibrosis through AsCas12a genome editing," *Nature Communications*, vol. 10, no. 1, p. 3556, 2019.

[98] P. Pettingill, G. A. Weir, T. Wei et al., "A causal role for TRESK loss of function in migraine mechanisms," *Brain*, vol. 142, no. 12, pp. 3852–3867, 2019.

[99] D. Sinha, B. Steyer, P. K. Shahi et al., "Human iPSC modeling reveals mutation-specific responses to gene therapy in a genotypically diverse dominant maculopathy," *American Journal of Human Genetics*, vol. 107, no. 2, pp. 278–292, 2020.

[100] K. G. Maxwell, P. Augsornworawat, L. Velazco-Cruz et al., "Gene-edited human stem cell-derived β cells from a patient with monogenic diabetes reverse preexisting diabetes in mice," *Science translational medicine*, vol. 12, no. 540, 2020.

[101] A. Moretti, L. Fonteyne, F. Giesert et al., "Somatic gene editing ameliorates skeletal and cardiac muscle failure in pig and human models of Duchenne muscular dystrophy," *Nature Medicine*, vol. 26, no. 2, pp. 207–214, 2020.

[102] H. Ledford, "Who should lead on genome-editing policy, advisers say," *Nature*, 2021.

[103] A. C. Eberherr, A. Maaske, C. Wolf et al., "Rescue of STAT3 function in hyper-IgE syndrome using adenine base editing," *The CRISPR Journal*, vol. 4, no. 2, pp. 178–190, 2021.

[104] F. Baylis, "ISSCR guidelines fudge heritable human-genome editing," *Nature*, vol. 594, no. 7863, p. 333, 2021.

[105] Q. Liang, C. Monetti, M. Shutova et al., "Linking a cell-division gene and a suicide gene to define and improve cell therapy safety," *Nature*, vol. 563, no. 7733, pp. 701–704, 2018.

[106] V. Wiebking, J. Patterson, R. Martin et al., "Metabolic engineering generates a transgene-free safety switch for cell therapy," *Nature Biotechnology*, vol. 38, no. 12, pp. 1441–1450, 2020.

[107] P. Gee, M. S. Y. Lung, Y. Okuzaki et al., "Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping," *Nature Communications*, vol. 11, no. 1, p. 1334, 2020.

[108] J. Zhuang, J. Tan, C. Wu et al., "Extracellular vesicles engineered with valency-controlled DNA nanostructures deliver CRISPR/Cas9 system for gene therapy," *Nucleic Acids Research*, vol. 48, no. 16, pp. 8870–8882, 2020.

[109] D. N. Nguyen, T. L. Roth, P. J. Li et al., "Polymer-stabilized Cas9 nanoparticles and modified repair templates increase genome editing efficiency," *Nature Biotechnology*, vol. 38, no. 1, pp. 44–49, 2020.

[110] M. Arbab, M. Shen, B. Mok et al., "Determinants of base editing outcomes from target library analysis and machine learning," *Cell*, vol. 182, no. 2, pp. 463–480.e30, 2020.

[111] M. H. Geurts, E. de Poel, G. D. Amatngalim et al., "CRISPR-based adenine editors correct nonsense mutations in a cystic fibrosis organoid biobank," *Cell Stem Cell*, vol. 26, no. 4, pp. 503–510.e7, 2020.

[112] J. T. Liu, J. L. Corbett, J. A. Heslop, and S. A. Duncan, "Enhanced genome editing in human iPSCs with CRISPR-Cas9 by co-targeting ATP1a1," *PeerJ*, vol. 8, article e9060, 2020.

[113] Y. W. Fu, X. Y. Dai, W. T. Wang et al., "Dynamics and competition of CRISPR-Cas9 ribonucleoproteins and AAV donor-mediated NHEJ, MMEJ and HDR editing," *Nucleic Acids Research*, vol. 49, no. 2, pp. 969–985, 2021.

[114] S. Iyer, S. Suresh, D. Guo et al., "Precise therapeutic gene correction by a simple nuclease-induced double- stranded break," *Nature*, vol. 568, no. 7753, pp. 561–565, 2019.

[115] I. F. Schene, I. P. Joore, R. Oka et al., "Prime editing for functional repair in patient-derived disease models," *Nature Communications*, vol. 11, no. 1, p. 5352, 2020.

[116] J. Qu, Y. Xie, Z. Guo et al., "Identification of a novel cleavage site and confirmation of the effectiveness of NgAgo gene editing on RNA targets," *Molecular Biotechnology*, vol. 63, no. 12, pp. 1183–1191, 2021.

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Research Article

A Novel High-Content Screening-Based Method for Anti-*Trypanosoma cruzi* Drug Discovery Using Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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Chagas disease is caused by *Trypanosoma cruzi* infection and remains a relevant cause of chronic heart failure in Latin America. The pharmacological arsenal for Chagas disease is limited, and the available anti-*T. cruzi* drugs are not effective when administered during the chronic phase. Cardiomyocytes derived from human-induced pluripotent stem cells (hiPSC-CMs) have the potential to accelerate the process of drug discovery for Chagas disease, through predictive preclinical assays in target human cells. Here, we aimed to establish a novel high-content screening- (HCS-) based method using hiPSC-CMs to simultaneously evaluate anti-*T. cruzi* activity and cardiotoxicity of chemical compounds. To provide proof-of-concept data, the reference drug benznidazole and three compounds with known anti-*T. cruzi* activity (a betulinic acid derivative named BA5 and two thiazolidinone compounds named GT5A and GT5B) were evaluated in the assay. hiPSC-CMs were infected with *T. cruzi* and incubated for 48 h with serial dilutions of the compounds for determination of EC50 and CC50 values. Automated multiparametric analyses were performed using an automated high-content imaging system. Sublethal toxicity measurements were evaluated through morphological measurements related to the integrity of the cytoskeleton by phalloidin staining, nuclear score by Hoechst 33342 staining, mitochondria score following MitoTracker staining, and quantification of NT-pro-BNP, a peptide released upon mechanical myocardial stress. The compounds showed EC_{50} values for anti-*T. cruzi* activity similar to those previously described for other cell types, and GT5B showed a pronounced trypanocidal activity in hiPSC-CMs. Sublethal changes in cytoskeletal and nucleus scores correlated with NT-pro-BNP levels in the culture supernatant. Mitochondrial score changes were associated with increased cytotoxicity. The assay was feasible and allowed rapid assessment of anti-*T. cruzi* action of the compounds, in addition to cardiotoxicity parameters. The utilization of hiPSC-CMs in the drug development workflow for Chagas disease may help in the identification of novel compounds.

1. Introduction

Chagas disease, caused by the hemoflagellate protozoan *Trypanosoma cruzi*, affects approximately 6 to 7 million people worldwide [1, 2]. During the chronic phase, cardiac involvement occurs in up to 30% of the cases, leading to chronic Chagas cardiomyopathy (CCC), a disease that continues to be ranked among the most frequent etiologies of chronic heart failure in Latin American countries [3, 4].

Currently, benznidazole and nifurtimox are the only medications available to treat Chagas disease, both with proven efficacy when administered during the acute phase of the disease, which is often underdiagnosed [2]. In addition, treatment with these drugs can lead to serious adverse effects in some patients [5]. Therefore, there is an urgent need to increase the therapeutic arsenal for chronic Chagas disease through drug discovery or repurposing [2, 6]. In this context, the incorporation of innovative approaches in the preclinical *in vitro* screening process of anti-*T. cruzi* may contribute to accelerate the drug discovery process.

In the past years, human-induced pluripotent stem cells (hiPSCs) have contributed to drug discovery and toxicological studies applied to cardiovascular diseases [7, 8]. hiPSCs can be differentiated into any adult cell type, including cardiomyocytes, thus representing an invaluable tool for cardiovascular research, disease modeling, cardiotoxicity screening, and drug discovery [9]. Cardiomyocyte infection and parasite persistence are key factors in the pathophysiology of Chagas heart disease [10]. Therefore, studies with cardiomyocytes produced from hiPSCs (hiPSC-CMs) hold the potential to advance current knowledge about the disease pathogenesis and accelerate drug discovery and development, by facilitating preclinical assessments of toxicity and efficacy in relevant human cells [11].

Unpredicted cardiotoxicity is one of the main causes of drug withdrawal from the market and is the result of the low predictive value of currently available methods for preclinical cardiac toxicity testing [12]. This can be partially attributed to significant interspecies genetic and functional differences critical to the cardiomyocytes, which may influence the results obtained from animal studies [13]. hiPSC-CM-based assays offer the possibility of simultaneous evaluation of antitrypanocidal activity and cardiotoxicity in human cells. In this study, we evaluated a novel *in vitro* drug discovery method using *T. cruzi*-infected hiPSC-CMs and multiparameter analyses using a high-content screening (HCS) platform.

2. Methods

2.1. Ethics Statement. Cell reprogramming and experiments with hiPSCs received approval from the Ethics and Research Committee (IRB) at São Rafael Hospital (CAAE 20032313.6.0000.0048).

2.2. hiPSC Culture. We used two hiPSC lines obtained from two donors, previously obtained by integration-free reprogramming of erythroblasts with episomal vectors [14]. The cells were plated in Matrigel-coated wells (Corning; New York, NY, USA) and cultured with mTeSR1™ (Stem Cell Technologies; Vancouver, Canada). The medium was exchanged daily, and the cells were passaged with ReleSR (Stem Cell Technologies; Vancouver, Canada) when 80% confluence was reached, followed by replating in a 1 : 10 split ratio.

2.3. Cardiomyocyte Differentiation. hiPSCs were differentiated into cardiomyocytes using the PSC Cardiomyocyte Differentiation Kit (Thermo Fisher Scientific; Waltham, MA, US). Briefly, the hiPSCs were dissociated with ReleSR (Stem Cell Technologies; Vancouver, Canada) into a single cell suspension and replated at a 1 : 8 ratio in 12-well Matrigel-coated wells, being cultured with mTeSR1 (Stem Cell Technologies; Vancouver, Canada). During the first 24 h after plating (Day -3), 10 μM Y27632 (ROCK Inhibitor, STEMCELL Technologies; Vancouver, Canada) was added to the culture medium. On Days -2 and -1, the hiPSC medium was exchanged, and on Day 0, mTeSR1 was replaced by Cardiomyocyte Differentiation Medium A, followed by Cardiomyocyte Differentiation Medium B on Day 2 and Cardiomyocyte Maintenance Medium on Day 4. The medium was then renewed every two days with Cardiomyocyte Maintenance Medium. Spontaneous beating clusters began to be observed on protocol Day 8.

For immunofluorescence, the cells were fixed with 4% paraformaldehyde (PFA), washed with PBS, and permeabilized with 0.05% Triton solution. The following primary antibodies and dilutions were used, with overnight incubation at 4°C: sarcomeric alpha-actinin (Sigma-Aldrich, St. Louis, MI, USA; 1 : 100), MF20 (DSHB, Iowa City, IA; 1 : 100), anti-cTNT (Thermo Fisher Scientific; 1 : 100), and GATA-4 (Santa Cruz Biotechnology, Dallas, TX, USA; 1 : 100). The cells were incubated with the secondary antibodies, for 1 h at room temperature with anti-mouse IgG Alexa Fluor 568-conjugated or anti-rabbit IgG Alexa Fluor 488-conjugated, both diluted at 1 : 500 (Thermo Fisher Scientific). Images were captured using a confocal microscope (Fluoview 1000, Olympus, Tokyo, Japan).

2.4. RT-qPCR Analysis. RNA was extracted from the cells with TRIzol reagent (Invitrogen, Carlsbad, CA) and the concentration determined by photometric measurement. A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from 1 μg of RNA, according to the manufacturer's recommendations. Synthesis of cDNA and RNA expression analysis was performed by Real-Time PCR using TaqMan Gene Expression Assay for *GATA4* (Hs 01034628_m1), *NKX2.5* (Hs 00231763_m1), and *TNNT2* (Hs 00943911_m1). All reactions were run in duplicate on an ABI 7500 Real-Time PCR System (Applied Biosystems) under standard thermal cycling conditions. A nontemplate control (NTC) and nonreverse transcription controls (No-RT) were also included. The samples were normalized with *GAPDH* (endogenous control). The threshold cycle ($2 - \Delta\Delta$Ct) method of comparative PCR was used to analyze the results [15]. Data was analyzed using GraphPad software version 9.

2.5. Flow Cytometry. The cells were dissociated into single-cell suspensions by incubation with trypsin-EDTA solution

(Thermo Fisher Scientific; Waltham, MA, US), at 37°C for 5 min, followed by centrifugation at 350 g for 5 min at room temperature. The cells were counted and stained with an APC-conjugated anti-TRA1-60 antibody (BD Biosciences; Franklin Lakes, USA) for pluripotency evaluation. The efficiency of cardiac differentiation was evaluated by staining with anticardiac troponin T (Thermo Fisher Scientific; Waltham, MA, US), after permeabilization with 0.3% Triton X-100 solution (Sigma-Aldrich; St. Louis, MI, United States). The secondary antibody anti-mouse IgG conjugated with Alexa Fluor 647 was then used (Thermo Fisher Scientific; Waltham, MA, US). Data acquisition was performed with the LSR Fortessa flow cytometer (BD Biosciences; Franklin Lakes, USA) using the FACSDiva v.6.3 acquisition and analysis software.

2.6. T. cruzi Infection. Infection of cardiomyocytes was performed using the Y strain *T. cruzi*. Trypomastigotes were obtained from *T. cruzi*-infected BALB/c mice at 7 d.p.i., as previously described [16] and maintained *in vitro* by infecting LLC-MK2 cells (ATCC CCL-7). The Animal Ethics Committee at Gonçalo Moniz Institute, Fiocruz, approved this protocol under the number 17/2017. The cells were cultured in DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/mL) (Thermo Fisher Scientific; Waltham, MA, US) and incubated at 37°C and 5% CO_2 for 7 days before harvesting of free trypomastigote forms in the supernatant.

T. cruzi infection experiments were performed with hiPSC-CMs obtained from three donors, two generated in our laboratory and the third one commercially obtained (Pluricell, São Paulo, Brazil). The characterization of commercially obtained hiPSC-CMs was previously published [17]. hiPSC-CMs (2×10^4 cells/well) were plated in 96-well plates coated with Matrigel (Corning; New York, NY, US) and kept at 37°C and 5% CO_2 for 24 h. Then, the cells were infected with 1×10^5 or 2×10^5 trypomastigotes/well (multiplicity of infection (MOI) 5 or 10, respectively). The assay was adapted to 384-well plates, using 7×10^3 cells/well and infection with 3.5×10^4 trypomastigotes/well (MOI 5) for 24 h. On the following day, the wells were washed, and fresh media was added with the selected compounds.

2.7. Compounds. Three compounds endowed with anti-T. cruzi activity previously determined in conventional assays were tested: a betulinic acid derivative named BA5 and two thiazolidinone compounds named GT5A and GT5B [18–22]. The compounds had a degree of purity > 95%, as previously described [20]. Stock solutions at 10 mM were prepared by dissolving the lyophilized compounds in dimethyl sulfoxide (DMSO, OriGen; Austin, TX, US). Benznidazole (Lafepe; Recife, PE, Brazil), a gold standard anti-*T. cruzi* compound, was used for comparison. Other molecules—doxorubicin and endothelin-1 (both from Sigma-Aldrich; St. Louis, MI, United States)—were used in the nonlethal toxicity standardization assay.

2.8. Pharmacological Assays and High-Content Imaging Analysis. hiPSC-CMs were plated in 96- or 384-well plates at densities of 2×10^4 cells/well and 7×10^3 cells/well, respectively, and infected for 24 h, as described in the previous section. After incubation with the compounds at different concentrations for 48 h, the cells were fixed with 4% PFA and labeled with Hoechst 33342 (Thermo Fisher Scientific; Waltham, MA, US) or DRAQ5 (eBioscience; Santa Clara, CA, US). The images were acquired with the Operetta High Content System (PerkinElmer; Waltham, MA, US). Nuclei were delimited by the Hoechst 33342 or DRAQ5 (segmentation) channel using the Harmony software. The cytoplasm was segmented through the Alexa 594 channel, corresponding to the troponin T labeling. Intracellular amastigotes were detected as spots stained with DRAQ5 in the cytoplasm. Mock-infected control cells were used to exclude other nonspecific cytoplasmic spots from the analysis, by using morphological and fluorescence intensity parameters for selection. The experiments were carried out in triplicate for each test condition. Following cell segmentation and parameter selection, the total number of cells, the number of infected cells, the total number of amastigotes, the number of amastigotes per cell, and the infection rate were calculated. The cytotoxic concentration for 50% of the cardiomyocyte population (CC_{50}), the inhibitory concentration for 50% of the amastigote population (EC_{50}), and the selectivity index were calculated for test compounds and standard drug (benznidazole). The calculation of nonlinear regression to obtain the EC_{50} value was evaluated using Prism 7.04 (GraphPad Software; San Diego, CA, US).

The cytotoxicity of the compounds was evaluated by counting the number of cells in each well (lethal toxicity) and by evaluating other parameters of nonlethal toxicity: cell and nuclear morphology, damage to the cytoskeleton using Phalloidin-488 staining, and mitochondria biomass using MitoTracker Red staining (both from Thermo Fisher Scientific; Waltham, MA, EUA). The parameters were plotted in a principal component panel, where the drugs are clustered, and it was observed what groups were closer to control groups, after 48 hours of treatment. The parameters used in the evaluation of the cytoskeleton score were area, roundness, fluorescent intensity, and coefficient of variation (CV) of intensity to assess homogeneity of markers in the cell. To evaluate mitochondria biomass, we used fluorescent intensity, CV of intensity, the texture index SER "Hole" and texture index SER "Saddle," and the texture-based analysis, to access the pixel intensity, showing the effects of the drugs on mitochondrial morphology (Figure S1).

2.9. NT-Pro-BNP Measurements. To evaluate cardiomyocyte mechanical stress and hypertrophy processes, the concentrations of the N-terminal prohormone of brain natriuretic peptide (NT-pro-BNP) in the cardiomyocyte culture supernatants were evaluated. Culture supernatants were collected 48 hours after incubation with the compounds, pooled, and frozen at -80°C until analysis with the commercially available kit Vidas® NT-pro-BNP (Biomerieux, Marcy, France), following the manufacturer's recommendations.

2.10. Statistical Analyses. Parametric data were evaluated using Student's *t*-test. Nonparametric data were assessed

using the Mann–Whitney test. For comparison between three or more groups, the ANOVA test with Tukey's posttest for parametric data and the Kruskal-Wallis with Dunn's posttest for nonparametric data were used. Values of $p < 0.05$ were considered statistically significant. The EC_{50} values were obtained through nonlinear regression analyses and the selectivity index by dividing the average of the CC_{50} values over EC_{50} ($IS = CC_{50} \div EC_{50}$). Correlations between continuous variables were evaluated by the Pearson or Spearman coefficients.

3. Results

Human iPSCs were induced to differentiate into hiPSC-CMs, generating a population of nearly 90% troponin T-positive beating cardiomyocytes at differentiation Day 14 (Figure 1). To evaluate the susceptibility of hiPSC-CMs to *T. cruzi* infection and to define the optimal MOI to be used in the assay, a preliminary test was performed using MOIs 5 and 10. hiPSC-CMs were highly permissive to *T. cruzi* infection, leading to similar infection rates (54% and 44% for MOIs 5 and 10, respectively). The average number of amastigotes per cell was significantly higher for MOI 10 compared to 5 (124 vs. 48, respectively) (Figure 2(a)). A nonstatistically significant tendency towards decreased hiPSC-CM numbers was found for MOI 10 (Figures 2(b) and 2(c)). MOI 5 was then selected for the following experiments.

Infected hiPSC-CMs were treated with the standard drug, benznidazole, in different concentrations. Benznidazole was effective in reducing the percentage of infection in a concentration-dependent manner (Figure 3(a)). None of the tested concentrations was associated with a reduction in the number of hiPSC-CMs, compared to untreated control cultures (Figure 3(b)).

Next, we used our hiPSC-CM-based infection model to evaluate the anti-*T. cruzi* activity of two synthetic and one semisynthetic compounds (GT5A, GT5B, and BA5), which were previously shown to exert potent anti-*T. cruzi* activities compared to the reference drug, benznidazole [22–24]. Among the compounds tested, benznidazole had the highest CC_{50} value, followed by GT5A, whereas compound GT5B had the lowest EC_{50} value (Table 1).

Nonlethal toxicity parameters were evaluated through morphological analysis of hiPSC-CMs incubated with the compounds in different concentrations, followed by staining with phalloidin, to evaluate the cytoskeleton/cell morphology, and MitoTracker, to evaluate mitochondria content/-morphology. Nuclei morphology was evaluated by Hoechst 33342 staining. NT-pro-BNP levels in the culture supernatant were also measured. In the first step, hiPSC-CMs were incubated with the cardiotoxic drug doxorubicin or the hypertrophic molecule endothelin-1 (ET-1). Incubation with ET-1 was associated with signs of cell hypertrophy (Figures S2A and B), with increased intensity of phalloidin-labeled cytoskeleton compared to the untreated control group and to doxorubicin-treated cells, which presented reduced intensity of phalloidin fluorescence and cell size. These results correlated with the detection of high NT-pro-BNP levels in culture supernatants of hiPSC-CMs treated with ET-1 and a reduction after treatment with doxorubicin, which was associated with a significant increase in cell death (Figures S2A and B).

Next, we evaluated the effects of the anti-*T. cruzi* compounds benznidazole, BA5, GT5A, and GT5B 48 hours following treatment in different concentrations (100, 50, 25, 12.5, and 6.25 μM). Different parameters were combined, and principal component analyses were conducted, defining a cytoskeleton score and a mitochondrial score (Figure S1). While treatment with DMSO did not alter significantly either the cytoskeleton or the mitochondria scores compared to untreated controls, treatments with all compounds slightly altered these parameters, which generated a cluster for most of the concentrations tested (Figure 4). In addition to doxorubicin, treatment with GT5A in the concentrations of 50 and 100 μM and BA5 in the concentrations of 12.5, 25, 50, and 100 μM led to higher dispersion and distancing from the values obtained for untreated and DMSO-treated hiPSC-CMs (Figure 4).

Treatment with all compounds tested led to an increase in the secretion of NT-pro-BNP compared to untreated hiPSC-CMs, but a concentration-dependent increase was observed in BA5-treated cells, leading to NT-pro-BNP levels that, at the concentrations 2.5, 5, 10, and 20 μM, surpassed the values observed after ET-1 stimulation (Figure 5(a)). Finally, we found that NT-pro-BNP levels in the culture supernatant showed a statistically significant correlation with the cytoskeleton score and nuclear morphology (Table 2 and Figure 5(b)).

To evaluate whether the high-content imaging strategy could also be applied to the study of compound effects in cell morphology of infected cells, we analyzed the experiments of *T. cruzi* infection generating a new score that combined measurements of spots, texture, and cytoskeleton staining (Figure 6). By principal component analysis, mock and *T. cruzi*-infected cells are displayed in separate regions of the plot, while the compounds brought the cell morphology parameters to an intermediate zone. Among the tested conditions, the cells treated with the compounds GT5B and GT5A were found to be more similar to mock-infected cells, while BA5 significantly altered cell morphology.

4. Discussion

hiPSC-CMs hold the potential to contribute to the anti-*T. cruzi* drug discovery process by increasing the predictive value of preclinical assays. Considering the need to develop new drugs for the treatment of Chagas disease, we established a multiparametric pharmacological assay for simultaneous evaluation of cardiotoxicity and anti-*T. cruzi* activity using hiPSC-CMs and a HCS platform with automated analysis, reducing bias and increasing confidence in the assay. Considering the role of parasite persistence in the myocardium, the preclinical confirmation that drugs with previously described antiparasitic actions (GT5A, GT5B, and BA5) are also safe and effective in cardiomyocytes brings valuable data for further development.

Treatment of infected hiPSC-CMs with the reference drug benznidazole resulted in an EC_{50} value of 5.9 μM, which is situated within the range of EC_{50} values reported in the

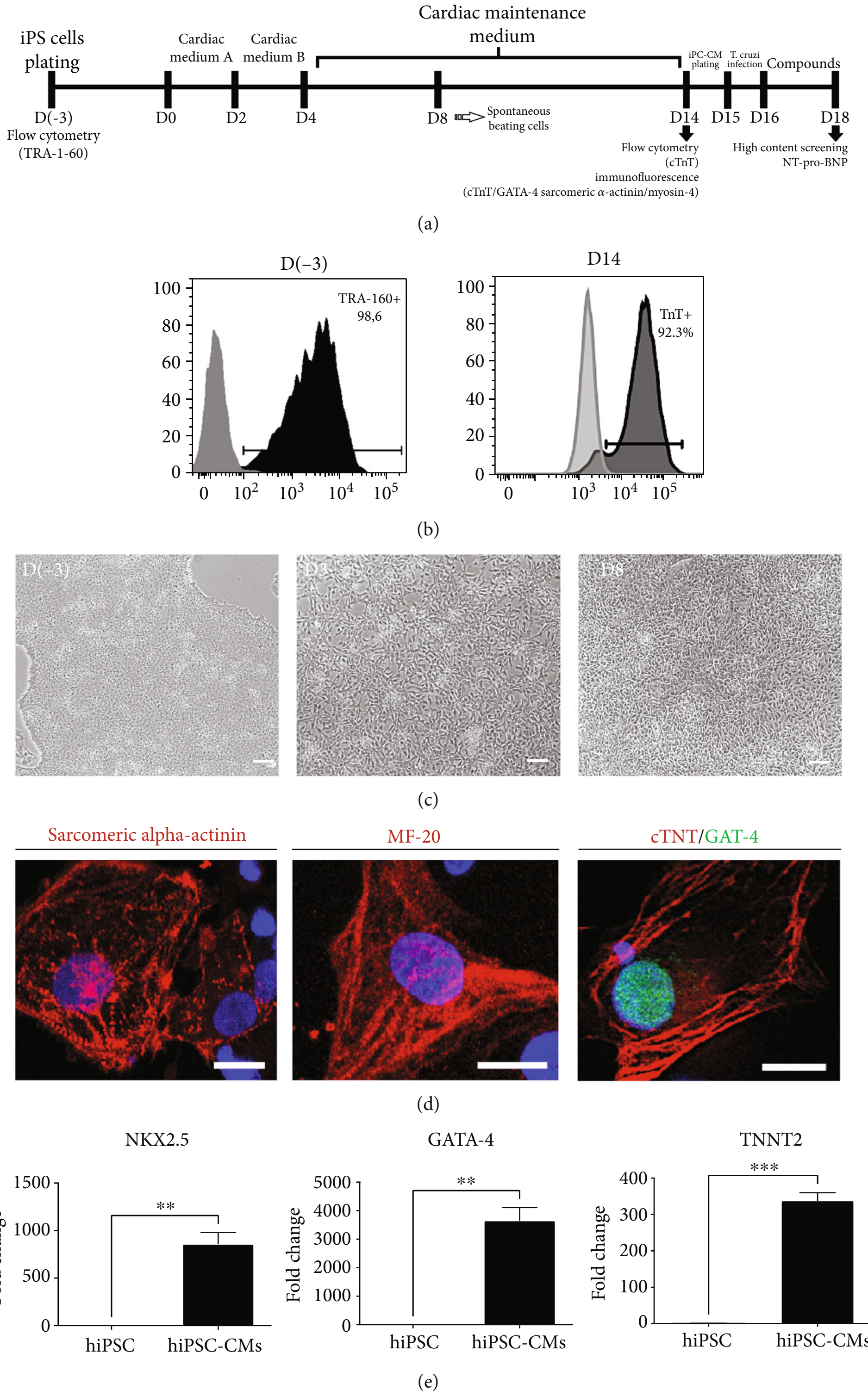


FIGURE 1: Cardiac differentiation induction in hiPSC and characterization of hiPSC-CMs. (a) Schematic experimental design. (b) Flow cytometry analysis of hiPSCs at D(-3), before plating for cardiac induction, for TRA-1-60 expression, and hiPSC-CM purity at differentiation D14 evaluated by cardiac troponin-T (cTNT) expression. Light grey histograms represent isotype controls. (c) Representative phase contrast micrographies of the different days postinduction of cardiac differentiation from hiPSCs. Bars = 50 μm. (d) Representative confocal microscopy images of hiPSC-CMs stained with sarcomeric alpha-actinin, sarcomeric myosin (MF-20), cardiac troponin T (cTNT), and GATA-4 (all in red). Nuclei were stained with DAPI (blue). Bars = 20 μm. (e) Gene expression analysis by RT-qPCR demonstrating mRNA expression of cardiac genes *NKX2.5*, *GATA-4*, and *TNNT2*, normalized to the levels of *GAPDH*. $^{**}p < 0.01$; $^{***}p < 0.001$.

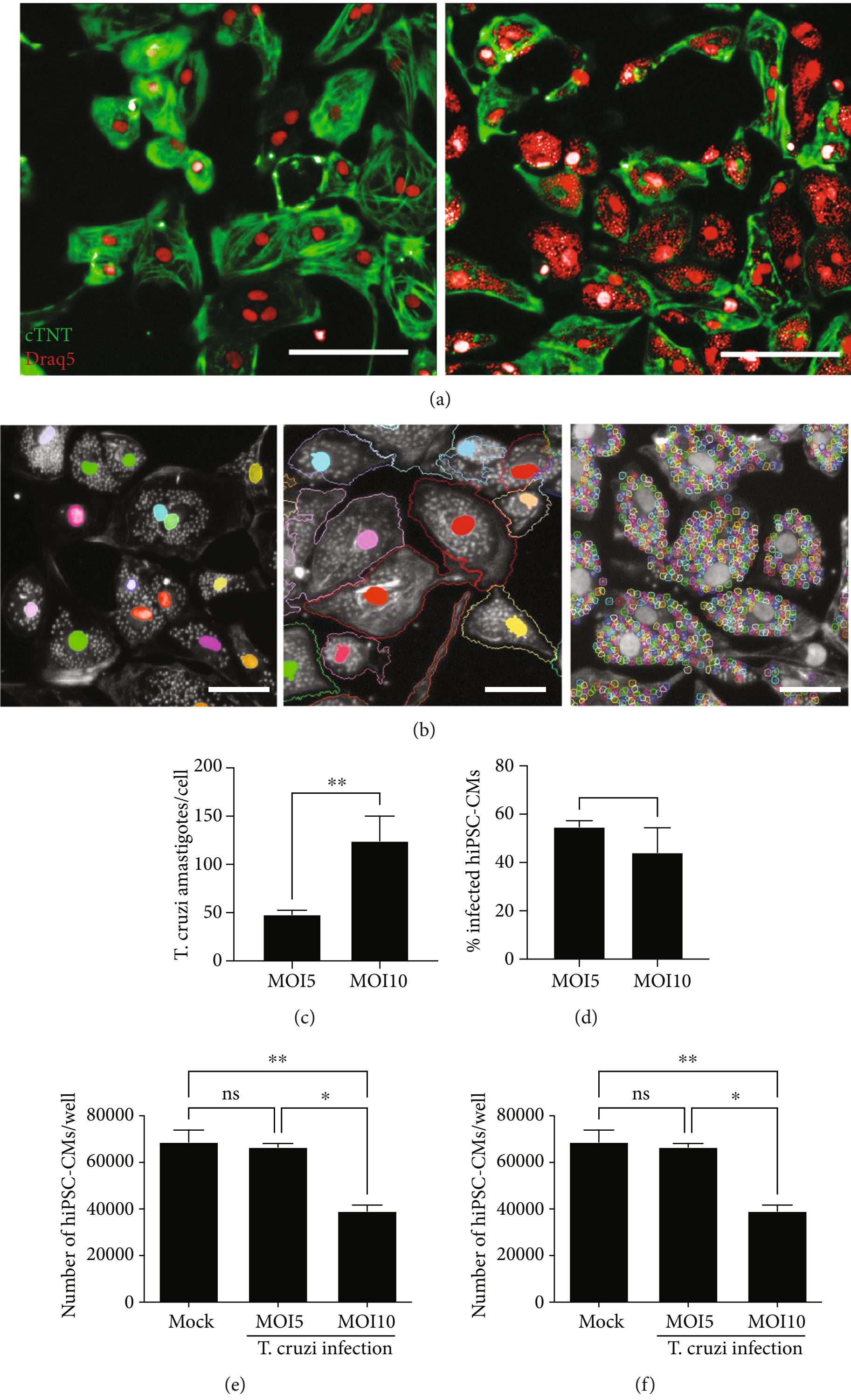


Figure 2: Infection of hiPSC-CMs with *T. cruzi* and high-content screening analysis. (a) Representative image of uninfected hiPSC-CM control, showing cTnT staining (green) and nuclei stained with Draq5 (red). (b) Representative image of hiPSC-CMs infected with *T. cruzi*, showing cTnT staining (green) nuclei and amastigotes stained with Draq5 (red). (c) Standardization of image analysis in Operetta High Content Imaging System, illustrating the steps of nuclei identification (left), followed by cytoplasm delimitation (middle), and *T. cruzi* amastigote spot identification (right). Quantification of the number of amastigotes/cell (d), percentage of infection (e), and number of hiPSC-CMs (f) 72 h following infection in MOI = 10 and MOI = 5. $^{*}p < 0.05$; $^{**}p < 0.01$; ns = not significant ($p \geq 0.05$). Bars = 50 μm.

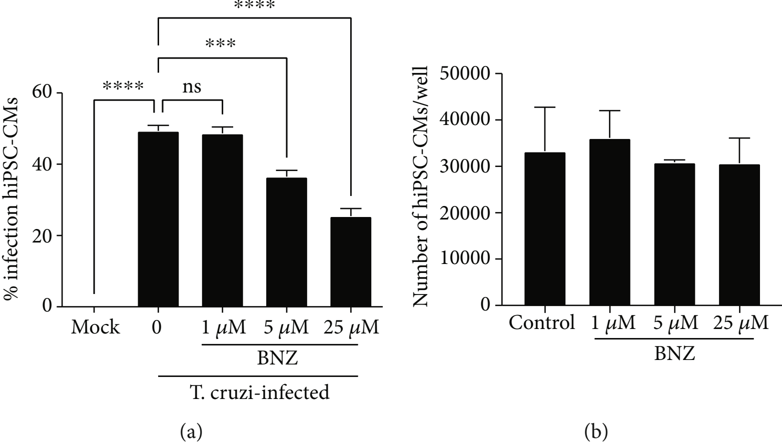


Figure 3: Effects of benznidazole (BNZ) on *T. cruzi*-infected hiPSC-CMs. (a) Percentage of infection BNZ-treated hiPSC-CMs. (b) Number of cardiomyocytes/well in cultures treated with BNZ and control. $^{***}p < 0.001$; $^{****}p < 0.0001$; ns = not significant ($p \geq 0.05$).

Table 1: Cytotoxicity against hiPSC-CMs and anti-*T. cruzi* activity against intracellular amastigotes.

Compound	CC_{50} (μM)	EC_{50} (μM)	SI
BA5	37 ± (1.9)	3.2 ± (0.8)	12
GT5A	87 ± (22)	1.9 ± (0.6)	46
GT5B	27 ± (3.5)	0.8 ± (0.2)	33
Benznidazole	>100	5.9 ± (0.5)	>17

CC_{50}: cytotoxicity concentration 50%; EC_{50}: effective concentration at 50% inhibitory concentration for inhibition of *T. cruzi* amastigotes. Values are means ± SD of three independent experiments.

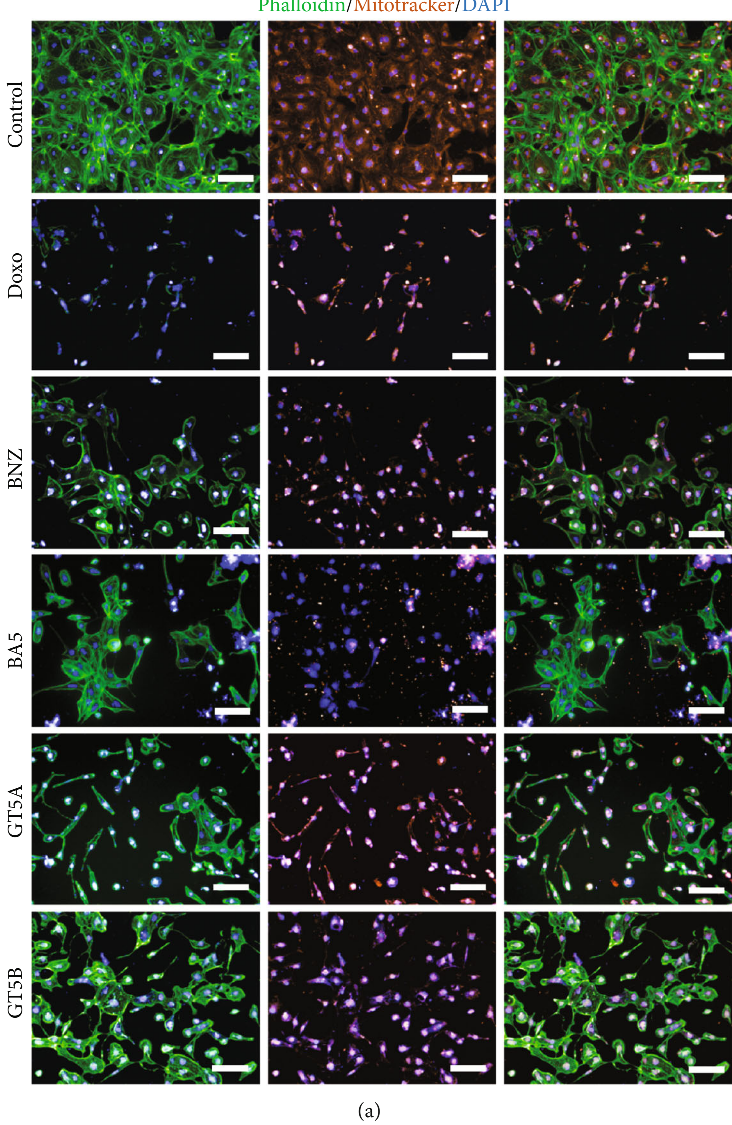
literature for benznidazole in other cell types [16, 18, 19]. Studies have shown that betulinic acid and its derivatives, such as BA5, inhibit the proliferation of epimastigotes and reduce the viability of trypomastigote forms [19, 20]. In our findings, BA5 had an EC_{50} of 3.2 μM for reducing *T. cruzi* amastigotes in hiPSC-CMs, half of the EC_{50} value found for the reference drug, benznidazole. Previously, an EC_{50} value of 1.8 μM was found for murine macrophages [20]. Thiazolidines are potent cruzain inhibitors and have been previously studied as anti-*T. cruzi* drugs [23]. Both GT5A and GT5B have been identified as potent agents with trypanocidal action with high selectivity index [24]. These compounds had EC_{50} values in hiPSC-CMs of 1.9 and 0.8 μM values, respectively, much lower compared to benznidazole. A previous study in macrophages reported higher values of EC_{50} for GT5A and GT5B, with 4.2 and 2.9 μM, respectively [24].

Our data also revealed cell type-specific toxicities for some of the compounds, which were not reported in previous studies with murine cells. BA5 showed lethal toxicity for hiPSC-CMs with a CC_{50} of 37 μM, which is discrepant and significantly lower than the values previously reported for murine macrophages [22]. At lower concentrations, hiPSC-CMs demonstrated changes in the cytoskeleton score, suggesting cytoskeleton disorganization, along with high levels of NT-pro-BNP, a biomarker of myocardial stress. Interestingly, we demonstrated that the levels of NT-pro-BNP showed a statistically significant correlation with the cytoskeleton and nuclear scores, but not with the mitochondria score or lethal toxicity parameters.

The mechanical stability of the cardiomyocyte depends on the integrity of the cytoskeleton. Geometric changes in the cell membrane can lead to changes in electrophysiology [25]. Some drugs can induce cytoskeletal disorganization and mechanical stress or even stimulate a hypertrophic response. In the present study, we used phalloidin staining to assess the cytoskeleton, and using positive controls, we observed that this analysis can demonstrate, quantitatively, a hypertrophic response (assessed with treatment with ET-1) and disruption of the cytoskeleton (observed with the doxorubicin treatment). Interestingly, these findings correlated with increased levels of release of the NT-pro-BNP biomarker to the culture medium in the hypertrophic response induced by ET-1, while reduced levels were observed in the case of treatment with doxorubicin, which is in agreement with previous observations in the literature [26].

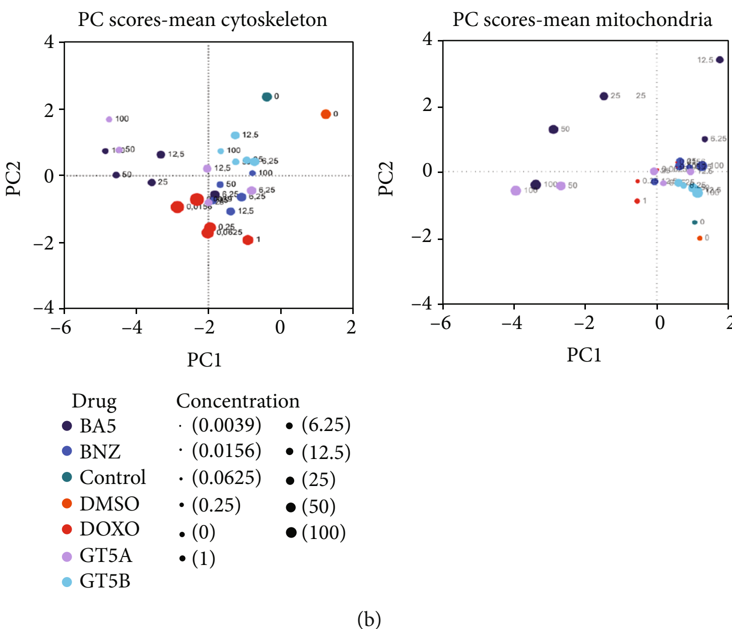
Dysfunctional mitochondria can compromise myocardial function [26, 27] as cardiomyocytes require high levels of ATP to function properly. Some drugs can be cardiotoxic by inducing mitochondrial damage, increasing oxidative stress, activating DNA damage response pathways, and increasing apoptosis [28]. By including mitochondria parameters in high-content analyses, the assay also allowed the identification of gross alterations in mitochondria that could lead to cardiotoxicity.

The compounds tested herein comprehend the two main classes of compounds currently explored as antiparasitic agents for Chagas disease: molecules with exclusive antiparasitic activity (GT5A and GT5B) and molecules with dual antiparasitic and anti-inflammatory/immunomodulatory activity (BA5). Another clinical significance of the compounds is their pharmacological profile. In cardiomyocytes, we found that all three compounds have IC50 values lower than the reference drug benznidazole, making them candidates for further development. However, the toxicity analyses



(a)

Figure 4: Continued.



(b)

Figure 4: High-content imaging analysis of sublethal cardiotoxicity of anti-*T. cruzi* compounds. (a) Representative images of hiPSC-CMs untreated (control) or treated with 0.5% DMSO, 1 μM doxorubicin (Doxo), benznidazole (BNZ), BA5, GT5A, or GT5B, all at the 25 μM concentration. Cytoskeleton was stained with phalloidin (green) and mitochondria with MitoTracker (orange), and nuclei were stained with DAPI (blue). Bars = 100 μm. (b) Principal component analysis demonstrating the effects of the compounds in different concentrations to the cytoskeleton and mitochondria scores.

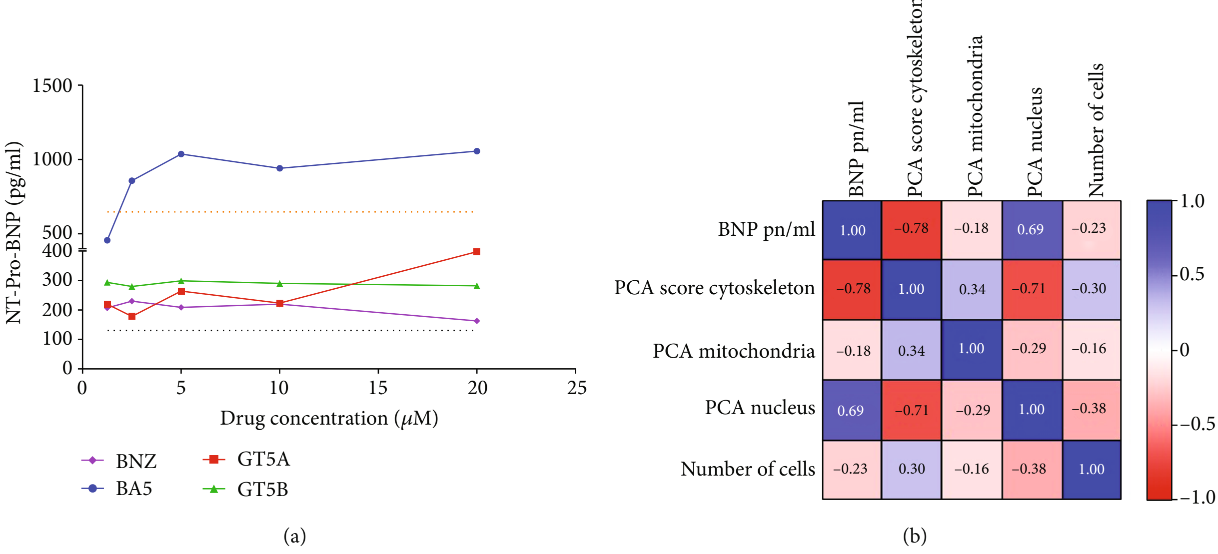


Figure 5: NT-pro-BNP analysis and correlations with morphology parameters. (a) NT-pro-BNP levels in the culture supernatant 48 h following treatment with the compounds in different concentrations. Dash lines represent NT-pro-BNP levels in the culture supernatant of untreated hiPSC-CMs (black) 48 h following treatment with endothelin-1 (orange). (b) Correlation matrix heat map demonstrating *R* values found for each comparison between the different variables.

Table 2: Correlations between NT-pro-BNP levels and morphological parameters.

	PCA cytoskeleton score	PCA mitochondria score	PCA nucleus morphology	Number of cells
NT-pro-BNP levels	$p = 0.006$ $R = -0.74$	$p = 0.792$ $R = -0.09$	$p = 0.027$ $R = 0.63$	$p = 0.473$ $R = -0.23$

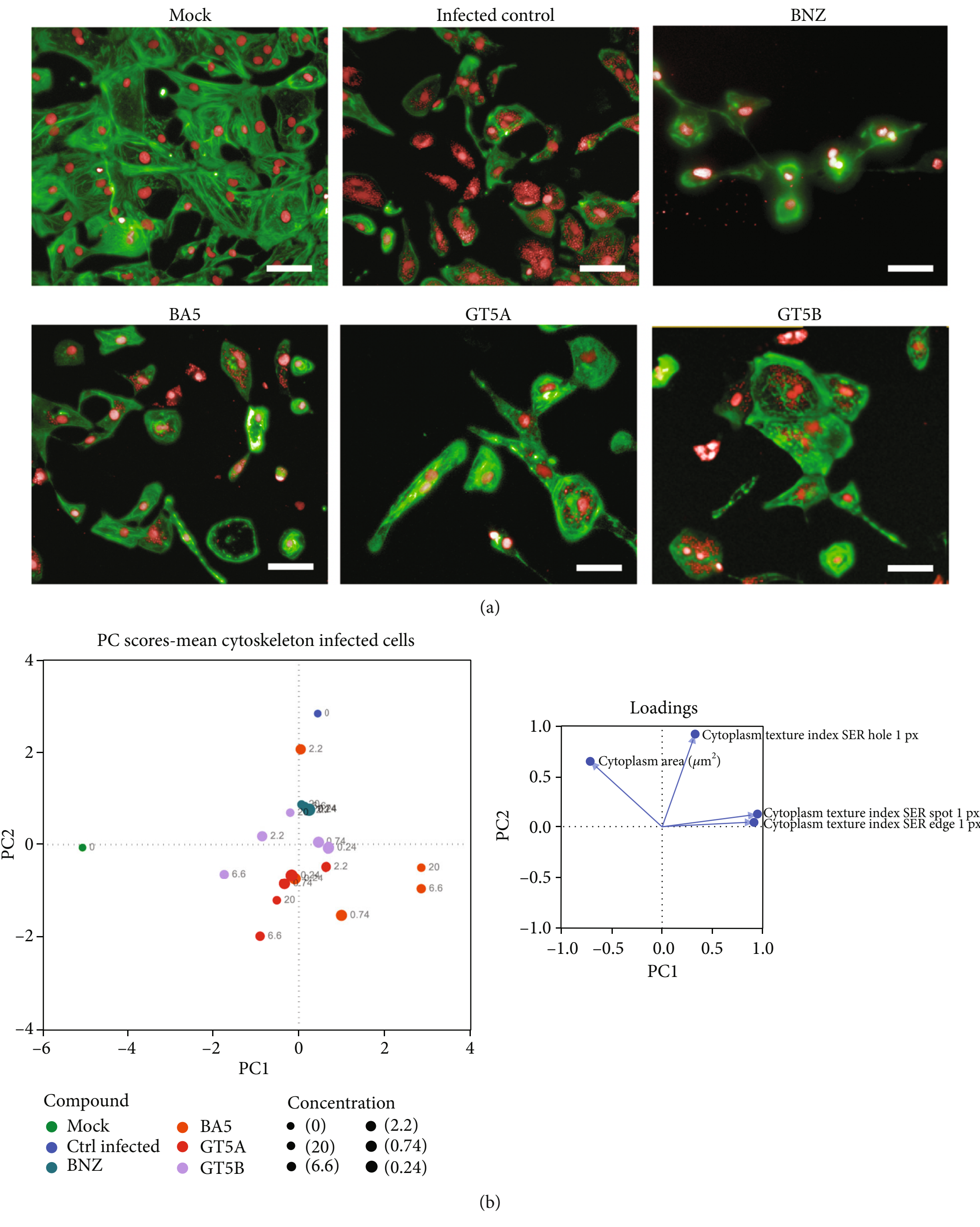


Figure 6: High-content imaging analysis of infected hiPSC-CMs treated with the compounds. (a) Representative images of hiPSC-CMs uninfected (mock) or infected with *T. cruzi* submitted to compound (BNZ (benznidazole), BA5, GT5A, or GT5B) testing at the concentration of 6.6 μm. Bars = 50 μm. (b) Principal component analysis demonstrating the effects of the compounds in different concentrations to the cytoskeleton-infected hiPSC-CM score.

showed that BA5 may present a cardiotoxicity profile, an observation similar to a high concentration of GT5A. But unlike GT5A, GT5B was not only the most potent compound in terms of anti-*T. cruzi* activity but also less toxic to hiPSC-CM, with less altered cell morphology, as demonstrated by multiparametric HCS analysis.

A limitation of the present study involves the degree of maturity of hiPSC-CMs. So far, hiPSC-CMs that have been used for the cardiotoxicity test show a structural phenotype compatible with fetal cardiomyocytes. The stage of development of hiPSC-CMs used in this study is in accordance with the literature, which reports that cardiac differentiation from

hiPSC routinely leads to the generation of cells with an immature structural and functional phenotype, of the fetal type [29]. However, the impact that the hiPSC-CMs' maturation status has on the reaction capacity to the compounds is not clear [28, 29]. Considering that the degree of maturation of hiPSC-CMs can be a critical factor for obtaining more predictive tests, further studies should be carried out to optimize the process of obtaining these cells. There are already reports in the literature of methods for inducing maturation, including through the modulation of mechanical strength (afterload) or by culturing hiPSC-CMs in 3D [30, 31], which could be evaluated in future studies. Doxorubicin binds to cardiolipin and inhibits the respiratory chain and the depolarization of the membrane potential, among other mechanisms [32]. Mitochondria are involved in several cardiomyocyte functions, including fatty acid metabolism, amino acids, and ATP generation [33].

5. Conclusions

The use of hiPSC-CMs in the drug development workflow for Chagas disease has the potential to assist in the identification of new compounds and to predict cardiotoxicity. In this work, we established the test for infection and screening compounds with anti-*T. cruzi* activity in hiPSC-CMs, using multiparametric analyses on a high-content screening platform. This assay was able to confirm the anti-*T. cruzi* activity of BA5, GT5A, and GT5B and identified the compound GT5B as promising, due to its potency and low toxicity in cardiomyocytes. In addition to having the advantage of being based on the use of human cardiac cells affected by Chagas disease, the assay has the advantage of allowing the rapid assessment of anti-*T. cruzi* actions in addition to parameters of lethal and sublethal cardiotoxicity, which can increase the predictive value of the tests.

Data Availability

Data is available upon reasonable request.

Conflicts of Interest

The authors claim no conflict of interest.

Acknowledgments

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Supplementary Materials

Figure S1: vectors included in the principal component analysis for cytoskeleton and mitochondria scores. Figure S2: morphology and NT-pro-BNP levels. (A) Representative images of hiPSC-CMs stained with phalloidin (green) and DAPI (blue) untreated (control) or 48 h following treatment with endothelin-1 (ET-1) or doxorubicin (Doxo). Bars = 50 μm. (B) Quantification of phalloidin fluorescence intensity, number of hiPSC-CMs, and levels of NT-pro-BNP in the supernatant. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns = not significant ($p \geq 0.05$). *(Supplementary Materials)*

References

[1] M. D. Flores-chavez, V. Sambri, V. Schottstedt et al., "Evaluation of the Elecsys Chagas assay for detection of *Trypanosoma cruzi*-specific antibodies in a multicenter study," *Journal of Clinical Microbiology*, vol. 56, no. 5, pp. 1–14, 2018.

[2] C. Bern, "Antitrypanosomal therapy for chronic Chagas' disease," *The New England Journal of Medicine*, vol. 364, no. 26, pp. 2527–2534, 2011.

[3] M. B. P. Soares, L. A. I. N. Pontes-de-Carvalho, and R. Ribeiro-Dos-Santos, "The pathogenesis of Chagas' disease: when autoimmune and parasite-specific immune responses meet," *Anais da Academia Brasileira de Ciências*, vol. 73, no. 4, pp. 547–559, 2001.

[4] E. A. Bocchi, A. Arias, H. Verdejo et al., "The reality of heart failure in Latin America," *Journal of the American College of Cardiology*, vol. 62, no. 11, pp. 949–958, 2013.

[5] D. L. Longo and C. Bern, "Chagas' disease," *The New England Journal of Medicine*, vol. 373, no. 5, pp. 456–466, 2015.

[6] B. S. Hall and S. R. Wilkinson, "Activation of benznidazole by trypanosomal type I nitroreductases results in glyoxal formation," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 1, pp. 115–123, 2012.

[7] S. Surendran and G. Sivamurthy, "Current applications and future prospects of stem cells in dentistry," *Dental Update*, vol. 42, no. 6, pp. 556–561, 2015.

[8] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[9] E. D. Cohen, Y. Tian, and E. E. Morrisey, "Wnt signaling: an essential regulator of cardiovascular differentiation, morphogenesis and progenitor self-renewal," *Development*, vol. 135, no. 5, pp. 789–798, 2008.

[10] F. R. S. Gutierrez, P. M. M. Guedes, R. T. Gazzinelli, and J. S. Silva, "The role of parasite persistence in pathogenesis of Chagas heart disease," *Parasite Immunology*, vol. 31, no. 11, pp. 673–685, 2009.

[11] F. M. Drawnel, S. Boccardo, M. Prummer et al., "Disease Modeling and Phenotypic Drug Screening for Diabetic Cardiomyopathy using Human Induced Pluripotent Stem Cells," *Cell Reports*, vol. 9, no. 3, pp. 810–820, 2014.

[12] D. Sinnecker, K. Laugwitz, and A. Moretti, "Induced pluripotent stem cell-derived cardiomyocytes for drug development and toxicity testing," *Pharmacology & Therapeutics*, vol. 143, no. 2, pp. 246–252, 2014.

[13] G. Gintant, P. T. Sager, and N. Stockbridge, "Evolution of strategies to improve preclinical cardiac safety testing," *Nature Reviews. Drug Discovery*, vol. 15, no. 7, pp. 457–471, 2016.

[14] B. D. Paredes, G. L. S. Martins, C. M. Azevedo et al., "Generation of three control iPS cell lines for sickle cell disease studies by reprogramming erythroblasts from individuals without hemoglobinopathies," *Stem Cell Research*, vol. 38, article 101454, 2019.

[15] T. Schmittgen and K. Livak, "Analyzing real-time PCR data by the comparative C_T method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[16] C. S. Meira, E. T. Guimarães, J. A. F. dos Santos et al., "*In vitro* and *in vivo* antiparasitic activity of *Physalis angulata* L.

concentrated ethanolic extract against *Trypanosoma cruzi*," *Phytomedicine*, vol. 22, no. 11, pp. 969–974, 2015.

[17] E. Cruvinel, I. Ogusuku, R. Cerioni et al., "Long-term single-cell passaging of human iPSC fully supports pluripotency and high-efficient trilineage differentiation capacity," *SAGE Open Medicine*, vol. 8, 2020.

[18] G. B. de Oliveira Filho, M. V. . O. Cardoso, J. W. P. Espíndola et al., "Structural design, synthesis and pharmacological evaluation of thiazoles against *Trypanosoma cruzi*," *European Journal of Medicinal Chemistry.*, vol. 141, pp. 346–361, 2017.

[19] A. Botero, S. Keatley, C. Peacock, and R. C. A. Thompson, "*In vitro* drug susceptibility of two strains of the wildlife trypanosome, *Trypanosoma copemani* : A comparison with *Trypanosoma cruzi*," *International Journal for Parasitology: Drugs and Drug Resistance*, vol. 7, no. 1, pp. 34–41, 2017.

[20] D. R. M. Moreira, A. C. L. Leite, M. V. O. Cardoso et al., "Structural design , synthesis and structure – activity relationships of thiazolidinones with enhanced anti- *Trypanosoma cruzi* activity," *ChemMedChem*, vol. 9, pp. 177-178, 2014.

[21] D. B. Domínguez-Carmona, F. Escalante-Erosa, K. García-Sosa et al., "Antiprotozoal activity of betulinic acid derivatives," *Phytomedicine*, vol. 17, no. 5, pp. 379–382, 2010.

[22] C. S. Meira, J. M. Barbosa-Filho, A. Lanfredi-Rangel, E. T. Guimarães, D. R. M. Moreira, and M. B. P. Soares, "Antiparasitic evaluation of betulinic acid derivatives reveals effective and selective anti- *Trypanosoma cruzi* inhibitors," *Experimental Parasitology*, vol. 166, pp. 108–115, 2016.

[23] M. Z. Hernandes, M. M. Rabello, A. C. Leite et al., "Studies toward the structural optimization of novel thiazolylhydrazone-based potent antitrypanosomal agents," *Bioorganic & Medicinal Chemistry*, vol. 18, no. 22, pp. 7826–7835, 2010.

[24] G. B. de Oliveira Filho, M. V. de Oliveira Cardoso, J. W. P. Espíndola et al., "Structural design, synthesis and pharmacological evaluation of 4-thiazolidinones against *Trypanosoma cruzi*," *Bioorganic & Medicinal Chemistry*, vol. 23, no. 23, pp. 7478–7486, 2015.

[25] X. Yang, J. I. Pedro, T. V. Pham et al., "Cytoskeletal actin microfilaments and the transient outward potassium current in hypertrophied rat ventriculocytes," *The Journal of Physiology*, vol. 541, no. 2, pp. 411–421, 2002.

[26] J. Louisse, R. C. I. Wüst, F. Pistollato et al., "Assessment of acute and chronic toxicity of doxorubicin in human induced pluripotent stem cell-derived cardiomyocytes," *Toxicology In Vitro*, vol. 42, pp. 182–190, 2017.

[27] Z. V. Varga, P. Ferdinandy, L. Liaudet, and P. Pacher, "Drug-induced mitochondrial dysfunction and cardiotoxicity," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 309, no. 9, pp. H1453–H1467, 2015.

[28] L. Sala, M. Bellin, and C. L. Mummery, "Integrating cardiomyocytes from human pluripotent stem cells in safety pharmacology: has the time come?," *British Journal of Pharmacology*, vol. 174, no. 21, pp. 3749–3765, 2017.

[29] T. J. Herron, A. M. D. Rocha, K. F. Campbell et al., "Extracellular matrix – mediated maturation of human pluripotent stem cell – derived cardiac monolayer structure and electrophysiological function," *Trends in Pharmacological Sciences*, vol. 9, no. 4, pp. 1–12, 2016.

[30] T. K. Feaster, A. G. Cadar, L. Wang et al., "Matrigel mattress: a method for the generation of single contracting human-induced pluripotent stem cell-derived cardiomyocytes," *Circulation Research*, vol. 117, no. 12, pp. 995–1000, 2015.

[31] A. Leonard, A. Bertero, J. D. Powers et al., "Afterload promotes maturation of human induced pluripotent stem cell derived cardiomyocytes in engineered heart tissues," *Journal of Molecular and Cellular Cardiology*, vol. 118, pp. 147–158, 2018.

[32] W. Shi, H. Deng, J. Zhang, Y. Zhang, X. Zhang, and G. Cui, "Mitochondria-targeting small molecules effectively Prevent Cardiotoxicity Induced by Doxorubicin," *Molecules*, vol. 23, no. 6, 2018.

[33] F. Fischer, A. Hamann, and H. D. Osiewacz, "Mitochondrial quality control: an integrated network of pathways," *Trends in Biochemical Sciences*, vol. 37, no. 7, pp. 284–292, 2012.

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Review Article

The Potential of Induced Pluripotent Stem Cells to Treat and Model Alzheimer's Disease

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An estimated 6.2 million Americans aged 65 or older are currently living with Alzheimer's disease (AD), a neurodegenerative disease that disrupts an individual's ability to function independently through the degeneration of key regions in the brain, including but not limited to the hippocampus, the prefrontal cortex, and the motor cortex. The cause of this degeneration is not known, but research has found two proteins that undergo posttranslational modifications: tau, a protein concentrated in the axons of neurons, and amyloid precursor protein (APP), a protein concentrated near the synapse. Through mechanisms that have yet to be elucidated, the accumulation of these two proteins in their abnormal aggregate forms leads to the neurodegeneration that is characteristic of AD. Until the invention of induced pluripotent stem cells (iPSCs) in 2006, the bulk of research was carried out using transgenic animal models that offered little promise in their ability to translate well from benchtop to bedside, creating a bottleneck in the development of therapeutics. However, with iPSC, patient-specific cell cultures can be utilized to create models based on human cells. These human cells have the potential to avoid issues in translatability that have plagued animal models by providing researchers with a model that closely resembles and mimics the neurons found in humans. By using human iPSC technology, researchers can create more accurate models of AD *ex vivo* while also focusing on regenerative medicine using iPSC *in vivo*. The following review focuses on the current uses of iPSC and how they have the potential to regenerate damaged neuronal tissue, in the hopes that these technologies can assist in getting through the bottleneck of AD therapeutic research.

1. Introduction

A common theme in current neurodegenerative biomedical research is collaboration and using an interdisciplinary approach to solve problems. These problems can be genetic, molecular, or cellular, so determining the root cause of the neurodegeneration is useful in helping create an effective treatment against the uncovered pathology. To accomplish this, a new field of biomedical research has emerged: Translational Medicine (TM). TM integrates basic sciences and clinical medicine with the aim of optimizing the preventative measures and patient care, as well as increasing the turnout and expediting the process of turning appropriate biological discoveries into efficacious treatments or appropriate medical devices [1].

The appropriate application of TM will be useful in overcoming the bottleneck associated with (1) the identification and validation of appropriate biomarkers for early or preclinical diagnosis as well as monitoring the clinical progression of the diseases, (2) promoting the innovative clinical technologies, such as neuroimaging, stem cell technology, and nanotechnology, and (3) expediting the development of novel drug candidates by using appropriate organisms to model clinical conditions [1]. These organisms include, but are not limited to, invertebrates such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Danio rerio* (zebrafish) [2–4] and mammalian vertebrates, such as rodents or mice [5, 6]. Although important molecular cascades have been uncovered using these model organisms, these KO/KD transgenic organisms do not translate well to the clinical setting [7]. The limitations associated with animal models include extrapolating rare, well-understood genetic variants of a disease to treating a more common, less-understood sporadic form of the same disease, artificial overexpression of proteins

in transgenic/AAV-mediated models that does not return to basal levels with the inclusion of a knock-in variant, the shorter lifespan of models that does not allow for the complete development of the pathogenesis in age-related neurodegenerative diseases, and the lack of complex brain development in these organisms the does not allow for interpreting behavioral deficits that are characteristic to human neurodegenerative diseases [5]. For a complete review on different animal models and their shortcomings, refer to Dawson et al. or Drummond et al. [5, 8]; see Dubey et al. for a complete review on cellular models [9].

One approach researchers attempted to overcome the hurdles associated with animal models was the use of pluripotent stem cells (PSC), such as murine embryonic stem cells (ESC), which are undifferentiated cells with self-renewal capabilities and the potential to differentiate into any cell type of the body, providing researchers an opportunity to model human diseases with human cells [10]. Prior to 2007, the only type of PSC being used in research was ESC, and these were limited in scope due to the ethical questions surrounding the use of ESC. In 2006, Takahashi and Yamanaka generated iPSC from mouse somatic cell lines and then later repeated this experiment with human cells, thus creating hiPSCs [11, 12]. These new cells behave similarly to ESC, in which they can differentiate into any cell types of the body.

However, without the ethical limitations associated with ESC, iPSC biotechnology gives a larger community of researchers access to technology that can be of great aid to biomedical and clinical research. Given this great leap in science, questions remain about the limitations that PSC possess, including what these cells can be utilized for. In this review, PSC will be broken down into the different types of stem cells, as well as the application that these stem cells may have for neurodegenerative diseases, such as Alzheimer's disease (AD). Through the use of stem cells, diseases can be modeled, therapeutics tested for efficacy, and the potential to regenerate lost tissue tested using translational models.

2. Pluripotent Stem Cells

When a sperm cell and an ovum fuse in the fallopian tube, fertilization begins, and a zygote is formed. As the zygote divides, it forms a ball of cells known as a blastocyst. This blastocyst contains an outer cell mass (OCM) and inner cell mass (ICM). The OCM forms the trophoblast, which differentiates into an inner layer called the cytotrophoblast and an outer layer called the syncytiotrophoblast, which protects the lacunae by secreting human chorionic gonadotropin (HcG) [13]. Together, these two layers form the placenta around the developing embryo. The ICM forms the embryoblast, the precursor to all the cells of the human body. Embryoblast cells are short-lived and begin their differentiation into more specialized cells as implantation occurs. Initially, they form a bilaminar disc, the epiblast, which gives rise to the mesoderm, endoderm, and ectoderm, and the hypoblast, which gives rise to the yolk sac and chorion [14]. If implantation is prevented, the ICM will not differentiate and these cells, derived from the assisted reproductive technology (ART) programs, can be cultured and studied in research laboratories.

It has been nearly 40 years since ESC were first isolated from the ICM of the developing mouse blastocyst and grown *in vitro* [15, 16]. However, it was not until 1998 when the first derivation of human ESC was reported in the literature [17]. ESC have been shown to contribute to the endoderm, ectoderm, and mesoderm, as well as the germ line, when incorporated into chimeras with intact embryos [18–29]. *In vitro*, ESC can be indefinitely propagated in the undifferentiated state by growth in the presence of the leukemia inhibitory factor (LIF) and/or layer of murine embryonic fibroblasts (MEF), yet they retain the ability to differentiate to all mature somatic phenotypes when induced by the correct set of transcriptional factors [30–32]. The initial isolation in 1981 ushered in a new era of developmental biology by providing researchers with an appropriate model to study processes of early cellular programming and differentiation. When ESC were derived from humans in 1998, regenerative medicine and tissue engineering in humans finally became a real possibility. ESC have the potential to be used in the treatment of a great number of diseases in which the body is not naturally able to fully repair organ damage or dysfunction properly, thus leading to life-threatening complications.

The ability to differentiate into different organs means that the safety and efficacy of drugs can be tested on more reliable human-cell-based models [33–36]. For example, patients with an inherited mutation in the *HERG* gene develop long QT syndrome, a cardiac repolarization disorder that predisposes affected individuals to arrhythmia which can lead to sudden fainting or even death [37]. Certain small-molecule therapeutics has the potential to block the potassium channel, which prevents the potassium from leaving the cell and can quickly lead to myocardial infraction in certain individuals; therefore, screening drugs early on to check their inhibition against these channels is crucial in the development of efficacious drugs. Myocardial cells that express these *HERG* channels can be cultured, and different drugs can be screened against them to test the cytotoxicity [38]. This approach saves resources by preventing researchers and large pharmaceutical companies from optimizing therapies that will not translate to the clinical setting.

However, a big wrench was thrown in ESC research when President Bush banned federal funding for research on newly created ESC lines and specified that research prior to August 9th, 2001, would still be eligible for funding [39]. This ban on funding limited the ability for researchers to investigate ethnic differences in cell populations and limited the ability for researchers to investigate new diseases [39]. The lines that remained were of poor therapeutic value due to inferior conditions in which the cells were cultured and maintained [40]. Luckily, when the new administration took over, President Obama signed an executive order that reversed the previous decision and allowed the federal funding of hundreds of viable stem cell lines that were previously restricted [39]. This funded new groups to investigate the previously unavailable lines, specifically unused embryos from ART fertility clinics, but it did not allow for funding embryos created specifically

for research purposes or derived from other sources [39]. This limit on funding means researchers have to utilize different methods to investigate diseases, such as animal models, which have their own swath of investigative issues, or the now revolutionary iPSC, which allows for the investigation of almost any human ailment using human-derived somatic cells.

Retrovirus-mediated transduction gives researchers the ability to transform single-stranded RNA into double-stranded DNA that can be incorporated into the DNA of dividing host cells. This technique has enabled researchers to infect target cells and reprogram their genetic makeup, forcing them to exhibit a specific biochemical response [41–45]. Retrovirus-mediated transduction of human fibroblasts with four transcriptional factors (Oct-3/4, Sox2, KLF4, and MYC), all of which are expressed in ESC, could induce the fibroblast into an iPSC [12]. The ectopic expression of these four transcription factors reverses the previous shutdown that occurred when the cell became specialized during development. OCT4 and SOX2 induce the pluripotent gene pathway and enhance the expression of NANOG, a critical transcriptional factor present in the morula-stage embryos, ICM, and the epiblast, but not the primordial germ cells (PGC), intraembryonic mesoderm, and extraembryonic endoderm [46]. A deficiency in NANOG triggers the differentiation of ESC to the extraembryonic endoderm lineage, suggesting that this DNA-binding protein acts in part by transcriptionally repressing key regulators of this alternative tissue fate [47]. NANOG-null embryos were unable to support the formation of the epiblast and subsequent ESC, producing an endodermal only derivatives [48]. MYC is not necessary for the pluripotency exhibited by the iPSCs. Instead, it is important to regulate chromatin structure to facilitate cellular reprogamming [49]. KLF4 interacts with pluripotency network proteins, including SOX2 and OCT4, and also inhibits cell death [47]. In normal cellular development, OCT4 is zygotically expressed in the four to eight cell stages and is continued to be expressed in the ICM of the blastocyst [50]. The downregulation of OCT4 leads a zone of trophoblastic specification in the outer edge cells of the morula [51]. This demonstrates that OCT4 acts as a negative regulator of differentiation in the trophectoderm and a critical regulator of the pluripotent capabilities of the ICM [51–54]. This further demonstrated failure of OCT4-null embryos to form the ICM, instead differentiating into trophoectoderm [50]. SOX2 mutants demonstrated limited differential capabilities, leaving only trophoblast giant cells and extraembryonic ectoderm [47]. These mutants allow the formation of the blastocyst cavity; however, it lacks the ICM. In murine SOX2-knockout (KO) models, failure of the ICM means ESC are not developed and the mice are not viable past early embryonic development; however, wild-type ESC injection into the SOX2 mutant can rescue expression and prevent epiblast defects [55, 56]. KLF4 promotes cell survival by suppressing the p53-dependent apoptotic pathway by directly inhibiting TP53 and suppressing BAX expression [57, 58]. Coupled together, these four transcription factors are capable of reprogramming almost any specialized cell.

The main benefit of using iPSC is the avoidance of using an oocyte, especially for use in patient-specific therapies because the patient would be able to donate their own cells for autotransplantation [47]. This also avoids issues associated with partial major histocompatibility (MHC) matches because the surface antigens from donors would match the patients and avoid elucidating an immune response. This is one of the benefits of using a patient's own cells to treat a patient-specific ailment.

An additional benefits of using fibroblast-derived iPSC are that they can be used to differentiate into different types of neuronal cells, such as forebrain acetylcholine neurons, dopaminergic progenitor cells (substantia nigra pars compacta (SN_{PC})), Purkinje cells, hippocampal cells, and striatal cells, managing to exhibit electrical responses characteristic of neuronal firing [59–68]. This potential for successful reprogramming might be possible because the nervous system and ectoderm originate from the same embryonic tissue, the neuroectoderm [69]. These iPSCs can be transplanted into the region of interest (ROI) in the brain tissue of transgenic animal models, and the effects on different cognitive abilities can be observed, such as learning, memory, arousal, motor function, and motivational response [70]. However, as previously stated, higher-level cognitive abilities that are characteristic of certain neurodegenerative diseases are difficult to study, even with the addition of iPSC technology in transgenic animal models. Nonetheless, iPSCs are being utilized and studied for their potential for patient-specific clinic treatments in different neurodegenerative diseases.

3. Alzheimer's Disease

3.1. Economic Impact of AD. In 2010, roughly 5 million individuals aged 65 years or older in the United States were diagnosed with AD, the leading cause of dementia [71]. By 2050, AD is predicted to affect just under 14 million individuals, almost tripling in impact in just 40 years [71]. Not only does AD have an economic impact on society but it also costs families 11-70 hours per week in care, doing tasks such as feeding, bathing, and caring for their affected family member [72]. The costs associated with care were just under $19,000 in 1998, owing to the costs associated with caregiving time and a caregiver's lost earnings [72]. Owing to inflation, that same amount would cost just over $30,000 in 2021. In 2015, it was estimated that approximately 18.1 billion hours of assistance was provided by roughly 16 million Americans, estimated to cost $221 billion dollars [73]. As the disease progresses, the family is not able to provide the adequate care that is necessary for the patient and they are then placed in an assisted living facility. These facilities alone have a median cost of $4,051 per month, or $48,612 per year [74]. The economic impact this disease will have on society will continue to grow until improved therapeutics and treatments arise.

3.2. Pathology of AD. Psychologically, AD is characterized by early progressive anterograde amnesia, followed by slow progressive retrograde amnesia. These symptoms coincide with impairments in executive functions and other behavioral disturbances, which include paranoia, agitation, and

impairment in spatial and temporal memory [5, 75]. Biologically, AD has three hallmark pathologies: insoluble extracellular senile plaques comprised of amyloid beta (Aβ), insoluble intracellular neurofibrillary tangles (NFT) comprised of hyperphosphorylated tau, and degeneration in the hippocampal formation and cerebral cortex [76–80]. The amyloid precursor protein (APP) is a type I transmembrane protein that is highly conserved in vertebrates and consists of three homologues: APP, amyloid precursor-like protein 1 (APLP-1), and amyloid precursor-like protein 2 (APLP-2) [81]. For autosomal dominant early-onset Alzheimer's disease (EOAD), mutations in the APP, presenilin 1 (PSEN1), or presenilin 2 (PSEN2) gene sequence are a major risk factor, while the APOE4 allele is a major risk factor for late-onset Alzheimer's disease (LOAD) [82]. Excess Aβ is believed to contribute to the dysfunction seen in AD by leading to the formation of senile plaques; however, amyloid plaques have been found in other diseases, including vascular dementia, Lewy body dementia, and Parkinson's disease with dementia, as well as in the brain of aged individuals without any cognitive deficits [83–86]. The presence of Aβ in otherwise healthy individuals demonstrates that Aβ may have an intrinsic property in the normal physiology of neurons that is not yet understood.

Briefly, APP can be cleaved by three enzymes: α-secretase, β-secretase, and γ-secretase. PSEN1/2 is a component of γ-secretase, and it is a combination of these three enzymes cleaving the carboxyl end of APP that results in the formation of different protein fragments. For example, cleavage of α-secretase followed by γ-secretase results in soluble amyloid precursor protein α (sAPPα) and P3 [87]. This cleavage is hypothesized to be beneficial to neurons against oxygen-glucose deprivation and cellular excitotoxicity by inhibiting calcium currents and increasing potassium currents which effectively stabilized the resting membrane potential of neurons [88, 89]. sAPPα was also shown to promote neurite outgrowth, synaptogenesis, and cell adhesion [90, 91]. The formation of sAPPα prevents the formation of Aβ because α-secretase cleaves the APP protein at a site within 10 amino acids of the location β-secretase would cleave [87]. Aβ is formed when β-secretase cleaves the APP protein to form soluble amyloid precursor protein β (sAPPβ), followed by cleavage by γ-secretase, resulting in insoluble Aβ. This insoluble Aβ has the potential to induce conformational changes in soluble APP fragments, resulting in the senile plaques that are seen postmortem.

Intracellularly, tau is a member of the microtubule-associated proteins (MAPs) that stabilize neuronal microtubules (MTs) for their role in the development of cell processes, establishment of cell polarity, and axonal intracellular transport, both anterograde and retrograde [77]. A single *tau* gene on chromosome 17 codes for the tau protein that has 6 isoforms due to alternative splicing [92]. KO of the gene in *Drosophila* was not detrimental to the behavior, survival, or neuronal function [93], possibly because other MAPs can be substituted to stabilize MTs and the subsequent wild-type (WT) function is not affected. Tau mRNA is transported to the proximal axon from the cell body where translation occurs, and a gradient exists of tau protein, with the highest concentration found in the proximal axon, decreasing the more distal tau is from the cell body [94, 95]. Tau itself is an intrinsically disordered protein (IDP) that adopts a conformation that allows it to stabilize the MTs without being relegated to a single, rigid conformation [96, 97]. Its ability to adopt multiple conformations depends on posttranslational modification activity from both kinases and phosphatases. Tau kinases are classified as proline-directed (PDPK) and non-proline-directed protein kinases (NPDPK) [98]. One example of PDPK is glycogen synthase kinase 3 (GSK-3), which phosphorylates numerous sites in the tau protein, as well as in murine models overexpressing GSK-3 [96, 99, 100]. In tau phosphatases, the most significant enzyme is protein phosphatase 2A (PP2A) which accounts for more than 70% of the total posttranslational modification activity found in the human brain [101, 102]. Excess activity in the kinases or decreased activity in the phosphatases at specific phosphorylation sites can result in hyperphosphorylated tau (p-tau) [96, 103]. Many of the abnormal phosphorylation sites are at Ser-Pro or Thr-Pro motifs [77], which might explain the difficulty phosphatase enzymes encounter when removing a phosphate group at the Ser or Thr amino acid. Pro contains a rigid 5-membered nitrogen ring that forms a peptide bond with the adjacent amino acid's carbonyl group, via a condensation reaction. The hyperphosphorylation of tau at PDPK sites may induce a conformational change in the normally fluid tau at the Pro site residue, possibly changing its conformation from a *cis* to a *trans*-conformation to reduce any steric hindrances that the additional of an electronegative phosphate group might have on the peptide bond between the two residues. The phosphate group can also form salt bridges with neighboring arginine groups [104], another example of a posttranslational modification that potentially impacts PP2A activity and ability to remove phosphate groups.

A high concentration of p-tau consequently results in the depolymerization of MTs when it loses its IDP properties and adopts a rigid conformation [104]. The depolymerization of the MTs results in the reduction of length and size of the axons and increases the concentration p-tau in the intracellular matrix. Eventually, p-tau aggregates to form paired helical filaments (PHF), which bundle to form the intracellular NFTs seen in the postmortem pathology of AD [105]. PHF are not characteristic of only AD and have been characterized in frontotemporal dementia (FTD) linked to a V337M *MAPT* mutation as well as D252V and G389_I392del mutations [106, 107]. These mutations and subsequent phenotypes demonstrate that MAPs play an important role in regulating intracellular activity in neurons found in various regions of not only the hippocampus but also the cerebral cortex.

The third and final pathological hallmark of AD is the degeneration of neurons in the hippocampal formation and cerebral cortex, findings that are studied with neuropsychological examination but only confirmed upon autopsy. Neuropsychological exams are able to assess the global cognitive ability, memory, and executive function of the patient [108] but offer little in the ability to monitor the atrophy of the actual brain tissue. Ideally, researchers want to

monitor the atrophy of the brain in a living person to test the effects of potential therapeutics against degeneration, and so they employ an array of biomarkers or imaging techniques [109–111]. However, in order to develop effective biomarkers, effective drugs that target AD pathology are needed to test the efficaciousness of the biomarkers. The best treatments that exist are acetyl cholinesterase inhibitors (AChEI) like donepezil and rivastigmine, which slow down the symptoms associated with AD by blocking the uptake of acetylcholine (ACh) into the postsynaptic neuron [112–115]. It has also been shown that donepezil may play a role in suppressing inflammatory responses in the brain [115, 116] and that this inhibition of the inflammatory system may slow down any damage caused by microglia in the hippocampus and cerebral cortex. Currently, it is not known what makes the hippocampus vulnerable to atrophy; however, a number of neurochemical and vascular alterations, such as deviations in the levels of glucocorticoids, serotonin, glutamate, and their subsequent receptors, have been implicated [64, 117]. Understanding what causes the atrophy in this region at the cellular level will elicit the biochemical processes that link Aβ pathology to NFT pathology, and this knowledge will enable the next generation of therapeutics to be developed that target the pathology instead of the symptoms.

4. Stem Cells in AD

4.1. Modeling

4.1.1. Genetics. iPSCs derived from peripheral blood mononuclear cells (PBMC) and fibroblasts have the potential to revolutionize the drug discovery process by providing researchers with a model that has the potential to usurp animal models as the model of choice among researchers through a translatable model derived directly from the cells of patients who have been diagnosed with neurodegenerative diseases [118, 119]. However, waiting for patients to develop symptoms associated with AD means that the pathology has developed past the point of preventative medicine and enters the realm of improving the quality of life. Therefore, genetic models of AD are utilized in the creation of effective iPSC models (Table 1). Cells derived from a patient with a double mutation in the APP gene (KM670/671NL) increased the total levels of Aβ, while cells derived from a patient with a duplicated APP gene revealed higher levels of Aβ (1–4) and p-tau (Thr231) and increased activity in GSK3B [120, 121]. As a side note, there is a gene mutation in *APP* (A673T) that was shown to be protective against cognitive decline by decreasing levels of sAPPβ [122]. iPSCs were generated from a patient with this mutation [123] and are being investigated to uncover the cellular processes that the increased polarity from this mutation might have on the shape, function, and environment of the APP protein.

Patients with trisomy 21 have an extra copy of *APP*, found on the 21st chromosome, which is associated with elevated levels of Aβ, an overaccumulation of which has been shown to lead to AD dementia in patients with Down syndrome [124–126]. iPSCs derived from mesenchymal stem cells (MSC) in amniotic fluid from individuals with trisomy 21 demonstrate the ability to model the pathology in AD, such as elevated levels of Aβ and increased levels of p-tau [127]. As individuals with Down syndrome represent a population that is at risk of developing AD, the iPSC cell lines can be used to screen different therapeutics for their ability to reduce the levels of p-tau and Aβ.

Mutations of *PSEN1/2*, the catalytic component of γ-secretase, have been linked to familial Alzheimer's disease (fAD). Patients with fAD have mutations in *PSEN1* (A246E) and *PSEN2* (N141I) [70]. A separate *PSEN1* exon 9 deletion (PSEN1δ9) produced mutant astrocytes that altered the calcium signaling activity of healthy neurons when AD astrocytes generated from iPSC from PSEN1δ9 donor cells were cocultured with healthy neurons [128]. Toxic Aβ42 secretion was seen in neurons derived from *PSEN1* mutation donor cells [129], demonstrating the potential that fAD iPSC models possess for modeling AD. However, abnormal issues with γ-secretase represent a small portion of patients diagnosed with AD, so these models might not be the most translatable.

Microtubule-associated protein tau (*MAPT*) gene mutations are the most prevalent cause of familial frontotemporal dementia (fFTD), a condition linked to mutations on chromosome 17 (p.A152T), which has also been implicated in AD and Parkinson's disease with dementia [130]. The mutation leads to an additional phosphorylation site that has the potential to form salt bridges with nearby amino acids. If post-translational modifications of this mutant tau by phosphorylation changes the 3D conformation to a more stable, rigid conformation, then understanding how this mechanism works is the key to reversing and developing therapeutics that prevents the formation of p-tau and its aggregates. iPSC-derived neurons were generated from individuals carrying the p.A152T variant, and it was established that upregulation of p-tau was coupled with enhanced stress-inducible markers and cell vulnerability to proteotoxic, excitotoxic, and mitochondrial stressors, which were rescued by CRISPR/Cas9-mediated targeting of tau or by pharmacological activation of autophagy [131]. With iPSCs producing mutant tau, it becomes possible to elucidate and uncover the cellular mechanisms that underpin protein misfolding in tauopathies, mainly by studying the effects seeding with p-tau has on microtubule formation in these derived neurons. A separate study used zinc finger nucleases (ZFNs) to introduce two *MAPT* mutations in healthy donor iPSC: an IVS10+16 mutant shown to increase the inclusion of exon 10 and a P301S point mutation in exon 10 [132]. The former mutation was selected for its potency to fasten the inclusion *MAPT* exon 10 while the latter mutation was chosen to generate an aggressive fFTD model [132]. This model would provide researchers with a genetic model of tauopathy that can be used in conjunction with other models to study the effects therapeutics have on p-tau without the presence of Aβ.

The largest population of patients diagnosed belong to the LOAD group, and of this group, the most prevalent genetic risk factor is *APOE4*, which is linked to the sporadic form of the disease, sporadic Alzheimer's disease (sAD). Apolipoprotein E (apoE) is produced primarily by astrocytes in the CNS as a carrier of cholesterol and other lipids that

Table 1: Current genetic iPSC models of Alzheimer's disease.

Gene	Model/mutation	Phenotype	References
APP	iPSC/KM670/671NL	Increased levels of Aβ p-tau (Thr231) GSK3B activity ↑ Neurodegeneration	[120, 121]
	iPSC/A673T	Decreases levels of sAPPβ Neurodegeneration	[123]
	MSC/trisomy 21	Aβ expression ↑ p-Tau expression ↑ Neurodegeneration	[127]
PSEN1	iPSC/PSEN1δ9	Mutant astrocytes Disrupted Ca2+ signaling in healthy neurons Toxic Aβ secretion Neurodegeneration	[128, 129]
MAPT	iPSC/IVS10+16, P301S	4R:3R tau expression increased Perturbations in Ca^{2+} burst frequency Reduced lysosomal acidity Tau oligomerization Neurodegeneration	[132]
APOE	iPSC/APOER (R = 2, 3, or 4)	Allelic expression of APOE influences APP transcription through an abnormal kinase cascade APOE4 astrocytes and microglia exhibited a decrease in Aβ clearance Accumulation of cholesterol in the intra- and extracellular matrices Aβ expression led to the activation of microglia; however, the length of processes was allelic dependent	[134, 137, 139]

support the membrane, synaptic integrity, and injury repair [133, 134]. In one experiment, ApoE4 secreted by glia cells stimulated Aβ formation by binding with APOER found on the extracellular surface of iPSC-derived neurons, initiating a noncanonical cascade that results in the upregulation of Mitogen-Activated Protein (MAP) kinase kinase kinase, also known as Dual Leucine zipper-bearing Kinase (DLK), an attractive candidate for neuronal signaling because it has been implicated in axonal regeneration, synaptogenesis, and neurodegeneration [134–136]. Mixed-lineage kinase (MLK) MKK7 was previously shown to be found in the same cellular compartment as DLK and phosphorylation target, and overexpression of DLK led to increased levels of phosphorylated MKK7 (pMKK7) and subsequent levels of phosphorylated ERK1/2 MAP kinase [134]. This cellular cascade led to the upregulation of APP independent of APLP1 and APLP2, by activating a DLK-dependent MAP kinase signaling pathway that induces cFos phosphorylation which stimulates AP-1 and enhances APP synthesis via a direct effect on the *APP* gene promoter [134].

A separate study derived neurons, astrocytes, and microglia-like cells from isogenic *APOE3* and *APOE4 iPSC* lines to examine the cellular differences exhibited between cells from donors with different alleles [137]. These results showed that *APOE4* astrocytes and microglia were less efficient in the uptake and clearance of Aβ compared to *APOE3* astrocytes, but it did not determine if ApoE is necessary for the clearance of Aβ from the extracellular matrix as reduced *APOE4* mRNA and protein levels were seen in iPSC-derived astrocytes, indicating the effect is specific to astrocytes [137]. The APOE4 variant was shown to regulate the expression of numerous lipid metabolism and transport genes, leading to the accumulation of cholesterol in the intracellular and extracellular space in the glial cell cultures [137].

In 2D cultures without Aβ, *APOE4* microglia exhibited fewer and shorter processes than *APOE3* microglia; however, after embedding in 3D neuronal cultures that produced Aβ, the same cells had longer processes than their APOE3 counterparts, consistent with impairment in the ability of APOE4 microglia-like cells to respond effectively to Aβ in the environment [137–139]. One of the upregulated immune genes seen in the microglia-like cells was *IRF8*, an immune-related gene that has been shown to induce transcription of many other immune-related genes, transforming the resting microglia into a reactive state [140]. The expression of TREM2 and its signaling adaptor TYROBP, proteins crucial for microglial function and a significant AD risk gene, was positively correlated with the APOE4 genotype [141, 142] and is consistent with recent studies that show increased levels of TREM2 in cerebrospinal fluid of AD patients [143], but further work is needed in order to determine the exact mechanism linking TREM2 and ApoE.

4.1.2. Organoids. Along with diseased neurons, researchers can generate astrocytes, oligodendrocytes, microglia, and the vasculature of the brain in 2D or 3D models in order to examine the cellular dysfunction that arises during the development and interaction of different cell types in AD [10, 144–146]. For a more encompassing and complete review on brain organoid protocols, current advances, and limitations, refer to Papaspyropoulos et al. [147] A scaffold-free 3D model generated from fibroblasts of controls and patients

with fAD, the result of a duplication in APP or a PSEN1 mutation, resulted in elevated levels of Aβ and p-tau in organoids from fAD cultures compared to controls [148]. These organoids were treated with a BACE-1 β-secretase inhibitor and a γ-secretase inhibitor that are well known to inhibit the aggregation of Aβ [149] or a DMSO vehicle that acted as the control. After 60 days of treatment, the particle counts of Aβ had decreased and immunoreactivity of p-tau decreased, signifying a decrease in the concentration of p-tau [148]. These results demonstrate that elevated Aβ levels correlates positively with levels of p-tau and that treatment that decreases Aβ levels subsequently decreases the concentration, potentially via a cellular mechanism that results in the reduction of GSK3β activity. By inhibiting β-secretase and γ-secretase, α-secretase and low levels of γ-secretase are cleaving APP into sAPPα which promotes neuroprotective factors and stimulates neurite outgrowth [91], a process that is mediated by the binding of tau to MTs in the axon [94].

In separate studies, sAPPα overexpression led to low levels of GSK3β activity and decreased levels of p-tau [121, 150], providing evidence that APP processing might underpin the pathology exhibited in AD through currently intracellular interactions. This hypothesis coincides with the evidence from studies of patients with elevated Aβ levels with no cognitive deficits. We investigated the intracellular matrix and subsequent proteome of the neurons of these types of patients compared to neurons of individuals homozygous for *APOE4* who have been diagnosed with MCI or AD. This comparison experiment could potentially uncover proteins or a signaling cascade that prevents the adverse effects that improper APP processing has on otherwise healthy cells by comparing the relative levels of protein expression between the two populations.

To examine the effects of the immune system on the brain organoid development and maturation, organoids were generated that included both neuronal cells and microglial cells [151]. The microglia, being the resident macrophages of the brain, have sparked interest in recent years as potential targets for immunotherapies that are aimed at reducing the inflammation and subsequent damage caused by the phagocytosis of neurons, both *in vivo* and *ex vivo* [152–155]. Microglia differ from peripheral blood monocytes that derive from myeloblastosis protooncogene and transcription factor-(MYB) dependent HSC in the bone marrow by originating from MYB-independent yolk sac-derived fetal macrophages that invade the brain around embryonic day 31 until the closure of the blood-brain barrier where they proliferate locally in the brain and are not replaced by peripheral macrophages in the body [156].

Since microglia have a distinct embryonic origin, microglia-derived iPSC from HSC will resemble the monocyte-derived cells found in the brain that have a morphology similar to that of resident microglia, but its function and transcriptome differ significantly from those of the native microglia [157]. A more in-depth analysis of microglia protocols is presented by Haenseler and Rajendran [156], the main takeaway being that a near-authentic microglia model should mimic the microglial ontogeny and neuronal environment by differentiating in an MYB-independent manner to yolk sac-derived fetal macrophages that are allowed to invade a neuronal environment where they can mature and adopt the healthy, resident microglia phenotype and avoid creating a "microglia-like" cell that does not imitate the interactions seen in neurodegenerative diseases. Coculturing microglia and neurons will not only improve preclinical models but also improve translatability from benchtop to bedside by improving drug screening. One such screen could be to examine synaptotoxicity of neurons with fluorescently tagged synapses (using synapsin I, synapsin II, or synaptophysin as markers) [158] and microglia containing an activation marker, such as allograft inflammation factor 1(AIF-1) [159]. First, conditions that induce microglia-driven synaptotoxicity would need to be identified, either *in vivo* or *ex vivo*. These conditions could be a prion protein that induces an inflammation response in the microglia or a pathogen that activates the microglia into phagocytizing the otherwise healthy neurons. Once a coculture system exists, small molecules can be assayed and screened to find potential hits, molecules that are capable of interrupting the interaction between activated microglia and neurons and preventing the induced synaptotoxicity and subsequent neuronal loss.

4.2. Reconstruction. iPSCs not only have the potential to be used for modeling diseases *ex vivo*, implanting autologous gene-edited iPSC-derived cells into patients opens up Pandora's box of new therapeutic potential. iPSC-derived microglia have been shown to integrate successfully into the brains of murine models [160–162]. Transplantation of human long-term neuroepithelial-like stem (lt-NES) cell-derived cortical neurons at two months into stroke injured rats produced from iPSC improved neurological deficits and established both afferent and efferent morphological and functional connections with host cortical neurons at 5 months, as demonstrated by the presence of cortical phenotype cells with pyramidal morphology and the presence of the cortex-specific marker TBR1 and lack of tumorigenesis [163–165]. At 6 months after transplantation into rats with ischemic lesions in the cerebral cortex, host neurons in the contralateral somatosensory cortex received monosynaptic inputs from grafted neurons [165]. Immunoelectron microscopy demonstrated the myelination of the graft-derived axons in the corpus callosum, and their terminals formed excitatory glutamatergic synapses on host cortical neurons [165]. Optogenetic inhibition of the lt-NES cells and the subsequent loss of motor function in the murine model demonstrated their involvement in the regulation of the stroke-induced animals' behavior [163, 164]. These experiments demonstrate that transplantation of hiPSC into a murine model is possible and that the recovery of lost motor function can be achieved in a live murine model.

Taking the previous experiment further, healthy neocortical tissue from the middle temporal gyrus of patients undergoing elective surgery for epilepsy was cocultured with lt-NES cells and was shown to form functional afferent and efferent connections with adult human cortical neurons in the slices, evidenced by electron microscopy, rabies virus retrograde monosynaptic tracing, and whole-cell patch clamp recordings [166]. This experiment provides evidence that

hiPSC can differentiate into layer-specific functional synaptic networks when implanted onto organotypic cultures. This finding supports the clinical translatability that neuronal replacement with iPSC-derived cells might possess in neurodegenerative diseases by strengthening the functional networks that are damaged due to the loss of tissue.

Furthermore, this grafting, in patients with AD, might ameliorate or even prevent the neurodegeneration seen in the cortex of AD patients. In a human trial of 50 patients living with Parkinson's disease (PD), autologous implantation of stem cells with highly selective arterial catheterization was performed into the posterior region of the circle of Willis and the quality of life (QOL), activities of daily living, depression, and disability were evaluated for two years [167]. No complications arose from this treatment, and improvements in all of the categories were seen in the patients, especially the QOL.

In a separate phase I clinical study, human umbilical cord blood MSC were stereotactically injected into the precuneus, the site where amyloid accumulation is believed to begin, and the hippocampus, the site where NFT aggregation is seen [168]. MSC are unlikely to differentiate into the neurons; however, they potentially secrete cytotropic factors into the brain that could decrease neuroinflammation by reducing total amyloid load and increasing endogenous neurogenesis [169]. The patients received a bilateral injection into the hippocampus or a lateral injection into the right side of the precuneus to compare the change in amyloid burden between the MSC-treated right precuneus region and the untreated left precuneus region. Adverse events were recorded, such as wound pain where the burr hole was created in all of the patients, headache, dizziness, delirium, nausea, and back pain which were noted in a small minority of the patients, but none of these symptoms were considered serious enough to halt the trial. The conclusion of this phase I trial determined that administration of MSC derived from umbilical cord blood into the hippocampus and precuneus was feasible, safe, and well tolerated in patients with mild-moderate AD [168]. One caveat with these results was the lack of a control group to compare these results to. Without a proper control, the efficacy could not be determined; however, further studies should be conducted to determine the clinical benefit of this treatment by comparing experimental MSC results with placebo treatments on a larger cohort.

5. Shortcomings

iPSC-derived cells from humans have been investigated for their potential in improving translatability from benchtop to bedside. These cells have the capabilities of modeling diseases, like AD, *ex vivo* and *in vivo*. *Ex vivo* experiments using hiPSC can be conducted at a faster rate than animal models, allowing for the rapid understanding of the effects of different KO and knock-ins. Behavioral assays on cell cultures cannot be accomplished, but hiPSCs that are xenotransplanted into the brains of murine models were shown to form functional synaptic connections with the native tissue, a finding that was recapitulated in hiPSC cultured with healthy neocortical tissue. Using organotypic slices preserves key cellular elements of the brain, such as glial cells and neurons, as well as morphological and electrophysiological properties that are consistent with pyramidal neurons *in vivo*, and provides a 3D architecture that preserves its synaptic connections and microenvironment [148, 170–172]. However, the use of these slices does not allow for the study of its mechanisms of interaction to be fully elicited due to the absence of components of the vascular and immune systems and the decreased survival of the neurons with long-term culturing [166]. Furthermore, an injury response involving reactive microglial cells and progressive neurodegeneration is seen in resected human tissue [173]. This injury response was a result of the procedure and not a pathological immune response of the grafting.

Brain organoids derived from hiPSC are capable of recapitulating key aspects of the human brain; however, they are not a perfect replica. Therefore, overcoming limitations of the organoid will expand the ability to investigate human brain development and disorders associated with abnormal development. Currently, one of the greatest pitfalls in organoid technology is the small number of current organoids as well as the batch-to-batch variability that arises from a diverse number of protocols being followed by researchers. The eventual establishment of a human brain atlas containing immunohistology data, *in situ* hybridization, and transcriptomics data will give researchers developing and engineering organoids a "gold standard" by which they can compare their lab organoids to the tissue of a "standard" human brain [174]. With a gold standard, organoid engineers will be able to further engineer a "gold standard" organoid to which further organoids that model neurodegenerative diseases can be compared, enabling researchers to test different therapeutics, such as iPSC regeneration treatment or small-molecule drug therapy, on a translatable model.

Considering that the development of iPSC technologies provides an attractive possibility of using differentiated somatic human cells as a platform to model diseases or regenerate tissue, one of the greatest shortcomings is the genomic instability exhibited by iPSCs [47, 132, 175–179]. Whole exome sequencing was done on the human foreskin fibroblast at two different passages to determine if the mutations seen in iPSC are due to stress associated with oncogene expression during reprogramming, and the researchers found that *in vitro* passaging contributed to 7% of the mutations; 19% of the mutations were preexisting and were derived from parental fibroblasts, suggesting that 74% of the mutations were acquired during cellular reprogramming [177]. Structural variations in the chromosome are also seen; the most recurrent are chromosome deletions, which cause a loss of heterozygosity, and duplications of chromosomes [175], which might be advantageous to the growth and survival of the culture, but at the same time, these chromosomal aberrations can confer a completely different phenotype to a cell, potentially creating a teratoma. One example of a beneficial duplication is trisomy 12. Chromosome 12 contains cell cycle-related genes and the pluripotency-associated gene *NANOG* [179]. Duplication of this chromosome has the potential to contribute to the selective advantage of

proliferation and reprogramming of iPSC by providing the cell with more NANOG. This additional NANOG might allow the cell to reprogram itself, making this mutation favorable for the reprogramming phase of iPSC and allow for the differentiation to a specific cell type.

Epigenetic genomic imprinting mechanisms, such as histone modification and DNA methylation, function to regulate chromosome architecture and the transcriptional repression of repetitive elements and regulate and repress gene activity during development [180, 181]. DNA methylation modifies CpG dinucleotides and is associated with a transcriptionally repressed state, effectively silencing the gene on either the maternal or the paternal allele [182]. Compared with ESC, iPSC generated from blood, fibroblast, and brain tissue exhibited a much greater tissue-specific epigenetic signature [183], due to incomplete reset of the tissue-specific epigenetic signature to the default embryonic stages during the process of reprogramming. These tissue-specific epigenetic signatures originate during the development of the embryo, at certain stages of somatic cell differentiation and dedifferentiation under tightly regulated gene expression [47]. The genomic instability of iPSC could result from (I) preexisting mutations in parental somatic cells, (II) reprogramming-induced mutations, and (III) mutations that arise during *in vitro* culture [184]. This genomic instability could hamper *in vitro* models of AD because the presence of genomic deletions and amplifications exhibited by the iPSC-derived neurons is suggestive of oncogene-induced DNA replication stress [185]. This replication stress, usually located in the common fragile sites (CFS), has the potential to alter the phenotypes exhibited by iPSC and prevent them from fully exhibiting their differentiated properties that are specific to the cells of interest; this could be caused by aneuploidy, an abnormality in chromosomal number, single-nucleotide variations (SNV), and subchromosomal copy number variation (CNV), all of which have the potential to promote the spontaneous loss of chromosomes [186]. If, for example, a researcher is trying to study APP, a protein coded on chromosome 21 in fibroblast-derived neurons, an abnormality in this chromosome could potentially impact the transcription and subsequent translation of the proteins of interest, resulting in a shift in production that would not be found in normal neuronal conditions, resulting in an *in vitro* experiment that provides results for a mutated phenotype, instead of the desired phenotype.

In addition, genomic instability can alter the ability of iPSC to reconstruct the cellular morphology *in vivo*. One such alteration that can arise involves the tumor suppressor P53 gene [187]. Normally, P53 induces cell cycle arrest, apoptosis, or senescence of the stressed somatic cells to prevent the passage of genetic abnormalities; in iPSC, p53 is silenced to allow the reprogramming transcriptional factors to revert the somatic cell into a cell that can be differentiated [188]. Given the importance p53 has on maintaining genetic stability, silencing this gene and then transplanting the cells for *in vivo* culture could result in the formation of a teratoma at the site of implantation.

One way to overcome the hurdle posed by transferring epigenetic markers to iPSC would be through the use of a nuclear transfer to an unnucleated oocyte (ntESC) [189]. These ntESC provide genetically identical and immunologically compatible stem cells for individual somatic cell donors; however, this process is arduous and inefficient. However, the lack of tissue-specific epigenetic memory seen in ntESC provides evidence that the ooplasm contains additional factors needed to competently erase tissue-specific epigenetic memory, and research is currently being undertaken to determine these additional factors. One study attempted to reverse the incomplete reprogramming status of iPSC after iPSC nuclear transfer to an enucleated oocyte [190]. They found that iPSC-nt-ESC showed even worse developmental potential compared with the original iPSC, indicating that aberrant gene expression pattern established during iPSC derivation cannot be reset by nuclear transfer [190], potentially because of genetic aberrations acquired during iPSC formation [175]. This experiment demonstrated that faulty gene expressions that existed previously in iPSC cannot be reset by nuclear transfer, nor can it reverse developmental deficiencies characteristic of iPSC.

Identification of differentially methylated regions (DMRs) between iPSC and ESC is an important starting point. High-resolution DNA methylation analysis identifies DMRs in iPSC and compares them with the findings in ESC and somatic cells, allowing the researchers to determine the source of the epigenetic change. Another technique used to abrogate the epigenetic differences exhibited by iPSC derived from different origins is continuous passaging [191]. They found that the RNA expression profile of 12 different iPSC lines was notably different at the fourth passage; however, by the 16th passage, the expression profile of the iPSC was reduced from between 500 and 2000 differentially expressed genes to less than 50 in the late passage cultures [191]. Extensive *in vitro* passaging has the ability to reduce the variability seen in iPSC derived from different origins. However, the use of earlier passages of iPSC is favored in a therapeutic application to avoid genetic and epigenetic changes that arise during the extended culturing process. A different approach would be to use a chromatin-modifying compound that enables a DNA demethylation agent, such as 5-aza-cytidine [192], to remove the methylation that is tissue specific, restoring the ability to differentiate to various tissue lineages [193]. However, this approach does not improve the pluripotency and potentially damages other regions of DNA that are susceptible to modifications.

Site-specific targeting of hiPSC is also important in regenerating damaged CNS tissue, so more research needs to be conducted that bridges the gap between biomarkers of the central nervous system (CNS) that differentiate neural lineages into the specific tissue [194] and the ability of hiPSC to differentiate into these specific brain regions without (1) generating an immune response, (2) forming cancerous teratomas *in vivo*, or (3) forming non-site-specific tissue, while also (4) regaining lost brain function, both physical (electrophysiological, histological) and psychological, and (5) being reproducible. These five pillars need to be followed if neurodegenerative diseases, like AD, are hoped to have any treatment that improves the quality of life while also treating the neurodegeneration that precedes the psychological

symptoms of the disease. These five pillars can be applied to any regenerative treatment that is aimed at successfully treating damaged tissue in the body, substituting item (4) for whatever organ the researcher aims to study, such as the liver, heart, or kidney and focusing on regaining its lost molecular functions.

6. Conclusion

To improve the QOL of patients diagnosed with Alzheimer's disease, the next generation of therapeutics needs to be developed. However, in order to develop effective therapeutics, model organisms that recapitulate the pathology of the disease need to be studied in order to ascertain the mechanisms that lead to neurodegeneration. The past 50 years have relied heavily on transgenic animal models that do not translate well to the phenotype's characteristic of the disease, relying heavily on silencing gene expression or overexpression of proteins to elicit a pathological response. These methods, although effective at inducing protein misfolding or aggregation, do not accurately represent the cascade of events that underlies the pathology seen in sporadic Alzheimer's disease, the leading cause of AD in patients. To better understand the pathology that underlies neurodegeneration seen in sAD patients, induced pluripotent stem cell models generated from the patient should be utilized to not only model the degeneration, thus elucidating the mechanisms that underlie the abnormal protein responses in sAD, but also reconstruct damaged or degenerated neural tissue. Once the kinks have been hammered out of iPSC, they have the potential to revolutionize the way we model and treat diseases of the body.

Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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References

[1] S. Chen and J. C. Zheng, "Translational neurodegeneration, a platform to share knowledge and experience in translational study of neurodegenerative diseases," *Translational Neurodegeneration*, vol. 1, no. 1, p. 1, 2012.

[2] S. Saleem and R. R. Kannan, "Zebrafish: an emerging realtime model system to study Alzheimer's disease and neurospecific drug discovery," *Cell Death Discovery*, vol. 4, no. 1, p. 45, 2018.

[3] C. D. Link, "Invertebrate models of Alzheimer's disease," *Genes, Brain and Behavior*, vol. 4, no. 3, pp. 147–156, 2005.

[4] K. Prüßing, A. Voigt, and J. B. Schulz, "Drosophila melanogaster as a model organism for Alzheimer's disease," *Molecular Neurodegeneration*, vol. 8, no. 1, p. 35, 2013.

[5] T. M. Dawson, T. E. Golde, and C. Lagier-Tourenne, "Animal models of neurodegenerative diseases," *Nature Neuroscience*, vol. 21, no. 10, pp. 1370–1379, 2018.

[6] A. M. Hall and E. D. Roberson, "Mouse models of Alzheimer's disease," *Brain Research Bulletin*, vol. 88, no. 1, pp. 3–12, 2012.

[7] P. McGonigle, "Animal models of CNS disorders," *Biochemical Pharmacology*, vol. 87, no. 1, pp. 140–149, 2014.

[8] E. Drummond and T. Wisniewski, "Alzheimer's disease: experimental models and reality," *Acta Neuropathologica*, vol. 133, no. 2, pp. 155–175, 2017.

[9] S. K. Dubey, M. S. Ram, K. V. Krishna et al., "Recent expansions on cellular models to uncover the scientific barriers towards drug development for Alzheimer's disease," *Cellular and Molecular Neurobiology*, vol. 39, no. 2, pp. 181–209, 2019.

[10] E. G. Z. Centeno, H. Cimarosti, and A. Bithell, "2D versus 3D human induced pluripotent stem cell-derived cultures for neurodegenerative disease modelling," *Molecular Neurodegeneration*, vol. 13, no. 1, p. 27, 2018.

[11] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[12] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[13] C. Nwabuobi, S. Arlier, F. Schatz, O. Guzeloglu-Kayisli, C. J. Lockwood, and U. A. Kayisli, "HCG: biological functions and clinical applications," *International Journal of Molecular Sciences*, vol. 18, no. 10, p. 2037, 2017.

[14] M. J. Gallego, P. Porayette, M. M. Kaltcheva, R. L. Bowen, S. Vadakkadath Meethal, and C. S. Atwood, "The pregnancy hormones human chorionic gonadotropin and progesterone induce human embryonic stem cell proliferation and differentiation into neuroectodermal rosettes," *Stem Cell Research & Therapy*, vol. 1, no. 4, p. 28, 2010.

[15] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, no. 5819, pp. 154–156, 1981.

[16] G. R. Martin, "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells," *Proceedings of the National Academy of Sciences*, vol. 78, no. 12, pp. 7634–7638, 1981.

[17] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro et al., "Embryonic stem cell lines derived from human blastocysts," *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[18] M. Mori, K. Furuhashi, J. A. Danielsson et al., "Generation of functional lungs via conditional blastocyst complementation using pluripotent stem cells," *Nature Medicine*, vol. 25, no. 11, pp. 1691–1698, 2019.

[19] A. Nagy, E. Gocza, E. M. Diaz et al., "Embryonic stem cells alone are able to support fetal development in the mouse," *Development*, vol. 110, no. 3, pp. 815–821, 1990.

[20] A. Bradley, M. Evans, M. H. Kaufman, and E. Robertson, "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines," *Nature*, vol. 309, no. 5965, pp. 255-256, 1984.

[21] B. Descamps and C. Emanueli, "Vascular differentiation from embryonic stem cells: novel technologies and therapeutic

promises," *Vascular Pharmacology*, vol. 56, no. 5-6, pp. 267–279, 2012.

[22] D. Später, E. M. Hansson, L. Zangi, and K. R. Chien, "How to make a cardiomyocyte," *Development*, vol. 141, no. 23, pp. 4418–4431, 2014.

[23] M. E. Hartman, D.-F. Dai, and M. A. Laflamme, "Human pluripotent stem cells: prospects and challenges as a source of cardiomyocytes for in vitro modeling and cell-based cardiac repair," *Advanced Drug Delivery Reviews*, vol. 96, pp. 3–17, 2016.

[24] Y.-K. Wang, W.-W. Zhu, M.-H. Wu et al., "Human Clinical-Grade Parthenogenetic ESC-Derived Dopaminergic Neurons Recover Locomotive Defects of Nonhuman Primate Models of Parkinson's Disease," *Stem Cell Reports*, vol. 11, no. 1, pp. 171–182, 2018.

[25] J. P. Weick, Y. Liu, and S.-C. Zhang, "Human embryonic stem cell-derived neurons adopt and regulate the activity of an established neural network," *Proceedings of the National Academy of Sciences*, vol. 108, no. 50, pp. 20189–20194, 2011.

[26] M. M. Daadi and G. K. Steinberg, "Manufacturing neurons from human embryonic stem cells: biological and regulatory aspects to develop a safe cellular product for stroke cell therapy," *Regenerative Medicine*, vol. 4, no. 2, pp. 251–263, 2009.

[27] M. Hohwieler, M. Müller, P.-O. Frappart, and S. Heller, "Pancreatic progenitors and organoids as a prerequisite to model pancreatic diseases and cancer," *Stem Cells International*, vol. 2019, Article ID 9301382, 11 pages, 2019.

[28] M. B. K. Petersen, A. Azad, C. Ingvorsen et al., "Single-cell gene expression analysis of a human ESC model of pancreatic endocrine development reveals different paths to β-cell differentiation," *Stem Cell Reports*, vol. 9, no. 4, pp. 1246–1261, 2017.

[29] S. J. Micallef, X. Li, M. E. Janes et al., "Endocrine cells develop within pancreatic bud-like structures derived from mouse ES cells differentiated in response to BMP4 and retinoic acid," *Stem Cell Research*, vol. 1, no. 1, pp. 25–36, 2007.

[30] H. J. Rippon and A. E. Bishop, "Embryonic stem cells," *Cell Proliferation*, vol. 37, no. 1, pp. 23–34, 2004.

[31] A. G. Smith, J. K. Heath, D. D. Donaldson et al., "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides," *Nature*, vol. 336, no. 6200, pp. 688–690, 1988.

[32] R. L. Williams, D. J. Hilton, S. Pease et al., "Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells," *Nature*, vol. 336, no. 6200, pp. 684–687, 1988.

[33] E. Raschi, I. Diemberger, B. Cosmi, and F. De Ponti, "ESC position paper on cardiovascular toxicity of cancer treatments: challenges and expectations," *Internal and Emergency Medicine*, vol. 13, no. 1, pp. 1–9, 2018.

[34] R. S. Deshmukh, K. A. Kovács, and A. Dinnyés, "Drug discovery models and toxicity testing using embryonic and induced pluripotent stem-cell-derived cardiac and neuronal cells," *Stem Cells International*, vol. 2012, Article ID 379569, 9 pages, 2012.

[35] S. Bremer and T. Hartung, "The use of embryonic stem cells for regulatory developmental toxicity testing in vitro–the current status of test development," *Current Pharmaceutical Design*, vol. 10, no. 22, pp. 2733–2747, 2004.

[36] A. Rolletschek, P. Blyszczuk, and A. M. Wobus, "Embryonic stem cell-derived cardiac, neuronal and pancreatic cells as model systems to study toxicological effects," *Toxicology Letters*, vol. 149, no. 1-3, pp. 361–369, 2004.

[37] M. C. Sanguinetti and M. Tristani-Firouzi, "HERG potassium channels and cardiac arrhythmia," *Nature*, vol. 440, no. 7083, pp. 463–469, 2006.

[38] Y. Qu, B. Gao, M. Fang, and H. M. Vargas, "Human embryonic stem cell derived cardiac myocytes detect hERG-mediated repolarization effects, but not Nav1.5 induced depolarization delay," *Journal of Pharmacological and Toxicological Methods*, vol. 68, no. 1, pp. 74–81, 2013.

[39] V. Murugan, "Embryonic stem cell research: a decade of debate from Bush to Obama," *Yale Journal of Biology and Medicine*, vol. 82, no. 3, pp. 101–103, 2009.

[40] G. Q. Daley, "Missed opportunities in embryonic stem-cell research," *New England Journal of Medicine*, vol. 351, no. 7, pp. 627-628, 2004.

[41] J. Sung Park, D.-Y. Chang, J.-H. Kim et al., "Retrovirus-mediated transduction of a cytosine deaminase gene preserves the stemness of mesenchymal stem cells," *Experimental & Molecular Medicine*, vol. 45, no. 2, pp. e10–e10, 2013.

[42] J. Liao, Q. Wei, J. Fan et al., "Characterization of retroviral infectivity and superinfection resistance during retrovirus-mediated transduction of mammalian cells," *Gene Therapy*, vol. 24, no. 6, pp. 333–341, 2017.

[43] J. M. Wilson, D. M. Jefferson, J. R. Chowdhury, P. M. Novikoff, D. E. Johnston, and R. C. Mulligan, "Retrovirus-mediated transduction of adult hepatocytes," *Proceedings of the National Academy of Sciences*, vol. 85, no. 9, pp. 3014–3018, 1988.

[44] J. D. Miller and T. M. Schlaeger, "Generation of induced pluripotent stem cell lines from human fibroblasts via retroviral gene transfer," *Methods in Molecular Biology*, vol. 767, pp. 55–65, 2011.

[45] M. Imamura, H. Okuno, I. Tomioka et al., "Derivation of induced pluripotent stem cells by retroviral gene transduction in mammalian species," *Methods in Molecular Biology*, vol. 925, pp. 21–48, 2012.

[46] K. Mitsui, Y. Tokuzawa, H. Itoh et al., "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells," *Cell*, vol. 113, no. 5, pp. 631–642, 2003.

[47] S. C. Tobin and K. Kim, "Generating pluripotent stem cells: differential epigenetic changes during cellular reprogramming," *FEBS Letters*, vol. 586, no. 18, pp. 2874–2881, 2012.

[48] I. Chambers, D. Colby, M. Robertson et al., "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells," *Cell*, vol. 113, no. 5, pp. 643–655, 2003.

[49] M. Nakagawa, M. Koyanagi, K. Tanabe et al., "Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts," *Nature Biotechnology*, vol. 26, no. 1, pp. 101–106, 2008.

[50] A. Bortvin, K. Eggan, H. Skaletsky et al., "Incomplete reactivation ofOct4-related genes in mouse embryos cloned from somatic nuclei," *Development*, vol. 130, no. 8, pp. 1673–1680, 2003.

[51] J. Nichols, B. Zevnik, K. Anastassiadis et al., "Formation of Pluripotent Stem Cells in the Mammalian Embryo Depends on the POU Transcription Factor Oct4," *Cell*, vol. 95, no. 3, pp. 379–391, 1998.

[52] H. Niwa, J. Miyazaki, and A. G. Smith, "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or

self-renewal of ES cells," *Nature Genetics*, vol. 24, no. 4, pp. 372–376, 2000.

[53] N. Plachta, T. Bollenbach, S. Pease, S. E. Fraser, and P. Pantazis, "Oct4 kinetics predict cell lineage patterning in the early mammalian embryo," *Nature Cell Biology*, vol. 13, no. 2, pp. 117–123, 2011.

[54] M.-E. Torres-Padilla, D.-E. Parfitt, T. Kouzarides, and M. Zernicka-Goetz, "Histone arginine methylation regulates pluripotency in the early mouse embryo," *Nature*, vol. 445, no. 7124, pp. 214–218, 2007.

[55] A. A. Avilion, S. K. Nicolis, L. H. Pevny, L. Perez, N. Vivian, and R. Lovell-Badge, "Multipotent cell lineages in early mouse development depend on SOX2 function," *Genes & Development*, vol. 17, no. 1, pp. 126–140, 2003.

[56] J. Rossant and J. C. Cross, "Placental development: lessons from mouse mutants," *Nature Reviews Genetics*, vol. 2, no. 7, pp. 538–548, 2001.

[57] A. M. Ghaleb, J. P. Katz, K. H. Kaestner, J. X. Du, and V. W. Yang, "Kruppel-like factor 4 exhibits antiapoptotic activity following _γ_ -radiation-induced DNA damage," *Oncogene*, vol. 26, no. 16, pp. 2365–2373, 2007.

[58] B. D. Rowland, R. Bernards, and D. S. Peeper, "The KLF4 tumour suppressor is a transcriptional repressor of P53 that acts as a context-dependent oncogene," *Nature Cell Biology*, vol. 7, no. 11, pp. 1074–1082, 2005.

[59] A. Ochalek, K. Szczesna, P. Petazzi, J. Kobolak, and A. Dinnyes, "Generation of cholinergic and dopaminergic interneurons from human pluripotent stem cells as a relevant tool for in vitro modeling of neurological disorders pathology and therapy," *Stem Cells International*, vol. 2016, Article ID 5838934, 16 pages, 2016.

[60] L. Duan, B. J. Bhattacharyya, A. Belmadani, L. Pan, R. J. Miller, and J. A. Kessler, "Stem cell derived basal forebrain cholinergic neurons from Alzheimer's disease patients are more susceptible to cell death," *Molecular Neurodegeneration*, vol. 9, no. 1, p. 3, 2014.

[61] D. Doi, H. Magotani, T. Kikuchi et al., "Pre-clinical study of induced pluripotent stem cell-derived dopaminergic progenitor cells for Parkinson's disease," *Nature Communications*, vol. 11, no. 1, p. 3369, 2020.

[62] J. E. Beevers, T. M. Caffrey, and R. Wade-Martins, "Induced pluripotent stem cell (IPSC)-derived dopaminergic models of Parkinson's disease," *Biochemical Society Transactions*, vol. 41, no. 6, pp. 1503–1508, 2013.

[63] X. Hu, C. Mao, L. Fan et al., "Modeling Parkinson's disease using induced pluripotent stem cells," *Stem Cells International*, vol. 2020, Article ID 1061470, 15 pages, 2020.

[64] T. Hiragi, M. Andoh, T. Araki et al., "Differentiation of human induced pluripotent stem cell (HiPSC)-derived neurons in mouse hippocampal slice cultures," *Frontiers in Cellular Neuroscience*, vol. 11, p. 143, 2017.

[65] Y. Pomeshchik, O. Klementieva, J. Gil et al., "Human IPSC-derived hippocampal spheroids: an innovative tool for stratifying Alzheimer disease patient-specific cellular phenotypes and developing therapies," *Stem Cell Reports*, vol. 15, no. 1, pp. 256–273, 2020.

[66] E. Arenas, M. Denham, and J. C. Villaescusa, "How to make a midbrain dopaminergic neuron," *Development*, vol. 142, no. 11, pp. 1918–1936, 2015.

[67] G. Kouroupi, E. Taoufik, I. S. Vlachos et al., "Defective synaptic connectivity and axonal neuropathology in a human IPSC-based model of familial Parkinson's disease," *Proceedings of the National Academy of Sciences*, vol. 114, no. 18, pp. E3679–E3688, 2017.

[68] N. Stanslowsky, P. Reinhardt, H. Glass et al., "Neuronal dysfunction in IPSC-derived medium spiny neurons from chorea-acanthocytosis patients is reversed by Src kinase inhibition and F-actin stabilization," *The Journal of Neuroscience*, vol. 36, no. 47, pp. 12027–12043, 2016.

[69] A. I. Abdullah, A. Pollock, and T. Sun, "The path from skin to brain: generation of functional neurons from fibroblasts," *Molecular Neurobiology*, vol. 45, no. 3, pp. 586–595, 2012.

[70] W. Wuli, S.-T. Tsai, T.-W. Chiou, and H.-J. Harn, "Human-induced pluripotent stem cells and herbal small-molecule drugs for treatment of Alzheimer's disease," *International Journal of Molecular Sciences*, vol. 21, no. 4, p. 1327, 2020.

[71] L. E. Hebert, J. Weuve, P. A. Scherr, and D. A. Evans, "Alzheimer disease in the United States (2010–2050) estimated using the 2010 census," *Neurology*, vol. 80, no. 19, pp. 1778–1783, 2013.

[72] M. J. Moore, C. W. Zhu, and E. C. Clipp, "Informal costs of dementia care: estimates from the National Longitudinal Caregiver Study," *The Journals of Gerontology Series B: Psychological Sciences and Social Sciences*, vol. 56, no. 4, pp. S219–S228, 2001.

[73] A. Wimo, M. Guerchet, G.-C. Ali et al., "The worldwide costs of dementia 2015 and comparisons with 2010," *Alzheimer's & Dementia*, vol. 13, no. 1, pp. 1–7, 2017.

[74] A. Deb, J. D. Thornton, U. Sambamoorthi, and K. Innes, "Direct and indirect cost of managing Alzheimer's disease and related dementias in the United States," *Expert Review of Pharmacoeconomics & Outcomes Research*, vol. 17, no. 2, pp. 189–202, 2017.

[75] A. Goyal, J. Miller, A. J. Watrous et al., "Electrical stimulation in hippocampus and entorhinal cortex impairs spatial and temporal memory," *The Journal of Neuroscience*, vol. 38, no. 19, pp. 4471–4481, 2018.

[76] A. Rauk, "Why is the amyloid beta peptide of Alzheimer's disease neurotoxic?," *Dalton Transactions*, vol. 10, no. 10, pp. 1273–1282, 2008.

[77] E.-M. Mandelkow and E. Mandelkow, "Tau in Alzheimer's disease," *Trends in Cell Biology*, vol. 8, no. 11, pp. 425–427, 1998.

[78] N.-V. Mohamed, T. Herrou, V. Plouffe, N. Piperno, and N. Leclerc, "Spreading of tau pathology in Alzheimer's disease by cell-to-cell transmission," *European Journal of Neuroscience*, vol. 37, no. 12, pp. 1939–1948, 2013.

[79] A. Fuster-Matanzo, M. Llorens-Martín, J. Jurado-Arjona, J. Avila, and F. Hernández, "Tau protein and adult hippocampal neurogenesis," *Frontiers in Neuroscience*, vol. 6, p. 104, 2012.

[80] M. R. Sabuncu, R. S. Desikan, J. Sepulcre et al., "The dynamics of cortical and hippocampal atrophy in Alzheimer disease," *Archives of Neurology*, vol. 68, no. 8, pp. 1040–1048, 2011.

[81] W. G. Tharp and I. N. Sarkar, "Origins of amyloid-β," *BMC Genomics*, vol. 14, no. 1, p. 290, 2013.

[82] S. Carmona, J. Hardy, and R. Guerreiro, "The genetic landscape of Alzheimer disease," *Handbook of Clinical Neurology*, vol. 148, pp. 395–408, 2018.

[83] M. Barrachina, E. Dalfó, B. Puig et al., "Amyloid-β deposition in the cerebral cortex in Dementia with Lewy bodies is accompanied by a relative increase in AβPP mRNA isoforms

containing the Kunitz protease inhibitor," *Neurochemistry International*, vol. 46, no. 3, pp. 253–260, 2005.

[84] P. T. Kotzbauer, N. J. Cairns, M. C. Campbell et al., "Pathologic accumulation of α-synuclein and Aβ in Parkinson disease patients with dementia," *Archives of Neurology*, vol. 69, no. 10, pp. 1326–1331, 2012.

[85] M. Yamada and H. Naiki, "Cerebral amyloid angiopathy," *Progress in Molecular Biology and Translational Science*, vol. 107, pp. 41–78, 2012.

[86] W. S. Liang, T. Dunckley, T. G. Beach et al., "Neuronal gene expression in non-demented individuals with intermediate Alzheimer's Disease neuropathology," *Neurobiology of Aging*, vol. 31, no. 4, pp. 549–566, 2010.

[87] V. W. Chow, M. P. Mattson, P. C. Wong, and M. Gleichmann, "An overview of APP processing enzymes and products," *Neuromolecular Medicine*, vol. 12, no. 1, pp. 1–12, 2010.

[88] K. Furukawa, S. W. Barger, E. M. Blalock, and M. P. Mattson, "Activation of K^+ channels and suppression of neuronal activity by secreted β-amyloid-precursor protein," *Nature*, vol. 379, no. 6560, pp. 74–78, 1996.

[89] M. P. Mattson, B. Cheng, A. R. Culwell, F. S. Esch, I. Lieberburg, and R. E. Rydel, "Evidence for excitoprotective and intraneuronal calcium-regulating roles for secreted forms of the β-amyloid precursor protein," *Neuron*, vol. 10, no. 2, pp. 243–254, 1993.

[90] M. P. Mattson, "Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives," *Physiological Reviews*, vol. 77, no. 4, pp. 1081–1132, 1997.

[91] N. Gakhar-Koppole, P. Hundeshagen, C. Mandl et al., "Activity requires soluble amyloid precursor protein α to promote neurite outgrowth in neural stem cell-derived neurons via activation of the MAPK pathway," *European Journal of Neuroscience*, vol. 28, no. 5, pp. 871–882, 2008.

[92] F. Liu and C.-X. Gong, "Tau exon 10 alternative splicing and tauopathies," *Molecular Neurodegeneration*, vol. 3, no. 1, p. 8, 2008.

[93] S. Burnouf, S. Grönke, H. Augustin et al., "Deletion of endogenous Tau proteins is not detrimental in *Drosophila*," *Scientific Reports*, vol. 6, no. 1, p. 23102, 2016.

[94] P. Litman, J. Barg, and I. Ginzburg, "Microtubules are involved in the localization of tau MRNA in primary neuronal cell cultures," *Neuron*, vol. 13, no. 6, pp. 1463–1474, 1994.

[95] J. W. Mandell and G. A. Banker, "A spatial gradient of tau protein phosphorylation in nascent axons," *The Journal of Neuroscience*, vol. 16, no. 18, pp. 5727–5740, 1996.

[96] Y.-T. Lin, J.-T. Cheng, L.-C. Liang, C.-Y. Ko, Y.-K. Lo, and P.-J. Lu, "The binding and phosphorylation of Thr231 is critical for tau's hyperphosphorylation and functional regulation by glycogen synthase kinase 3β," *Journal of Neurochemistry*, vol. 103, no. 2, pp. 802–813, 2007.

[97] S. Zhu, A. Shala, A. Bezginov, A. Sljoka, G. Audette, and D. J. Wilson, "Hyperphosphorylation of intrinsically disordered tau protein induces an amyloidogenic shift in its conformational ensemble," *PLoS One*, vol. 10, no. 3, pp. e0120416–e0120416, 2015.

[98] M. Morishima-Kawashima, M. Hasegawa, K. Takio et al., "Proline-directed and Non-proline-directed Phosphorylation of PHF-tau (∗)," *Journal of Biological Chemistry*, vol. 270, no. 2, pp. 823–829, 1995.

[99] B. R. Sperbera, S. Leight, M. Goedert, and V. M.-Y. Lee, "Glycogen synthase kinase-3β phosphorylates tau protein at multiple sites in intact cells," *Neuroscience Letters*, vol. 197, no. 2, pp. 149–153, 1995.

[100] J. J. Lucas, F. Hernández, P. Gómez-Ramos, M. A. Morán, R. Hen, and J. Avila, "Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice," *The EMBO Journal*, vol. 20, no. 1, pp. 27–39, 2001.

[101] C. X. Gong, T. J. Singh, I. Grundke-Iqbal, and K. Iqbal, "Phosphoprotein phosphatase activities in Alzheimer disease brain," *Journal of Neurochemistry*, vol. 61, no. 3, pp. 921–927, 1993.

[102] F. Liu, I. Grundke-Iqbal, K. Iqbal, and C.-X. Gong, "Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation," *European Journal of Neuroscience*, vol. 22, no. 8, pp. 1942–1950, 2005.

[103] C.-X. Gong and K. Iqbal, "Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease," *Current Medicinal Chemistry*, vol. 15, no. 23, pp. 2321–2328, 2008.

[104] M. Schwalbe, H. Kadavath, J. Biernat et al., "Structural impact of tau phosphorylation at threonine 231," *Structure*, vol. 23, no. 8, pp. 1448–1458, 2015.

[105] J. P. Brion, "Neurofibrillary tangles and Alzheimer's disease," *European Neurology*, vol. 40, no. 3, pp. 130–140, 1998.

[106] S. Spina, D. R. Schonhaut, B. F. Boeve et al., "Frontotemporal dementia with the V337MMAPTmutation," *Neurology*, vol. 88, no. 8, pp. 758–766, 2017.

[107] R. Shafei, I. O. C. Woollacott, C. J. Mummery et al., "Two pathologically confirmed cases of novel mutations in the *MAPT* gene causing frontotemporal dementia," *Neurobiology of Aging*, vol. 87, pp. 141.e15–141.e20, 2020.

[108] C. Zarow, H. V. Vinters, W. G. Ellis et al., "Correlates of hippocampal neuron number in Alzheimer's disease and ischemic vascular dementia," *Annals of Neurology*, vol. 57, no. 6, pp. 896–903, 2005.

[109] H. Hampel, S. E. O'Bryant, S. Durrleman et al., "A precision medicine initiative for Alzheimer's disease: the road ahead to biomarker-guided integrative disease modeling," *Climacteric*, vol. 20, no. 2, pp. 107–118, 2017.

[110] H. Hampel, R. Frank, K. Broich et al., "Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives," *Nature Reviews Drug Discovery*, vol. 9, no. 7, pp. 560–574, 2010.

[111] J. Cummings, "The role of biomarkers in Alzheimer's disease drug development," *Advances in Experimental Medicine and Biology*, vol. 1118, pp. 29–61, 2019.

[112] M. B. Colović, D. Z. Krstić, T. D. Lazarević-Pašti, A. M. Bondžić, and V. M. Vasić, "Acetylcholinesterase inhibitors: pharmacology and toxicology," *Current Neuropharmacology*, vol. 11, no. 3, pp. 315–335, 2013.

[113] D. Quinn and M. Acetylcholinesterase, "Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition states," *Chemical Reviews*, vol. 87, no. 5, pp. 955–979, 1987.

[114] S. M. Stahl, "The new cholinesterase inhibitors for Alzheimer's disease, part 2: illustrating their mechanisms of action," *The Journal of Clinical Psychiatry*, vol. 61, no. 11, pp. 813-814, 2000.

[115] Y. Takada-Takatori, S. Nakagawa, R. Kimata et al., "Donepezil modulates amyloid precursor protein endocytosis and

reduction by up-regulation of SNX33 expression in primary cortical neurons," *Scientific Reports*, vol. 9, no. 1, p. 11922, 2019.

[116] Y. Yoshiyama, A. Kojima, C. Ishikawa, and K. Arai, "Anti-inflammatory action of donepezil ameliorates tau pathology, synaptic loss, and neurodegeneration in a tauopathy mouse model," *Journal of Alzheimer's Disease*, vol. 22, no. 1, pp. 295–306, 2010.

[117] V. Dhikav and K. S. Anand, "Is hippocampal atrophy a future drug target?," *Medical Hypotheses*, vol. 68, no. 6, pp. 1300–1306, 2007.

[118] D. Das, J. Li, S. Liu et al., "Generation and characterization of a novel human iPSC line from a resilient Alzheimer's disease patient," *Stem Cell Research*, vol. 48, article 101979, 2020.

[119] J. Penney, W. T. Ralvenius, and L.-H. Tsai, "Modeling Alzheimer's disease with iPSC-derived brain cells," *Molecular Psychiatry*, vol. 25, no. 1, pp. 148–167, 2020.

[120] M. Oksanen, I. Hyötyläinen, J. Voutilainen et al., "Generation of a human induced pluripotent stem cell line (LL008 1.4) from a familial Alzheimer's disease patient carrying a double KM670/671NL (Swedish) mutation in *APP* gene," *Stem Cell Research*, vol. 31, pp. 181–185, 2018.

[121] M. A. Israel, S. H. Yuan, C. Bardy et al., "Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells," *Nature*, vol. 482, no. 7384, pp. 216–220, 2012.

[122] A. Kokawa, S. Ishihara, H. Fujiwara et al., "The A673T mutation in the amyloid precursor protein reduces the production of β-amyloid protein from its β-carboxyl terminal fragment in cells," *Acta Neuropathologica Communications*, vol. 3, no. 1, p. 66, 2015.

[123] Š. Lehtonen, I. Höytyläinen, J. Voutilainen et al., "Generation of a human induced pluripotent stem cell line from a patient with a rare A673T variant in amyloid precursor protein gene that reduces the risk for Alzheimer's disease," *Stem Cell Research*, vol. 30, pp. 96–99, 2018.

[124] H. Potter, A. Granic, and J. Caneus, "Role of Trisomy 21 Mosaicism in Sporadic and Familial Alzheimer's Disease," *Current Alzheimer Research*, vol. 13, no. 1, pp. 7–17, 2015.

[125] I. T. Lott and E. Head, "Down syndrome and Alzheimer's disease: a link between development and aging," *Mental Retardation and Developmental Disabilities Research Reviews*, vol. 7, no. 3, pp. 172–178, 2001.

[126] D. J. Colacurcio, A. Pensalfini, Y. Jiang, and R. A. Nixon, "Dysfunction of autophagy and endosomal-lysosomal pathways: Roles in pathogenesis of Down syndrome and Alzheimer's Disease," *Free Radical Biology & Medicine*, vol. 114, pp. 40–51, 2018.

[127] C.-Y. Chang, S.-M. Chen, H.-E. Lu et al., "N-butylidenephthalide Attenuates Alzheimer's Disease-Like Cytopathy in Down Syndrome Induced Pluripotent Stem Cell-Derived Neurons," *Scientific Reports*, vol. 5, no. 1, p. 8744, 2015.

[128] M. Oksanen, A. J. Petersen, N. Naumenko et al., "*_PSEN1_* Mutant iPSC-Derived Model Reveals Severe Astrocyte Pathology in Alzheimer's Disease," *Stem Cell Reports*, vol. 9, no. 6, pp. 1885–1897, 2017.

[129] T. Yagi, D. Ito, Y. Okada et al., "Modeling familial Alzheimer's disease with induced pluripotent stem cells," *Rinshō Shinkeigaku*, vol. 52, no. 11, pp. 1134–1136, 2012.

[130] E. Kara, H. Ling, A. M. Pittman et al., "The *_MAPT_* p.A152T variant is a risk factor associated with tauopathies with atypical clinical and neuropathological features," *Neurobiology of Aging*, vol. 33, no. 9, pp. 2231.e7–2231.e14, 2012.

[131] M. C. Silva, C. Cheng, W. Mair et al., "Human IPSC-derived neuronal model of tau-A152T frontotemporal dementia reveals tau-mediated mechanisms of neuronal vulnerability," *Stem Cell Reports*, vol. 7, no. 3, pp. 325–340, 2016.

[132] A. Verheyen, A. Diels, J. Reumers et al., "Genetically Engineered iPSC-Derived FTDP-17 _MAPT_ Neurons Display Mutation- Specific Neurodegenerative and Neurodevelopmental Phenotypes," *Stem Cell Reports*, vol. 11, no. 2, pp. 363–379, 2018.

[133] C.-C. Liu, C. C. Liu, T. Kanekiyo, H. Xu, and G. Bu, "Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy," *Nature Reviews. Neurology*, vol. 9, no. 2, pp. 106–118, 2013.

[134] Y.-W. A. Huang, B. Zhou, M. Wernig, and T. C. Südhof, "ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Aβ Secretion," *Cell*, vol. 168, no. 3, pp. 427–441.e21, 2017.

[135] A. Tedeschi and F. Bradke, "The DLK signalling pathway–a double-edged sword in neural development and regeneration," *EMBO Reports*, vol. 14, no. 7, pp. 605–614, 2013.

[136] Y. Lu, S. Belin, and Z. He, "Signaling regulations of neuronal regenerative ability," *Current Opinion in Neurobiology*, vol. 27, pp. 135–142, 2014.

[137] Y.-T. Lin, J. Seo, F. Gao et al., "*_APOE4_* Causes Widespread Molecular and Cellular Alterations Associated with Alzheimer 's Disease Phenotypes in Human iPSC-Derived Brain Cell Types," *Neuron*, vol. 98, no. 6, pp. 1141–1154.e7, 2018.

[138] H. Konttinen, M. E. C. Cabral-da-Silva, S. Ohtonen et al., "*_PSEN1_ ΔE9*, *_APPswe_* , and *_APOE4_* Confer Disparate Phenotypes in Human iPSC- Derived Microglia," *Stem Cell Reports*, vol. 13, no. 4, pp. 669–683, 2019.

[139] Y.-W. A. Huang, B. Zhou, A. M. Nabet, M. Wernig, and T. C. Südhof, "Differential signaling mediated by ApoE2, ApoE3, and ApoE4 in human neurons parallels Alzheimer's disease risk," *The Journal of Neuroscience*, vol. 39, no. 37, pp. 7408–7427, 2019.

[140] T. Masuda, M. Tsuda, R. Yoshinaga et al., "IRF8 is a critical transcription factor for transforming microglia into a reactive phenotype," *Cell Reports*, vol. 1, no. 4, pp. 334–340, 2012.

[141] Z.-T. Wang, C.-C. Tan, L. Tan, and J.-T. Yu, "Systems biology and gene networks in Alzheimer's disease," *Neuroscience and Biobehavioral Reviews*, vol. 96, pp. 31–44, 2019.

[142] J. D. Ulrich and D. M. Holtzman, "TREM2 function in Alzheimer's disease and neurodegeneration," *ACS Chemical Neuroscience*, vol. 7, no. 4, pp. 420–427, 2016.

[143] A. Heslegrave, W. Heywood, R. Paterson et al., "Increased cerebrospinal fluid soluble TREM2 concentration in Alzheimer's disease," *Molecular Neurodegeneration*, vol. 11, no. 1, p. 3, 2016.

[144] B. De Strooper and E. Karran, "The Cellular Phase of Alzheimer's Disease," *Cell*, vol. 164, no. 4, pp. 603–615, 2016.

[145] N. Hattori, "Cerebral organoids model human brain development and microcephaly," *Movement Disorders*, vol. 29, no. 2, p. 185, 2014.

[146] M. Simian and M. J. Bissell, "Organoids: a historical perspective of thinking in three dimensions," *The Journal of Cell Biology*, vol. 216, no. 1, pp. 31–40, 2017.

[147] A. Papaspyropoulos, M. Tsolaki, N. Foroglou, and A. A. Pantazaki, "Modeling and targeting Alzheimer's disease with organoids," *Frontiers in Pharmacology*, vol. 11, p. 396, 2020.

[148] W. K. Raja, A. E. Mungenast, Y.-T. Lin et al., "Self-organizing 3D human neural tissue derived from induced pluripotent stem cells recapitulate Alzheimer's disease phenotypes," *PLoS One*, vol. 11, no. 9, article e0161969, 2016.

[149] S. J. Stachel, C. A. Coburn, T. G. Steele et al., "Structure-based design of potent and selective cell-permeable inhibitors of human beta-secretase (BACE-1)," *Journal of Medicinal Chemistry*, vol. 47, no. 26, pp. 6447–6450, 2004.

[150] J. Deng, A. Habib, D. F. Obregon et al., "Soluble amyloid precursor protein alpha inhibits tau phosphorylation through modulation of GSK3β signaling pathway," *Journal of Neurochemistry*, vol. 135, no. 3, pp. 630–637, 2015.

[151] P. R. Ormel, R. Vieira de Sá, E. J. van Bodegraven et al., "Microglia innately develop within cerebral organoids," *Nature Communications*, vol. 9, no. 1, p. 4167, 2018.

[152] S. F. Yanuck, "Microglial phagocytosis of neurons: diminishing neuronal loss in traumatic, infectious, inflammatory, and autoimmune CNS disorders," *Frontiers in Psychiatry*, vol. 10, p. 712, 2019.

[153] P. D. Wes, F. A. Sayed, F. Bard, and L. Gan, "Targeting microglia for the treatment of Alzheimer's disease," *Glia*, vol. 64, no. 10, pp. 1710–1732, 2016.

[154] E. Janda, L. Boi, and A. R. Carta, "Microglial phagocytosis and its regulation: a therapeutic target in Parkinson's disease?," *Frontiers in Molecular Neuroscience*, vol. 11, p. 144, 2018.

[155] G. C. Brown and J. J. Neher, "Microglial phagocytosis of live neurons," *Nature Reviews. Neuroscience*, vol. 15, no. 4, pp. 209–216, 2014.

[156] W. Haenseler and L. Rajendran, "Concise review: modeling neurodegenerative diseases with human pluripotent stem cell-derived microglia," *Stem Cells*, vol. 37, no. 6, pp. 724–730, 2019.

[157] G. Kronenberg, R. Uhlemann, N. Richter et al., "Distinguishing features of microglia- and monocyte-derived macrophages after stroke," *Acta Neuropathologica*, vol. 135, no. 4, pp. 551–568, 2018.

[158] G. Thiel, "Synapsin I, synapsin II, and synaptophysin: marker proteins of synaptic vesicles," *Brain Pathology*, vol. 3, no. 1, pp. 87–95, 1993.

[159] A. M. Jurga, M. Paleczna, and K. Z. Kuter, "Overview of general and discriminating markers of differential microglia phenotypes," *Frontiers in Cellular Neuroscience*, vol. 14, p. 198, 2020.

[160] R. Xu, X. Li, A. J. Boreland et al., "Human IPSC-derived mature microglia retain their identity and functionally integrate in the chimeric mouse brain," *Nature Communications*, vol. 11, no. 1, p. 1577, 2020.

[161] D. S. Svoboda, M. I. Barrasa, J. Shu et al., "Human IPSC-derived microglia assume a primary microglia-like state after transplantation into the neonatal mouse brain," *Proceedings of the National Academy of Sciences*, vol. 116, no. 50, pp. 25293–25303, 2019.

[162] E. M. Abud, R. N. Ramirez, E. S. Martinez et al., "IPSC-derived human microglia-like cells to study neurological diseases," *Neuron*, vol. 94, no. 2, pp. 278–293.e9, 2017.

[163] D. Tornero, S. Wattananit, M. Grønning Madsen et al., "Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery," *Brain*, vol. 136, no. 12, pp. 3561–3577, 2013.

[164] D. Tornero, O. Tsupykov, M. Granmo et al., "Synaptic inputs from stroke-injured brain to grafted human stem cell-derived neurons activated by sensory stimuli," *Brain*, vol. 140, no. 3, pp. aww347–aww706, 2017.

[165] S. Palma-Tortosa, D. Tornero, M. Grønning Hansen et al., "Activity in grafted human IPS cell-derived cortical neurons integrated in stroke-injured rat brain regulates motor behavior," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 117, no. 16, pp. 9094–9100, 2020.

[166] M. Grønning Hansen, C. Laterza, S. Palma-Tortosa et al., "Grafted human pluripotent stem cell-derived cortical neurons integrate into adult human cortical neural circuitry," *Stem Cells Translational Medicine*, vol. 9, no. 11, pp. 1365–1377, 2020.

[167] A. Brazzini, R. Cantella, A. De la Cruz et al., "Intraarterial autologous implantation of adult stem cells for patients with Parkinson disease," *Journal of Vascular and Interventional Radiology*, vol. 21, no. 4, pp. 443–451, 2010.

[168] H. J. Kim, S. W. Seo, J. W. Chang et al., "Stereotactic brain injection of human umbilical cord blood mesenchymal stem cells in patients with Alzheimer's disease dementia: a phase 1 clinical trial," *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, vol. 1, no. 2, pp. 95–102, 2015.

[169] H. J. Lee, J. K. Lee, H. Lee et al., "The therapeutic potential of human umbilical cord blood-derived mesenchymal stem cells in Alzheimer's disease," *Neuroscience Letters*, vol. 481, no. 1, pp. 30–35, 2010.

[170] S. Cho, A. Wood, and M. R. Bowlby, "Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics," *Current Neuropharmacology*, vol. 5, no. 1, pp. 19–33, 2007.

[171] F. Cavaliere, M. Benito-Muñoz, and C. Matute, "Organotypic cultures as a model to study adult neurogenesis in CNS disorders," *Stem Cells International*, vol. 2016, 3540566 pages, 2016.

[172] C. L. Croft, H. S. Futch, B. D. Moore, and T. E. Golde, "Organotypic brain slice cultures to model neurodegenerative proteinopathies," *Molecular Neurodegeneration*, vol. 14, no. 1, p. 45, 2019.

[173] R. W. H. Verwer, A. A. Sluiter, R. A. Balesar et al., "Injury response of resected human brain tissue in vitro," *Brain Pathology*, vol. 25, no. 4, pp. 454–468, 2015.

[174] X. Qian, H. Song, and G. Ming, "Brain organoids: advances, applications and challenges," *Development*, vol. 146, no. 8, 2019.

[175] P. Rebuzzini, M. Zuccotti, C. A. Redi, and S. Garagna, "Achilles' heel of pluripotent stem cells: genetic, genomic and epigenetic variations during prolonged culture," *Cellular and Molecular Life Sciences*, vol. 73, no. 13, pp. 2453–2466, 2016.

[176] M. Yoshihara, Y. Hayashizaki, and Y. Murakawa, "Genomic instability of IPSCs: challenges towards their clinical applications," *Stem Cell Reviews and Reports*, vol. 13, no. 1, pp. 7–16, 2017.

[177] J. Ji, S. H. Ng, V. Sharma et al., "Elevated coding mutation rate during the reprogramming of human somatic cells into induced pluripotent stem cells," *Stem Cells*, vol. 30, no. 3, pp. 435–440, 2012.

[178] M. Sugiura, Y. Kasama, R. Araki et al., "Induced pluripotent stem cell generation-associated point mutations arise during the initial stages of the conversion of these cells," *Stem Cell Reports*, vol. 2, no. 1, pp. 52–63, 2014.

[179] Y. Mayshar, U. Ben-David, N. Lavon et al., "Identification and classification of chromosomal aberrations in human induced pluripotent stem cells," *Cell Stem Cell*, vol. 7, no. 4, pp. 521–531, 2010.

[180] A. D. Riggs, "X inactivation, differentiation, and DNA methylation," *Cytogenetic and Genome Research*, vol. 14, no. 1, pp. 9–25, 1975.

[181] V. Ngo, Z. Chen, K. Zhang, J. W. Whitaker, M. Wang, and W. Wang, "Epigenomic analysis reveals DNA motifs regulating histone modifications in human and mouse," *Proceedings of the National Academy of Sciences*, vol. 116, no. 9, pp. 3668–3677, 2019.

[182] A. Bird, "DNA methylation patterns and epigenetic memory," *Genes & Development*, vol. 16, no. 1, pp. 6–21, 2002.

[183] K. Kim, A. Doi, B. Wen et al., "Epigenetic memory in induced pluripotent stem cells," *Nature*, vol. 467, no. 7313, pp. 285–290, 2010.

[184] M. Yoshihara, A. Oguchi, and Y. Murakawa, "Genomic instability of IPSCs and challenges in their clinical applications," *Adv. Exp. Med. Biol.*, vol. 1201, pp. 23–47, 2019.

[185] C. E. Pasi, A. Dereli-Öz, S. Negrini et al., "Genomic instability in induced stem cells," *Cell Death and Differentiation*, vol. 18, no. 5, pp. 745–753, 2011.

[186] G. Liang and Y. Zhang, "Genetic and epigenetic variations in IPSCs: potential causes and implications for application," *Cell Stem Cell*, vol. 13, no. 2, pp. 149–159, 2013.

[187] K. H. Vousden and C. Prives, "Blinded by the light: the growing complexity of P53," *Cell*, vol. 137, no. 3, pp. 413–431, 2009.

[188] A. Gore, Z. Li, H.-L. Fung et al., "Somatic coding mutations in human induced pluripotent stem cells," *Nature*, vol. 471, no. 7336, pp. 63–67, 2011.

[189] T. Wakayama, "Establishment of nuclear transfer embryonic stem cell lines from adult somatic cells by nuclear transfer and its application," *Ernst Schering Research Foundation Workshop*, vol. 60, pp. 111–123, 2006.

[190] J. Jiang, G. Ding, J. Lin et al., "Different developmental potential of pluripotent stem cells generated by different reprogramming strategies," *Journal of Molecular Cell Biology*, vol. 3, no. 3, pp. 197–199, 2011.

[191] J. M. Polo, S. Liu, M. E. Figueroa et al., "Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells," *Nature Biotechnology*, vol. 28, no. 8, pp. 848–855, 2010.

[192] J. K. Christman, "5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy," *Oncogene*, vol. 21, no. 35, pp. 5483–5495, 2002.

[193] Z. Yao, Y. Chen, W. Cao, and N. Shyh-Chang, "Chromatin-modifying drugs and metabolites in cell fate control," *Cell Proliferation*, vol. 53, no. 11, article e12898, 2020.

[194] F. Ghanavatinejad, Z. P. Fard Tabrizi, S. Omidghaemi, E. Sharifi, S. G. Møller, and M.-S. Jami, "Protein biomarkers of neural system," *The Journal of Otolaryngology*, vol. 14, no. 3, pp. 77–88, 2019.

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Review Article

The Opportunities and Challenges regarding Induced Platelets from Human Pluripotent Stem Cells

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As a standard clinical treatment, platelet transfusion has been employed to prevent hemorrhage in patients with thrombocytopenia or platelet dysfunctions. Platelets also show therapeutic potential for aiding liver regeneration and bone healing and regeneration and for treating dermatological conditions. However, the supply of platelets rarely meets the rising clinical demand. Other issues, including short shelf life, strict storage temperature, and allogeneic immunity caused by frequent platelet transfusions, have become serious challenges that require the development of high-yielding alternative sources of platelets. Human pluripotent stem cells (hPSCs) are an unlimited substitution source for regenerative medicine, and patient-derived iPSCs can provide novel research models to explore the pathogenesis of some diseases. Many studies have focused on establishing and modifying protocols for generating functional induced platelets (iPlatelets) from hPSCs. To reach high efficiency production and eliminate the exogenous antigens, media supplements and matrix have been optimized. In addition, the introduction of some critical transgenes, such as *c-MYC*, *BMI1*, and *BCL-XL*, can also significantly increase hPSC-derived platelet production; however, this may pose some safety concerns. Furthermore, many novel culture systems have been developed to scale up the production of iPlatelets, including 2D flow systems, 3D rotary systems, and vertical reciprocal motion liquid culture bioreactors. The development of new gene-editing techniques, such as CRISPR/Cas9, can be used to solve allogeneic immunity of platelet transfusions by knocking out the expression of *B2M*. Additionally, the functions of iPlatelets were also evaluated from multiple aspects, including but not limited to morphology, structure, cytoskeletal organization, granule content, DNA content, and gene expression. Although the production and functions of iPlatelets are close to meeting clinical application requirements in both quantity and quality, there is still a long way to go for their large-scale production and clinical application. Here, we summarize the diverse methods of platelet production and update the progresses of iPlatelets. Furthermore, we highlight recent advances in our understanding of key transcription factors or molecules that determine the platelet differentiation direction.

1. Introduction

In mammals, platelets are produced by mature megakaryocytes (MKs) in the bone marrow and differentiate from pluripotent stem cells in hematopoietic tissues. The primary function of platelets is coagulation and hemostasis; once blood vessel injury occurs, platelets are rapidly activated, adhere to the wound, and aggregate to form a platelet clot. As a result, they are known as the "band-aids" of the bloodstream. Platelets play an executive role in the clinical

treatment of blood diseases, such as acute myeloid leukemia, immune thrombocytopenia, and idiopathic thrombocytopenic purpura [1]. Platelets are overlooked immune regulators; they play significant roles in inflammation and infection [2] as they can recognize exterior pathogens and produce many chemoattractants to activate and recruit leukocytes into the site of infection and inflammation, thereby enhancing their lethality to pathogens [3].

The roles of platelets in assisting liver regeneration, bone regeneration, and in the treatment of dermatological conditions, have also increased the demand for platelets in clinical treatment [4–6]. The discovery of platelet-derived serotonin involved in hepatic regeneration and the correlation between impaired platelets and liver cell proliferation suggest that platelets play a significant role in liver regeneration [7, 8]. Platelet transfusion can improve CCl4-induced liver fibrosis in mice with severe combined immune deficiency [9]. The transfer of coding and regulatory RNA between platelets and hepatocytes can promote hepatocyte proliferation and liver regeneration [10–12]. After hepatectomy, platelets coordinate with liver sinusoidal endothelial cells and Kupffer cells via the release of various growth factors, including human growth factor, insulin-like growth factor, and vascular endothelial growth factor (VEGF), or through direct contact with hepatocytes [13–15]. As the therapeutic role of platelets in many diseases is being studied, the application of platelet-rich plasma (PRP) products has gained extensive attention in regenerative medicine. PRP is an autologous biological product derived from centrifuging or apheresis of blood and is a solution with high concentration of platelets [16, 17]. PRP treatment utilizes platelets with abundant biological factors and chemoattractive cytokines associated with tissue regeneration and remodeling.

Moreover, the hydrogel properties of activated PRP make it a suitable medicine delivery vehicle [7, 8, 18]. Platelets dynamically regulate the process of bone remodeling by releasing proinflammatory cytokines to activate the inflammatory phase of early bone healing and then enhance the repair phase of the healing process [19, 20]. PRP treatment has been widely studied in orthopedic and oral/maxillofacial injuries to aid hemostasis and musculoskeletal regeneration [5, 18, 21, 22]. Moreover, in aesthetic dermatology, PRP has been reported to have a therapeutic effect in treating hair loss caused by androgenetic alopecia [23]. Combining platelets with fractional laser or fat grafting can improve scar revision [24, 25] and may provide benefits in skin rejuvenation and dermal augmentation [26, 27]. Thus, platelet therapy is expected to be a new therapeutic avenue for regenerative medicine and tissue engineering.

Previously, donor-derived platelets were the primary platelet source for the treatment of certain clinical diseases such as idiopathic thrombocytopenic purpura (ITP). However, the insufficient supply of donor blood limits its application worldwide. The complexity and doubts surrounding platelet donation have discouraged many donors, and current blood supplies do not meet clinical needs, causing severe shortages [28]. In addition to this problem, there are also several inevitable challenges in platelet transfusion. The first is platelet preservation; platelets can only be stored at room temperature for a short time; otherwise, there is a significant risk of bacterial contamination. Although cold storage can reduce bacterial reproduction and prolong shelf life of the platelets, it also changes platelet structure, molecules, and metabolism [29]. Second, exogenous platelets may cause excessive immune rejection in platelet recipients. Frequent platelet transfusions will cause allogeneic immunity, which results from the generation of multiple antibodies, such as human leukocyte antigen (HLA) antibodies and human platelet antigen antibodies in patients. Residual red blood cells (RBCs) in platelets can also induce RBC antibody production after transfusion [30]. Exploring safe and high-quality alternative sources of platelets for clinical use will markedly benefit the field of regenerative medicine.

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which have the advantages of unlimited self-renewal and multiple directional differentiation capabilities, have become reliable platelet sources in regenerative medicine. Numerous studies have demonstrated that iPSCs can differentiate into various functional cell types, such as cardiomyocytes, nephron progenitor cells, kidney organoids, oligodendrocyte progenitor cells, and melanocytes [31–34]. Systems for generating induced platelets (iPlatelets) from human PSCs (hPSCs) have also been established using various methods [35–38]. Using gene-editing techniques, such as CRISPR/-CAS9, PSCs with great genetic maneuverability can be developed; this makes PSCs more convenient and useful for overcoming some difficulties currently encountered by the use of platelets, such as allogeneic immunity. Therefore, hPSC-derived iPlatelets can overcome the limitations in the current blood donor-dependent system and solve a series of problems in platelet production for clinical application in the near future. However, there are still many challenges to overcome.

This review summarizes current approaches for generating hPSC-derived iPlatelets, presents the current status, compares the advantages and disadvantages, limitations, and defects, and suggests future research direction.

2. The Progress and the Current Approaches for iPlatelets

Many previous studies have reported that MKs are an essential intermediate product during hPSC differentiation into platelets, providing a new perspective for research and blood transfusion medicine. These studies are listed in Table 1; they describe MK differentiation and platelet generation in vitro (Figure 1).

As early as 2006, Gaur and his team established a genetically tractable system to differentiate human ESCs (hESCs) into MKs [39]. A coculture of OP9 stromal cells and hESCs was used to explore MK production in vitro for the first time. It was also called the conventional method or multiround replating method. On days 7 and 11, single cells derived from differentiated hESC colonies were transferred onto fresh OP9 cells and further cultured up to 17 days. Fluorescence analysis showed that approximately 20–60% of floating and loosely adherent cells expressed CD41a/CD42b, characteristic of

Table 1: Summary of current approaches from human PSCs to iPlatelets.

Cell source	Feeder cells	Multiple stages	Intervention factors	Specific markers	Production	Year
hESCs	OP9	MK	TPO	Not reported	Hardly	2006 [39]
hESCs	C3H10T1/2, OP9	HPC, MK	VEGF, TPO, SCF, heparin	$CD41a^{+}CD42b^{+}$	48 ± 0.2 platelets/hESC	2008 [35]
hESCs	According to stage	Hemangioblasts/blast, MK	BMP4, VEGF, SCF, TPO	$CD41a^{+}CD42b^{+}$	6.7 ± 0.4 platelets/MK	2011 [40]
hESCs	/	HPC, MK	BMP4, SCF, VEGF, FGF2	$CD41a^{+}CD42b^{+}$	Not provided	2013 [41]
hPSCs	C3H10T1/2	HPC, MK	HUVECs (2D bioreactor)	$CD41a^{+}$ or $CD42b^{+}$	Higher than static condition	2013 [42]
hiPSCs	/	HPC, HEC, MKP, MK	Multiple cytokines	$CD41a^{+}CD42b^{+}$	About 30 platelets/MK	2014 [38]
hPSCs	C3H10T1/2, OP9	HPC	*TAL1*, *GATA2*	Not reported	Not provided	2014 [43]
hiPSCs	C3H10T1/2	imMKCLs	*BMI1*, *BCL-XL*, *c-MYC*	$CD41a^{+}CD42b^{+}$	250 MKs/imMKCL	2014 [44]
hPSCs	/	HPC, MK	*GATA1*, *FLI1*, *TAL1*	$CD41a^{+}/CD42b^{+}$	About 7 platelets/MK	2016 [45]
hPSCs	/	MK	Shear stress (3D bioreactor)	β1-Tubulin1^{+}Hoechst^{-}CD41^{+}CD42b^{+}	~42 platelets/MK, ~350 platelets/h	2014 [46] 2016 [47]
hiPSCs	/	HG/CD42b^{+}MK	SCF, TPO, IL-9, IL-6	$FV^{+}CD42b^{+}$	Not provided	2017 [48]
hiPSCs	C3H10T1/2	imMKCLs	Turbulent flow, shear stress	$CD41^{+}CD42b^{+}$	~70–80 platelets/MK	2018 [49]
hiPSCs	C3H10T1/2, OP9	HSC, HPC, MK	*B2M* KO	$CD41^{+}CD42b^{+}$	Not provided	2020 [50]

hESCs: human embryonic stem cells; hPSCs: human pluripotent stem cells; hiPSCs: human induced pluripotent stem cells; MK: megakaryocyte; HPC: hematopoietic progenitor cell; HEC: hematopoietic endothelial cell; MKP: megakaryocyte progenitor; HSC: hematopoietic stem cell; imMKCL: immortalized megakaryocyte progenitor cell line; TPO: thrombopoietin; VEGF: vascular endothelial growth factor; BMP4: bone morphogenetic protein 4.

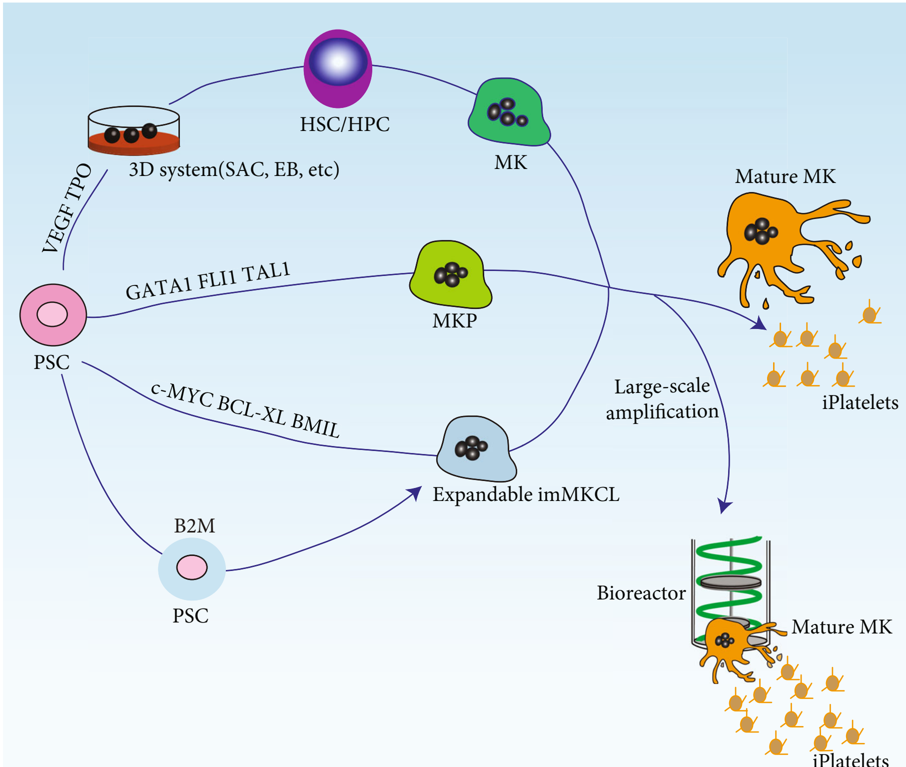

Figure 1: Schema to generate induced platelets from human pluripotent stem cells. HSC/HPC were induced from PSCs via intermediate stage (ESC-sac or EB) under stimulation of multiple cytokines and finally differentiated into mature MK for platelet release. Transgene combination (GATA1/FLI1/TAL1 or C-MYC/BCL-XL/BMI1) provides novel insights for expandable and cryopreserved MK. B2M knockout by CRISPR/Cas9 helped to diminish the allogeneic response caused by HLA mismatch. Furthermore, a 3D bioreactor can be applied for large-scale iPlatelet production. EB: embryoid body; HLA: human leukocyte antigen; HPC: hematopoietic progenitor cell; HSC: hematopoietic stem cell; imMKCL: immortalized megakaryocyte progenitor cell line; MK: megakaryocyte; TPO: thrombopoietin; VEGF: vascular endothelial growth factor.

the megakaryocyte lineage. However, this system yielded few platelets. Furthermore, hematopoietic progenitor cells (HPCs) derived from hPSCs were differentiated into MKs by adding cytokines SCF, Flt3L, and TPO [51, 52]. However, platelet production capacity was still limited. Since then, in attempts to address low yields, different differentiation systems have been established for obtaining hESC-derived platelets. Growth factors such as VEGF and ESC/iPSC-derived sacs (ES/iPS-sacs) differentiated into HPCs, thereby inducing the development of mature MKs and released platelets [35]. In this system, hESCs were cocultured with 10T1/2 or OP9 stromal cells. On day 14 of culture, ES/iPS-sacs were collected to concentrate HPCs, and the latter were then transferred onto fresh 10T1/2 or OP9 stromal cells and cultured up to day 26. With this protocol, a large number of mature platelets were efficiently obtained.

Another successful system was established by Lu et al. who found that hemangioblasts/blast cells (BCs) acted as intermediates in hPSC-derived platelet production and further produced functional MKs on a large scale [40]. In detail, hESCs were cultured in ultra-low attached plates with the addition of multiple cytokine combinations, including BMP4, VEGF, SCF, TPO, and FLT3L, in a serum-free medium for four days. This process involved the embryoid body (EB) formation, which showed excellent potential for industrial iPlatelet production and improvement in differentiation efficiency [40, 53]. The single cells generated from the EB stage were cultured in serum-free medium with multiple cytokines added for blast colony formation on day 8 and further differentiated into MKs in the presence of SCF, TPO, and IL-11 up to days 10–14. For platelet generation, OP9 stromal cells and cytokines, including SCF, TPO, and heparin, were used up to day 22 [40]. The number of platelets derived from these methods is considerable. It has been demonstrated that platelets generated from hESC-derived MKs displayed comparable ultrastructure and morphology with natural platelets and had similar characteristic properties to those of functional platelets [54, 55].

Since the feeder cells and serum addition used in the above culture systems have potential risk for introducing foreign pathogens and induce an immunogenic response in patients, substituting feeder cells and serum was researched. Salvagiotto et al. used collagen IV and a serum-free medium to induce feeder-free iPSCs into early hematopoietic progenitors, displaying the MK lineage characteristics [36]. Further technological innovation, a “spin embryoid body” method, was established for hESC differentiation towards the MK lineage in feeder-free and serum-free differentiation medium

[41]. In this protocol, hESCs were cultured in a serum-free medium supplemented with BMP4, VEGF, SCF, and FGF2 to form EBs and commit cells to hematopoiesis. The cells were cultured for an additional 3–10 days in a serum-free medium with TPO, SCF, and IL-3 to stimulate megakaryopoiesis [41]. This serum- and feeder-free culture system enabled the formation of MK progenitors generated from hPSCs and then that of induced platelet-like particles; however, the platelet yield was not provided in this method.

As mentioned earlier, iPSCs provide a promising opportunity to study the ontogeny of hematopoiesis; however, xeno components, such as feeder cells or serum from foreign species, limit the clinical application of iPlatelets. Therefore, Lanza and his team established a three-step protocol that may provide scalable and fully functional platelets for clinical use [38]. This protocol was performed under feeder-free and xeno-free culture conditions, significantly reducing the contamination and immunogenicity caused by foreign serum. Human iPSCs (hiPSCs) were suspended and seeded on human collagen IV-coated plates and incubated for 24 h at 37°C under normoxic conditions in a specific medium. The next day, the cells were transferred to hypoxic conditions and cultured for 4 days, followed by normoxia. The medium contained BMP4, VEGF, and FGF. The cells were cultured in a medium containing TPO, SCF, FLT3L, IL-3, IL-6, and heparin for up to 7 days to generate MK progenitors (MKPs). Finally, MKPs were collected and cultured in an MK maturation medium containing TPO, SCF, IL-6, IL-9, and heparin in ultra-low attachment plates at 39°C for MK maturation and platelet formation. This feeder-free system produced approximately 30 platelets per iPSC-derived MK [38]. In this culture method, proportion of $CD41a^+CD42b^+$ double-positive mature MKs was as high as 80%. The subsequently produced platelets were of high purity, with a further advantage that MKP cells can be cryopreserved [38].

With the rapid breakthrough in technological manipulation, the safety and purity of platelets have improved. At the same time, a method for stable output still needs to be explored for clinical or commercial use. These developed protocols suggest that feeder cells are dispensable for platelet generation in vitro and highlight the importance of media supplements and matrix optimization for future investigation.

2.1. Key Genetic Factors during iPlatelet Generation. In addition to improving the differentiation methods mentioned above, specific genetic modifications directly affect platelet production. Previous studies have demonstrated that various tissue lineages can be differentiated from hPSCs by regulating the expression of master transcription factors [56]. Therefore, by manipulating the gene expression of hiPSCs, Takayama et al. found that the expression pattern of *c-Myc* was a crucial factor in determining platelet production during hiPSC differentiation into MKs in vitro [57]. Transient activation of *c-Myc* followed by *c-Myc* expression reduction was critical for MK maturation and functional platelet production. Without this intervention, the constant expression of *c-Myc* decreased the expression of *GATA1* and nuclear factor erythroid-derived 2 p45 unit (p45NF-E2), leading to impaired production of functional platelets [57, 58]. In 2014, Igor Slukvin and his team found that transcription factors *GATA2* and *TAL1* induced hESC differentiation into designated erythrocyte MKPs through hemogenic endothelium intermediates [43]. Two years later, Moreau and his colleagues found that the overexpression of *GATA1*, *FLI1*, and *TAL1* promoted the proliferation and differentiation of MKs. Very high cell yields and purity of MKs could be achieved using entirely chemically defined nonheterogeneous culture conditions [45].

Similarly, immortalized megakaryocyte progenitor cell lines (imMKCLs) were produced after the overexpression of exogenous genes *BMI1*, *BCL-XL*, and *c-MYC* under the control of the Tet-on system in PSCs, and these imMKCLs could expand for a long time and be frozen and thawed [44]. Although these methods involving viral transduction may have specific safety concerns with regards to clinical-grade manufacturing, genetic manipulations offer novel insights into the mechanisms behind megakaryopoiesis; to help develop clinically safe protocols, nonviral or nonintegrated methods or alternative small-molecule compounds should be used in the future.

CD42b (GPIba) is a key marker of functional platelets that can bind to vWF and then initially mediate the adhesion of circulating platelets to an injured site [59, 60]. A study at the Children's Hospital of Philadelphia showed that the expression of CD42b was associated with MK maturation [48]. MKs can be classified into three subsets: low granular MKs (LGMKs), high granular MKs (HGMKs) with CD42b expression, and HGMKs without CD42b expression. A gradual decrease in the percentage of LGMKs and $HG/CD42b^{+-}$ MKs and accumulation of apoptotic $HG/CD42b^-$MKs indicates a reduction in platelet yield, and apoptosis inhibition plays a protective effect on MK apoptosis and CD42b exfoliation. The same study also found that $HG/CD42b^+$MKs were more likely to induce a response in platelet activators. These MKs may be close to the peak of maturation and have the ability to endocytose coagulation factor FV into alpha-granules. Interestingly, $CD42b^{high/}FV^+$ MKs represent a subpopulation of $HG/CD42b^+$ MKs with larger size and granularity; this indicates that FV uptake may be one of the final markers of the full MK maturity. FV-labeled MKs can release functional platelets after infusion in immunodeficient NOD/Shi-scid/IL-2Rγ^{null} (NSG) mice, and the same phenomenon can be found in iPSC-derived MKs [45]. Besides, the uptaken FV platelets have increased clot formation and aggregation ability compared to platelets without FV. These results provide new insight into the specific stage of MK maturation and show an experimental basis for PSC differentiation into platelets. FV labeling could be a useful tool to screen mature $HG/CD42b^+$MKs during iPlatelet generation and promote high platelet yield if further combined with bioreactors [42, 61].

Since mismatched HLA antigens are one reason for platelet transfusion failure, a new system was constructed to solve alloimmunity. Using the CRISPR/Cas9 technology to knock out the expression of the β2-microglobulin gene (*B2M*), an essential component of HLA class I molecules, the HLA I

expression on the cell surface was successfully eliminated [38, 50]. As a result, the functional platelets produced could escape antibody-mediated cytotoxicity both in vitro and in vivo.

2.2. Scale-Up System for iPlatelet Production. The efficiency of platelet production from iPSCs in vitro under static culture conditions is lower than that observed in vivo. Many bioreactors or novel culture systems have been developed and refined to scale up iPSC-derived platelet production. A two-dimensional flow culture system was proposed by Nakagawa et al. [42], and it was a biomimetic artificial vascular system consisting of a biodegradable scaffold with an ordered array of holes that were arranged to mimic bone marrow in vivo through salt leaching. In the method, two different flows (the angle between them was 60° instead of 90°) helped to apply appropriate pressure and shear stress to the MKs, thereby promoting platelet production. Platelets derived from hESCs or hiPSCs at a 60° angle through this bioreactor showed complete integrin αIIbβ3 activation after agonist stimulation.

A rotary cell culture system was developed and applied to potentiate megakaryopoiesis, which significantly enhanced platelet generation [62]. This 3D dynamic culture system has advantages in supplying shear force, simulated microgravity, and better diffusion of nutrients and oxygen. By screening chemical compounds, growth factors, and the rotary suspension culture system, the platelet yield was ~3.7-fold higher than that under static conditions.

Interestingly, turbulence energy, which acts as a physical regulator in thrombopoiesis in vivo, can be applied in platelet production ex vivo. Ito et al. [49] successfully generated highly efficient iPSC-derived platelets using another newly developed vertical reciprocal motion liquid culture bioreactor, VerMES. An optimal level of turbulent energy and shear stress was included in this system, which improved the platelet yield for the generation of 100 billion functional platelets from hiPSC-MKs in an 8 L VerMES independent of the cultivation scale size. The morphology and function of iPSC-derived platelets were comparable to those of donor-derived platelets. The possible explanation was that turbulent energy might promote proplatelet shedding via a cell-autonomous mechanism by soluble factors IGFBP2, MIF, and NRDC [49].

The platelet tracer technology has been further refined. It was found that the level of microtubule component molecule β1-tubulin (TUBB1), which is the main component of the cytoplasm of MKs, gradually increases with the maturation of MKs. Therefore, using CRISPR/Cas9 in β1-tubulin tagging can help monitor MK development and the generation of platelet-like particles. CRISPR/Cas9 can be applied to the high-throughput identification and validation of novel inducers of large-scale ex vivo platelet production [63]. These highly efficient and controllable methodologies represent a considerable leap in large-scale platelet production for future biomedical and clinical applications.

2.3. Functionality of iPlatelets. Many studies have been designed to evaluate whether hPSC-derived MKs and platelets have good purity and appropriate quality as normal human platelets and identify their essential functions both in vitro and in vivo. The assessment criteria for in vitro functionality and safety validation of platelets include, but are not limited to, morphology, ultrastructure, cytoskeletal organization, granule content, ploidy, gene expression, and biomarker expression [64]. Ultrastructural/morphological analyses showed that imMKCLs or other iPSC-derived MKs had similar polyploid states as primary human MKs [44]. iPlatelets have similar surface markers as blood-derived platelets; however, they have fewer platelet granules and larger cell sizes [38, 44]. iPSC-derived MKs expressed many functional specific markers such as CD41a, CD42b, and CD61, which can be used for platelet identification [35, 41]. Further, the relative expression of megakaryocytic marker gene mRNAs, such as *c-Myc*, *GATA1*, *TAL1*, and *RUNX1*, was higher in hPSC-derived MKs [41, 43, 44]. Typically, platelets show aggregated responsiveness after activation by the agonist adenosine diphosphate (ADP) because there are sites on platelets that can bind to ADP and cause a conformational change in integrin αIIbβ3 [35]. Flow cytometry analysis showed the high binding ability of iPlatelets to PAC-1 in the absence of ADP. Upon activation, iPlatelets on immobilized fibrinogen exhibited aggregates, lamellipodia, filopodia, and actin stress fibers, which induced cytoskeletal reorganization [44, 57].

Undoubtedly, the strongest evidence comes from in vivo experiments. Human normal platelets and iPSC-platelets were intravenously infused into macrophage-depleted mice to analyze the iPlatelet function in live animals. It was shown that iPSC-platelets were incorporated into developing mouse thrombus, similar to blood-derived platelets [38]. There is also evidence that iPSC-platelet kinetics is the same as that of fresh normal platelets. With high spatiotemporal resolution confocal laser microscopy, the behavior of iPlatelets upon initiation of adhesion to the injured vessel wall was dynamically observed in immunodeficient NSG mice irradiated with 2.0 Gy to induce thrombocytopenia [57, 65]. Several studies have evaluated the in vivo functions of iPlatelets, and it has been shown that iPlatelets adhered to the injured site initially, leading to thrombus formation and clot retraction [38, 40, 45]. However, some of these studies showed a limited iPlatelet thrombosis capacity compared to normal platelets [44, 45, 57].

2.4. Advantages, Limitations, and Potential Applications. Due to the unlimited self-renewal capacity of hPSCs, iPlatelets can be an ideal substitution for current donor-dependent systems. Besides, combination with CRISPR/Cas9 or other gene-editing technologies can help reduce alloimmune rejection [38, 45]. In terms of safety, iPSC-derived platelets can be irradiated to eliminate pathogens and other cell contaminations. As an intermediate, imMKCLs can expand for a long time and can also be frozen and thawed for utilization in emergencies [44]. However, there are still limitations to iPlatelet production, such as potential tumorigenicity, low yield, low functionality, and high cost [37, 38, 42, 44, 45]. The advantages and limitations of iPlatelets have been summarized and listed in Table 2.

Table 2: Advantages and limitations of current approaches for iPlatelet production.

Methods	Advantages	Limitations
OP9/C3H10T1/2 feeder system [39]	The cornerstone of hPSC-MK generation	Low platelet production Long induction period Potential xenogenous contamination
ES-sac system [35]	Identifies most effective cytokines during hPSC-MK generation The basis for efficient production of platelets	Long induction period Potential xenogenous contamination Low platelet production
EB formation system [40, 66]	Improves the efficiency of MK generation based on the ES-sac system Combined with defined serum- and animal feeder-free conditions Provides evidence for the functionality of iPlatelets in vivo	Limited efficiency in platelet production
Feeder- or serum-free system [36]	Without pathogen contamination	Limited efficiency in platelet production
HLA-universal iPlatelets [38, 50, 67]	Shortens platelet production time Increases MKP yield Reduces the immunoreactivity of iPlatelets	Inevitable off-target effects or genome toxicity effects Limited efficiency in platelet production
imMKCLs [44, 49]	High stability and cryopreserved storage Widely used in future clinical applications Combined with a bioreactor system	High cost The potential risk of exogene integration
Other genetic manipulation [43]	Feasibility in genetic manipulation Discovers new critical factors that determine the fate of iPlatelets	Inevitable off-target effects Exogene integration may have some specific safety concerns in clinical treatment Limited efficiency in platelet production

Lack of proper models largely restricts the exploration of pathogenesis of many diseases. Thus, patient-derived iPSCs can provide great research models that may address the above issue. For example, the induction of PSCs from patients with familial thrombocytopenia/acute myeloid leukemia showed that their ability to differentiate into HPCs and MKs was affected, which is associated with *RUNX1* germline mutations in these diseases [68]. Further, Paris-Trousseau syndrome (PTSx) is caused by the lack of FLI transcription factor, an essential factor in the process of MK differentiation. To better understand the role of *FLI* in this disease, the platelet production ability of iPSCs derived from PTSx patients was compared with that of wild-type iPSCs, and it was suggested that FLI might influence MK clonogenic potential and the production of platelets [69].

Similarly, because MKs are rare in the bone marrow, it is difficult to explore the differences between healthy and pathological states and their effect on platelet production, especially in patients with severe megakaryocytopenia, including congenital amegakaryocytic thrombocytopenia (CAMT). In this case, iPSCs provide a useful model that mimics thrombocytopenia for patients with CAMT. Although these patient-derived iPSCs cannot produce MKs and platelets, the overexpression of MPL can restore their hematopoietic function [70].

3. Conclusion and Future Perspectives

Stable and reliable stem cells are ideal sources for advancing fundamental scientific discoveries and cell therapy in the context of megakaryopoiesis and platelet production. Many studies have demonstrated that platelets derived from hPSCs have certain features and functions analogous to normal platelets, such as the *PAC-1* binding activity, the surface marker expression, and adhesion ability.

This review provides an overview of the possibilities and challenges regarding the production and use of hPSC-derived platelets. Firstly, autologous iPlatelet transfer becomes an effective avenue for averting allogeneic immune rejection. Moreover, the latest knockout technology, CRISPR/Cas9, can also effectively diminish alloimmune rejection due to the mismatched HLA. Then, platelet bioreactors are designed to mimic platelet production in vivo, exposing platelet progenitors to the architecture and intravascular shear stresses characteristic of their native microenvironment [61]. The combination of iPSCs and 3D bioreactors is a useful tool for improving platelet yields [49, 66]. Industrialized production of iPSC-derived platelets is an irresistible general trend for clinical application. With continuous efforts, platelets derived from PSCs have remarkable improvement both in quality and quantity; safety assessment and full functionality evaluation of iPlatelet transfusion are still essential, and the cost needs to be controlled. There is still a long way to go for the large-scale production and clinical application of iPlatelets. iPSC-derived platelets need further technological innovation to realize optimization in terms of scalable production and clinical feasibility.

Conflicts of Interest

There is no conflict of interest regarding the publication of this paper.

Authors' Contributions

LPL and YWZ conceived and designed the study. MXX drafted the manuscript. LPL, YWZ, and MXX contributed to reviewing, discussing, and revising the manuscript. All authors approved the final manuscript. LPL and MXX contributed equally to this work. YML supplied resources and materials. YML and YWZ are senior authors and co-correspondents of this work.

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References

[1] P. J. Vinholt, "The role of platelets in bleeding in patients with thrombocytopenia and hematological disease," *Clinical Chemistry and Laboratory Medicine (CCLM)*, vol. 57, no. 12, pp. 1808–1817, 2019.

[2] R. Storey and M. Thomas, "The role of platelets in inflammation," *Thrombosis and Haemostasis*, vol. 114, no. 9, pp. 449–458, 2015.

[3] C. N. Jenne and P. Kubes, "Platelets in inflammation and infection," *Platelets*, vol. 26, no. 4, pp. 286–292, 2015.

[4] X. Wang, Y. Zhang, J. Choukroun, S. Ghanaati, and R. J. Miron, "Effects of an injectable platelet-rich fibrin on osteoblast behavior and bone tissue formation in comparison to platelet-rich plasma," *Platelets*, vol. 29, no. 1, pp. 48–55, 2018.

[5] L. C. Garbin and C. S. Olver, "Platelet-rich products and their application to osteoarthritis," *J Equine Vet Sci*, vol. 86, article 102820, 2020.

[6] P. Samadi, M. Sheykhhasan, and H. M. Khoshinani, "The use of platelet-rich plasma in aesthetic and regenerative medicine: a comprehensive review," *Aesthetic Plastic Surgery*, vol. 43, no. 3, pp. 803–814, 2019.

[7] M. Lesurtel, R. Graf, B. Aleil et al., "Platelet-derived serotonin mediates liver regeneration," *Science*, vol. 312, no. 5770, pp. 104–107, 2006.

[8] S. Murata, N. Ohkohchi, R. Matsuo, O. Ikeda, A. Myronovych, and R. Hoshi, "Platelets promote liver regeneration in early period after hepatectomy in mice," *World Journal of Surgery*, vol. 31, no. 4, pp. 808–816, 2007.

[9] K. Takahashi, S. Murata, K. Fukunaga, and N. Ohkohchi, "Human platelets inhibit liver fibrosis in severe combined immunodeficiency mice," *World Journal of Gastroenterology*, vol. 19, no. 32, pp. 5250–5260, 2013.

[10] M. Kirschbaum, G. Karimian, J. Adelmeijer, B. N. G. Giepmans, R. J. Porte, and T. Lisman, "Horizontal RNA transfer mediates platelet-induced hepatocyte proliferation," *Blood*, vol. 126, no. 6, pp. 798–806, 2015.

[11] T. Lisman, M. Kirschbaum, and R. J. Porte, "The role of platelets in liver regeneration - what don't we know?," *Journal of Hepatology*, vol. 63, no. 6, pp. 1537-1538, 2015.

[12] J. Meyer, E. Lejmi, P. Fontana, P. Morel, C. Gonelle-Gispert, and L. Bühler, "A focus on the role of platelets in liver regeneration: do platelet-endothelial cell interactions initiate the regenerative process?," *Journal of Hepatology*, vol. 63, no. 5, pp. 1263–1271, 2015.

[13] T. Kurokawa, Y. W. Zheng, and N. Ohkohchi, "Novel functions of platelets in the liver," *Journal of Gastroenterology and Hepatology*, vol. 31, no. 4, pp. 745–751, 2016.

[14] M. Bilzer, F. Roggel, and A. L. Gerbes, "Role of Kupffer cells in host defense and liver disease," *Liver International*, vol. 26, no. 10, pp. 1175–1186, 2006.

[15] R. Malik, C. Selden, and H. Hodgson, "The role of non-parenchymal cells in liver growth," *Seminars in Cell & Developmental Biology*, vol. 13, no. 6, pp. 425–431, 2002.

[16] R. E. Marx, "Platelet-rich plasma: evidence to support its use," *Journal of Oral and Maxillofacial Surgery*, vol. 62, no. 4, pp. 489–496, 2004.

[17] S. Lang, M. Loibl, and M. Herrmann, "Platelet-rich plasma in tissue engineering: hype and hope," *European Surgical Research*, vol. 59, no. 3-4, pp. 265–275, 2018.

[18] J. Etulain, "Platelets in wound healing and regenerative medicine," *Platelets*, vol. 29, no. 6, pp. 556–568, 2018.

[19] A. Moshiri, A. Oryan, and A. Meimandi-Parizi, "Role of embedded pure xenogenous bovine platelet gel on experimental tendon healing, modelling and remodelling," *BioDrugs*, vol. 28, no. 6, pp. 537–556, 2014.

[20] G. Y. Li, J. M. Yin, H. Ding, W. T. Jia, and C. Q. Zhang, "Efficacy of leukocyte- and platelet-rich plasma gel (L-PRP gel) in treating osteomyelitis in a rabbit model," *Journal of Orthopaedic Research*, vol. 31, no. 6, pp. 949–956, 2013.

[21] S. Ahmadipour, J. Varshosaz, B. Hashemibeni, L. Safaeian, and M. Manshaei, "Polyhedral oligomeric silsesquioxane /platelets rich plasma/gelrite-based hydrogel scaffold for bone tissue engineering," *Current Pharmaceutical Design*, vol. 26, no. 26, pp. 3147–3160, 2020.

[22] A. Oryan, S. Alidadi, and A. Moshiri, "Platelet-rich plasma for bone healing and regeneration," *Expert Opinion on Biological Therapy*, vol. 16, no. 2, pp. 213–232, 2016.

[23] K. W. Badran and J. P. Sand, "Platelet-rich plasma for hair loss: review of methods and results," *Facial Plastic Surgery Clinics of North America*, vol. 26, no. 4, pp. 469–485, 2018.

[24] V. Cervelli, F. Nicoli, D. Spallone et al., "Treatment of traumatic scars using fat grafts mixed with platelet-rich plasma, and resurfacing of skin with the 1540 nm nonablative laser," *Clinical and Experimental Dermatology*, vol. 37, no. 1, pp. 55–61, 2012.

[25] J. T. ZHU, M. XUAN, Y. N. ZHANG et al., "The efficacy of autologous platelet-rich plasma combined with erbium fractional laser therapy for facial acne scars or acne," *Molecular Medicine Reports*, vol. 8, no. 1, pp. 233–237, 2013.

[26] A. Trink, E. Sorbellini, P. Bezzola et al., "A randomized, double-blind, placebo- and active-controlled, half-head study to evaluate the effects of platelet-rich plasma on alopecia areata," *The British Journal of Dermatology*, vol. 169, no. 3, pp. 690–694, 2013.

[27] M. S. Leo, A. S. Kumar, R. Kirit, R. Konathan, and R. K. Sivamani, "Systematic review of the use of platelet-rich plasma in aesthetic dermatology," *Journal of Cosmetic Dermatology*, vol. 14, no. 4, pp. 315–323, 2015.

[28] X. H. Liang, S. H. Zhou, Y. X. Fan et al., "A survey of the blood supply in China during 2012-2014," *Transfusion Medicine*, vol. 29, no. 1, pp. 28–32, 2019.

[29] T. M. Getz, "Physiology of cold-stored platelets," *Transfusion and Apheresis Science*, vol. 58, no. 1, pp. 12–15, 2019.

[30] P. Moncharmont, "Platelet component transfusion and alloimmunization: where do we stand?," *Transfusion Clinique et Biologique*, vol. 25, no. 3, pp. 172–178, 2018.

[31] P. Douvaras and V. Fossati, "Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells," *Nature Protocols*, vol. 10, no. 8, pp. 1143–1154, 2015.

[32] L. P. Liu, Y. M. Li, N. N. Guo et al., "Therapeutic potential of patient iPSC-derived iMelanocytes in autologous transplantation," *Cell Reports*, vol. 27, no. 2, pp. 455–466.e5, 2019, e5.

[33] C. L. Mummery, J. Zhang, E. S. Ng, D. A. Elliott, A. G. Elefanty, and T. J. Kamp, "Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes," *Circulation Research*, vol. 111, no. 3, pp. 344–358, 2012.

[34] R. Morizane and J. V. Bonventre, "Generation of nephron progenitor cells and kidney organoids from human pluripotent stem cells," *Nature Protocols*, vol. 12, no. 1, pp. 195–207, 2017.

[35] N. Takayama, H. Nishikii, J. Usui et al., "Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors," *Blood*, vol. 111, no. 11, pp. 5298–5306, 2008.

[36] G. Salvagiotto, S. Burton, C. A. Daigh, D. Rajesh, I. I. Slukvin, and N. J. Seay, "A defined, feeder-free, serum-free system to generate in vitro hematopoietic progenitors and differentiated blood cells from hESCs and hiPSCs," *PLoS One*, vol. 6, no. 3, article e17829, 2011.

[37] N. Takayama and K. Eto, "In vitro generation of megakaryocytes and platelets from human embryonic stem cells and induced pluripotent stem cells," *Methods in Molecular Biology*, vol. 788, pp. 205–217, 2012.

[38] Q. Feng, N. Shabrani, J. N. Thon et al., "Scalable generation of universal platelets from human induced pluripotent stem cells," *Stem Cell Reports*, vol. 3, no. 5, pp. 817–831, 2014.

[39] M. GAUR, T. KAMATA, S. WANG, B. MORAN, S. J. SHATTIL, and A. D. LEAVITT, "Megakaryocytes derived from human embryonic stem cells: a genetically tractable system to study megakaryocytopoiesis and integrin function," *Journal of Thrombosis and Haemostasis*, vol. 4, no. 2, pp. 436–442, 2006.

[40] S.-J. Lu, F. Li, H. Yin et al., "Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice," *Cell Research*, vol. 21, no. 3, pp. 530–545, 2011.

[41] M. Pick, L. Azzola, E. Osborne, E. G. Stanley, and A. G. Elefanty, "Generation of megakaryocytic progenitors from human embryonic stem cells in a feeder- and serum-free medium," *PLoS One*, vol. 8, no. 2, article e55530, 2013.

[42] Y. Nakagawa, S. Nakamura, M. Nakajima et al., "Two differential flows in a bioreactor promoted platelet generation from human pluripotent stem cell-derived megakaryocytes," *Experimental Hematology*, vol. 41, no. 8, pp. 742–748, 2013.

[43] I. Elcheva, A. Kumar, P. Liu et al., "Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators," *Nature Communications*, vol. 5, no. 1, p. 4372, 2014.

[44] S. Nakamura, N. Takayama, S. Hirata et al., "Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells," *Cell Stem Cell*, vol. 14, no. 4, pp. 535–548, 2014.

[45] T. Moreau, A. L. Evans, L. Vasquez et al., "Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming," *Nature Communications*, vol. 7, no. 1, article 11208, 2016.

[46] J. N. Thon, L. Mazutis, S. Wu et al., "Platelet bioreactor-on-a-chip," *Blood*, vol. 124, no. 12, pp. 1857–1867, 2014.

[47] A. Blin, A. le Goff, A. Magniez et al., "Microfluidic model of the platelet-generating organ: beyond bone marrow biomimetics," *Scientific Reports*, vol. 6, no. 1, article 21700, 2016.

[48] X. Sim, D. Jarocha, V. Hayes et al., "Identifying and enriching platelet-producing human stem cell-derived megakaryocytes using factor V uptake," *Blood*, vol. 130, no. 2, pp. 192–204, 2017.

[49] Y. Ito, S. Nakamura, N. Sugimoto et al., "Turbulence Activates Platelet Biogenesis to Enable Clinical Scale _Ex Vivo_ Production," *Cell*, vol. 174, no. 3, pp. 636–648.e18, 2018.

[50] P. Norbnop, P. Ingrungruanglert, N. Israsena, K. Suphapeetiporn, and V. Shotelersuk, "Generation and characterization of HLA-universal platelets derived from induced pluripotent stem cells," *Scientific Reports*, vol. 10, no. 1, p. 8472, 2020.

[51] C. E. Murry and G. Keller, "Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development," *Cell*, vol. 132, no. 4, pp. 661–680, 2008.

[52] J. A. Mills, P. Paluru, M. J. Weiss, P. Gadue, and D. L. French, "Hematopoietic differentiation of pluripotent stem cells in culture," *Methods in Molecular Biology*, vol. 1185, pp. 181–194, 2014.

[53] N.-N. Guo, L.-P. Liu, Y.-W. Zheng, and Y.-M. Li, "Inducing human induced pluripotent stem cell differentiation through embryoid bodies: a practical and stable approach," *World J Stem Cells*, vol. 12, no. 1, pp. 25–34, 2020.

[54] S. J. Lu, C. Luo, K. Holton, Q. Feng, Y. Ivanova, and R. Lanza, "Robust generation of hemangioblastic progenitors from human embryonic stem cells," *Regenerative Medicine*, vol. 3, no. 5, pp. 693–704, 2008.

[55] S. J. Lu, Q. Feng, S. Caballero et al., "Generation of functional hemangioblasts from human embryonic stem cells," *Nature Methods*, vol. 4, no. 6, pp. 501–509, 2007.

[56] R. Sugimura, D. K. Jha, A. Han et al., "Haematopoietic stem and progenitor cells from human pluripotent stem cells," *Nature*, vol. 545, no. 7655, pp. 432–438, 2017.

[57] N. Takayama, S. Nishimura, S. Nakamura et al., "Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells," *The Journal of Experimental Medicine*, vol. 207, no. 13, pp. 2817–2830, 2010.

[58] S. Masuda, M. Li, and J. C. Izpisua Belmonte, "In vitro generation of platelets through direct conversion: first report in my knowledge (iMK)," *Cell Research*, vol. 23, no. 2, pp. 176–178, 2013.

[59] J. Ware, S. Russell, and Z. M. Ruggeri, "Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 6, pp. 2803–2808, 2000.

[60] W. Bergmeier, P. C. Burger, C. L. Piffath et al., "Metalloproteinase inhibitors improve the recovery and hemostatic function of in vitro-aged or -injured mouse platelets," *Blood*, vol. 102, no. 12, pp. 4229–4235, 2003.

[61] J. N. Thon, B. J. Dykstra, and L. M. Beaulieu, "Platelet bioreactor: accelerated evolution of design and manufacture," *Platelets*, vol. 28, no. 5, pp. 472–477, 2017.

[62] Y. Yang, C. C. Liu, X. Lei et al., "Integrated biophysical and biochemical signals augment megakaryopoiesis and thrombopoiesis in a three-dimensional rotary culture system," *Stem Cells Translational Medicine*, vol. 5, no. 2, pp. 175–185, 2016.

[63] H. Seo, S. J. Chen, K. Hashimoto et al., "A β1-tubulin–based megakaryocyte maturation reporter system identifies novel drugs that promote platelet production," *Blood Advances*, vol. 2, no. 17, pp. 2262–2272, 2018.

[64] J. N. Thon, D. A. Medvetz, S. M. Karlsson, and J. E. Italiano Jr., "Road blocks in making platelets for transfusion," *Journal of Thrombosis and Haemostasis*, vol. 13, pp. S55–S62, 2015.

[65] H. Takizawa, S. Nishimura, N. Takayama et al., "Lnk regulates integrin alphaIIbbeta3 outside-in signaling in mouse platelets, leading to stabilization of thrombus development in vivo," *The Journal of Clinical Investigation*, vol. 120, no. 1, pp. 179–190, 2010.

[66] S.-J. Lu, T. Kelley, and Q. Feng, "3D microcarrier system for efficient differentiation of human induced pluripotent stem cells into hematopoietic cells without feeders and serum," *Regenerative Medicine*, vol. 8, no. 4, pp. 413–424, 2013.

[67] C. Gras, K. Schulze, L. Goudeva, C. A. Guzman, R. Blasczyk, and C. Figueiredo, "HLA-universal platelet transfusions prevent platelet refractoriness in a mouse model," *Human Gene Therapy*, vol. 24, no. 12, pp. 1018–1028, 2013.

[68] M. Sakurai, H. Kunimoto, N. Watanabe et al., "Impaired hematopoietic differentiation of RUNX1-mutated induced pluripotent stem cells derived from FPD/AML patients," *Leukemia*, vol. 28, no. 12, pp. 2344–2354, 2014.

[69] K. K. Vo, D. J. Jarocha, R. B. Lyde et al., "FLI1 level during megakaryopoiesis affects thrombopoiesis and platelet biology," *Blood*, vol. 129, no. 26, pp. 3486–3494, 2017.

[70] S. Hirata, N. Takayama, R. Jono-Ohnishi et al., "Congenital amegakaryocytic thrombocytopenia iPS cells exhibit defective MPL-mediated signaling," *Journal of Clinical Investigation*, vol. 123, no. 9, pp. 3802–3814, 2013.

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Review Article

Advances in Female Germ Cell Induction from Pluripotent Stem Cells

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Germ cells are capable of maintaining species continuity through passing genetic and epigenetic information across generations. Female germ cells mainly develop during the embryonic stage and pass through subsequent developmental stages including primordial germ cells, oogonia, and oocyte. However, due to the limitation of using early human embryos as *in vivo* research model, *in vitro* research models are needed to reveal the early developmental process and related mechanisms of female germ cells. After birth, the number of follicles gradually decreases with age. Various conditions which damage ovarian functions would cause premature ovarian failure. Alternative treatments to solve these problems need to be investigated. Germ cell differentiation from pluripotent stem cells *in vitro* can simulate early embryonic development of female germ cells and clarify unresolved issues during the development process. In addition, pluripotent stem cells could potentially provide promising applications for female fertility preservation after proper *in vitro* differentiation. Mouse female germ cells have been successfully reconstructed *in vitro* and delivered to live offspring. However, the derivation of functional human female germ cells has not been fully achieved due to technical limitations and ethical issues. To provide an updated and comprehensive information, this review centers on the major studies on the differentiation of mouse and human female germ cells from pluripotent stem cells and provides references to further studies of developmental mechanisms and potential therapeutic applications of female germ cells.

1. Introduction

Currently, female infertility caused by various reasons is becoming an exacerbating reproductive problem. Assisted reproductive technology (ART) is an effective treatment for non-germ cells (GCs-) caused infertility. However, infertility caused by GCs abnormalities has not yet had a good alternative treatment [1]. Treating infertility among these patients requires a precisely detailed understanding of female GCs differentiation and pathological defects which occurred in abnormal female GCs. However, female GCs formation mainly occurs during the embryonic stage. Due to the limited acquisition and ethical inhibition to early human embryos for research purpose, early female GCs development have not been revealed deliberately [2]. Therefore, establishing an appropriate *in vitro* model is necessary for the investigations on female GCs development and fertility reconstruction.

A mouse model is most commonly used to study mammalian female GCs formation, specialization, and differentiation [3]. Significant achievements have been gained in inducing mouse female GCs from pluripotent stem cells (PSCs) which provide remarkable references for reconstructing human female GCs *in vitro* from PSCs [4–8]. Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have competence for self-renewal and multilineage differentiation including female GCs [9–12]. However, the induction protocols are slightly different between mouse PSCs and human PSCs based on the differences of *in vivo* female GCs formation between mice and humans [4–8].

2. Female Germ Cells Development *In Vivo*

Mouse primordial germ cells (PGCs) were first discovered at the posterior end of the primitive streak in the extraembryonic mesoderm at embryonic day 6.25 (E6.25) [13], followed by PGC specification at E7.25 and migration at E9.5. At around E10.5, PGCs reach the genital ridge and enter embryonic gonads at E11.5 [14]. The ultimate sexual fate is not only regulated by the chromosomal constitution but also by the gonadal somatic cells [14]. Before interweaving with the signals from embryonic gonadal somatic cells, PGCs are "bipotential," which means PGCs could adapt either male or female fate [14]. After colonizing embryonic ovaries, PGCs begin sex differentiation at E12.5 and develop into oogonia at E13.5 [15]. Afterward, at E14.5, oogonia enter meiosis I and form primary oocytes which are arrested at the meiosis I diplotene stage until ovulation. At around birth, the primary oocytes were surrounded by granulosa cells and sequentially generate primordial, primary, secondary, and antral follicles [16]. Primary oocytes complete meiosis I around six weeks after birth and form secondary oocytes. Secondary oocytes are ovulated and arrested at metaphase of meiosis II (MII) before fertilization. MII oocytes are considered as the functional oocytes that could be fertilized with spermatozoa (Figure 1).

Mouse PGCs differentiation occurs under the regulation of sequential transcription factors (TFs) (Figure 2). Bone morphogenetic protein (BMP) and Wingless/Integrated (WNT) pathways trigger a set of downstream TFs [17]. BMP4 activates WNT3, which is located at the upstream of a mesodermal TF- BRACHYURY (T) [17]. T activates critical early GCs markers BLIMP1, PRDM14, and TFAP2C synergistically with BMP4 [17]. BLIMP1 is expressed in the precursors of mouse PGCs, induces PRDM14 and TFAP2C, activates the germline pathway, and robustly represses a somatic mesodermal pathway [15, 18]. PRDM14 is specifically expressed in mouse PGCs. Studies showed PRDM14 is essential for epigenetic reprogramming in mouse PGCs [19]. Thus, these interactions between TFs are essential for the subsequent differentiation of female GCs. During specification, at around E7.25, mouse PGCs express pluripotency markers OCT4, NANOG, SOX2, KLF2, and PGCs-specific markers SSEA1 and STELLA (Figure 1) [18, 20]. OCT4, which is critical for the specialization and maintenance of mouse PGCs exhibited high expression until sex differentiation [15]. SOX2 directly contributes to the survival and proliferation of mouse PGCs [21]. Migratory mouse PGCs mainly express SSEA1 and CXCR4 [19, 20]. DAZL and VASA begin to be expressed when sex differentiation is imminent. DAZL is considered as a germ cell-intrinsic competence factor, which is necessary for receiving signals from extrinsic factors in embryonic gonads. After sex differentiation, meiosis I is initiated by retinoic acid (RA) at around E12.5. RA induces premeiotic gene STRA8 and meiosis-associated gene REC8 expression in embryonic ovaries. STRA8 upregulates synaptonemal complex protein3 (SCP3) and DMC1, both of which represent meiosis initiation at E13.5 [22]. In conclusion, these stage-specific markers not only provided insights into GCs developmental mechanisms but also offered specific markers for assessing differentiated cells during female GCs development, as well as inducing female GCs differentiation through overexpression.

In humans, PGCs differentiation is similar in broad strokes with mouse PGCs, but varies in developmental timing (Figure 1), TFs interactions, and certain specific markers (Figure 2). Human PGCs were first identified by Fuss and Felix in the dorsal wall of the yolk sac endoderm at developmental week 3 (Wk3) [23, 24]. In later studies, researchers detected human PGCs are specified in the posterior epiblast of early postimplantation embryos approximately at Wk2 [25, 26]. Then, human PGCs start migration around Wk4 [27, 28] and enter genital gonads around Wk5-6 [29]. During Wk6-8, PGCs sex-differentiated with the gonadal somatic cells in embryonic ovaries [30]. With the interactions between PGCs and gonadal somatic cells, oogonia cells are formed at Wk9 and respond to RA signals around Wk11 to differentiate into primary oocytes at Wk14 [26, 31]. Afterward, primary oocytes assembled into primordial follicles with a layer of granulosa cells [32]. At birth, there are approximately 300,000 primordial follicles, and this number mostly declines with age after birth [33]. The subsequent folliculogenesis, completion of meiosis I, and generation of MII oocytes proceeded in a mostly analogous way with different point-in-time to mice.

Human PGCs specification occurred under BMP4, EOMES, SOX17, BLIMP1, and TFAP2C transcriptional network approximately similar to that of mice (Figure 2). EOMES, which is a critical factor in human mesodermal precursor cells, is located at the downstream of Activin and WNT signaling, meanwhile at the upstream of SOX17, BLIMP1, and TFAP2C [34]. SOX17, an essential specifier of human PGCs, also activates BLIMP1 and TFAP2C, both of which in turn activate the germline pathway and repress mesoderm, endoderm, and neural pathway [34]. The crucial mesodermal TF-T during mouse PGCs formation, however, is not essential for humans [34]. KLF4, a naive pluripotency factor, is expressed in human PGCs while in mice repressed by BLIMP1 [35]. In contrast, SOX2 and PRDM14, which are critical for mouse PGCs differentiation, are not highly expressed in human PGCs [7, 36]. Migrating human PGCs mainly express early GCs markers BLIMP1, TFAP2C, and SSEA1 as well as pluripotency markers OCT4 and NANOG. At the end of migration, DAZL and VASA are expressed at a lower level [26]. RA responsive genes STRA8, RDH10, and CYP26A1 begin to express as early as Wk11, indicating the imminent initiation of meiosis. The meiotic prophase female GCs mainly express SCP1, TEX12, and SPO11. The primary oocytes are characterized by ZP1-3, NOBOX, and OOSP2 expressions [26].

These abovementioned transcriptional factors and female GCs markers corresponding to different developmental stages provided important references for the establishment of the differentiation system *in vitro*. Meanwhile, the established *in vitro* models, in turn, elucidated the abovementioned mechanisms during female GCs formation. Continuing the described studies will elucidate precisely how mouse and human PSCs are induced into female GCs, respectively.

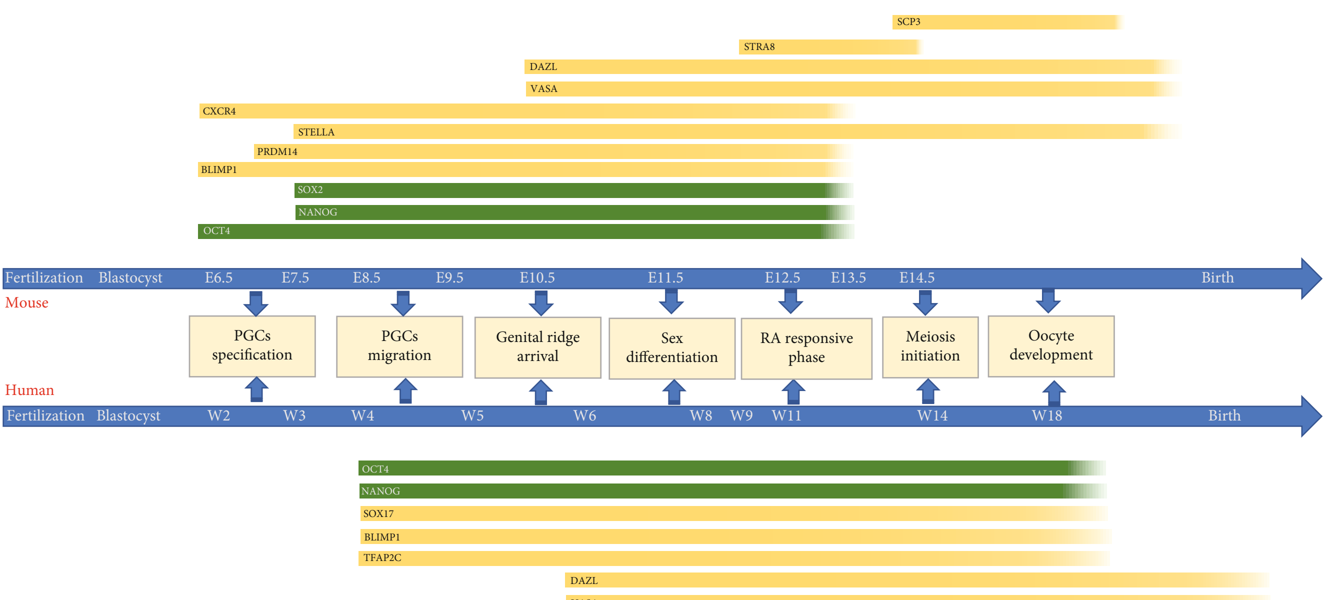

Figure 1: Schematic of female germ cell development and key gene expressions during the development. Developmental timelines and stages of mouse and human female germ cell development are shown in the center. PGCs undergo specification and migration then arrive at the genital ridge. After sex differentiation, PGCs subsequently undertake RA responsive phase, meiosis initiation, and oocyte development. Key gene expressions corresponding to different developmental stages are shown in yellow bars.

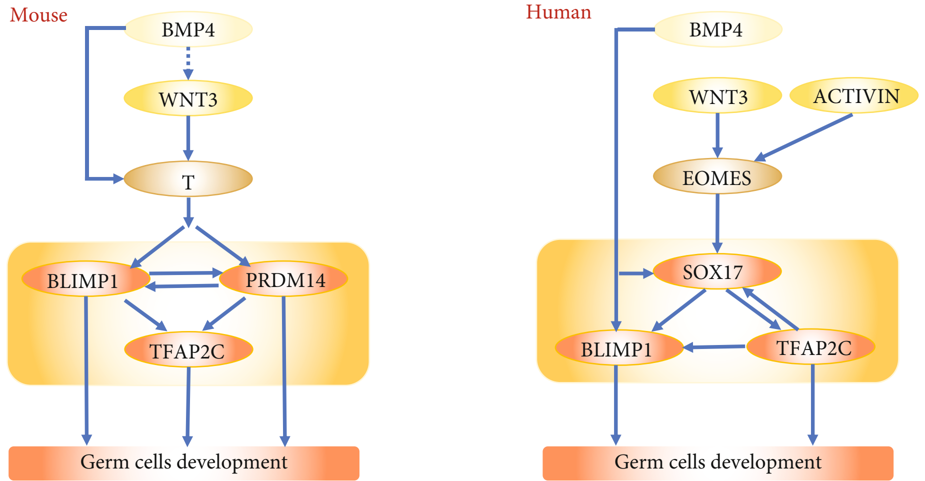

Figure 2: Transcriptional regulatory network models for mouse and human PGCs specification. Full and dashed arrows indicate direct and indirect regulations, respectively.

3. Female GCs Induction from PSCs *In Vitro*

The most commonly used PSCs are ESCs and iPSCs. Mouse and human ESCs were derived from inner cell mass (ICM) of the blastocyst in 1981 and 1998, respectively [37, 38]. ESCs have competence for self-renewal and multilineage differentiation potential to cells of three germ layers. However, the establishment of human ESCs needs to destroy early human embryos, thus resulting in ethical concerns. Also, xenotransplantation of ESCs-derived cells may probably cause immunological rejection. These concerns were relieved by the establishment of iPSCs. In 2006, scientists induced mouse iPSCs through the introduction of four key transcription factors—OCT3/4, SOX2, KLF4, and c-MYC—into mouse adult fibroblasts [9]. Subsequently, human iPSCs were generated from adult human fibroblasts [10, 39]. These iPSCs have become attractive alternatives of ESCs for their analogous biological characteristics to ESCs in cell morphology, gene expressions, and surface antigens. iPSCs were acquired *in vitro* without damaging early embryos, which could dispel ethical concerns about ESC acquisition and application. Furthermore, autologous cell transplantation derived from individual iPSCs avoids allogeneic immune rejection from ESCs. More importantly, they are also capable of differentiating into multilineage cells including female GCs [10]. Therefore, PSCs were studied to generate female GCs, especially iPSCs

were regarded as relatively ideal stem cell sources for regenerative medicine.

Generally, ESCs/iPSCs were induced into the germline pathway through spontaneous differentiation, direct induction with some cytokines, or overexpression of germline-specific genes. Induced female GCs were identified by the expression of stage-specific markers as well as the morphology or the functions. Scientists achieved great advances in inducing female GCs from PSCs [4, 6–8]. The induction schemes are slightly different between mouse and human PSCs based on their female GCs development discrepancies.

3.1. Female GCs Induction from Mouse PSCs In Vitro. Studies about mouse female GCs induction from PSCs acquired significant achievements in the recent two decades (Table 1). *In vitro* female GC induction was first evidenced from mouse ESCs in 2003 [40]. In this study, mouse ESCs were spontaneously differentiated in suspension condition without LIF and feeder cells. On the 12th day of culture, high GFP+/VASA+ expressions were detected in large colonies, which most likely represent postmigratory PGCs. These GFP+/VASA+ PGCs spontaneously formed oogonia-like cells, entered meiosis around the 16th day, and produced oocyte-like cells up to 20% at around the 26th day. Oocyte-like cells were characterized by zona pellucida (ZP) like coats, oocyte markers ZP2 and ZP3 expression. Subsequently, they formed small follicle-like cells (FLCs), which could be cultured into organized structures morphologically similar to primordial follicles. At around the 43rd day, some oocytes that completed meiosis I even could form blastocyst-like structures through parthenogenic activation [40]. These results indicated that mouse ESCs have the potential to spontaneously proceed beyond sex determination and differentiate into mouse female GCs approximately following the development phase and timing *in vivo* [41]. This pioneering study has revealed that mouse ESCs could be a new cell source for oocyte generation. However, in this study, oocyte-like cells have not been evidenced as mature oocytes. Besides, they were generated without directed differentiation and the induction efficiency is rather low. The addition of several growth factor signals was considered to directly differentiate the PSCs to germline and enhance the differentiation efficiency [42]. Researchers collected conditioned medium from testicular cell cultures since testis contain numerous growth factors like BMP4, SCF, LIF, βFGF, and GDF9. Mouse ESCs generated embryonic bodies (EBs) in suspension culture and were further induced into oocyte-like cells surrounded by one or two layers of flatted cells which resemble granulosa cells *in vivo*. This indicated testicular cell cultures could provide essential growth factors also for follicle formation [42]. However, in their study, the oocyte-like cells expressed oocyte markers SCP3, ZP3, and FIGα but not ZP1 and ZP2, indicating these oocyte-like cells are at an early stage of oocyte growth. Besides, they did not found synapsis despite the SCP3 existence. Regarding oocytes are generated under the interactions between PGCs and gonadal somatic cells *in vivo*, the spontaneous differentiation of oocytes from mouse ESCs was seen as a rare event, and gonadal somatic cells were considered necessary for oocyte-like cell induction [43]. Regarding this, researchers used a two-step method to induce oocyte-like cells from mouse ESCs [43]. First, PGCs were induced through EB formation in 4 days. They cultured mouse ESCs in LIF-free DMEM containing 10% FBS to form EB. EBs expressed OCT4, C-KIT, FRAGILIS, STELLA, and MVH. They sorted SSEA-1 and C-KIT positive cells which represent early PGCs then cocultured with gonadal somatic cells for further 10 days. The differentiated cells expressed female oocyte-specific markers FIGα, NOBOX, GDF9, and ZP1-3. However, these markers could not be detected when EBs were cultured alone. This is demonstrating that granulosa cells could enhance the female GCs induction. However, like previous studies [42], oocytes are still arrested at an early meiosis stage even after being cocultured with granulosa cells. Therefore, oocyte growth might require some additional factors that have not been included in these studies [42, 43]. Researchers assumed RA addition might contribute to meiosis completion since it could stimulate STRA8 and REC8 to enter meiosis *in vivo* [44]. Then, mouse ESCs-derived EBs were cultured under RA supplement for 10-15 days [45]. After RA treatment, researchers detected FLCs and presumptive germinal vesicle (GV) oocytes. Furthermore, these GV oocytes could be fertilized with sperms and develop into blastocysts. Thus, RA was confirmed critical for female GCs reconstitution.

In 2009, after the successful establishment of iPSCs, the chimaeric mouse was formed from mouse iPSCs by tetraploid complementation, demonstrating that mouse iPSCs have female GCs competency [46]. Similar to mouse ESCs induction in previous studies, mouse iPSCs were induced into round-shaped oogonia-like cells through EB formation in suspension culture supplemented with RA, BMP4, SCF, EGF, and GDNF [42, 45, 47]. This demonstrated that iPSCs and ESCs could be induced into female GCs through analogous induction methods.

The abovementioned studies established some useful approaches for female GCs induction; however, they failed to provide sequential systematic induction protocols with the clear transition from PSCs to PGCs and to later stage female GCs. Since PGCs are the natural precursors to the gametes [24], induction of functional PGC-like cells (PGCLCs) from PSCs is a significant procedure in reconstituting gametes *in vitro*. Mouse epiblast-like cells (EpiLCs) possess cellular characteristics similar to pregastrulating epiblasts and act as appropriate precursors for the induction of mouse PGCLCs [16]. Researchers found 2iLIF medium, which contained LIF and MAPK/GSK3 pathway inhibitors, could enable mouse ESCs to exhibit characteristics similar to the ICM and reveal more efficient germline competency [4, 48]. Mouse ground-state PSCs in 2iLIF medium, with further induction in ActA, βFGF, and KSR conditions for 2 days formed mouse EpiLCs. These mouse EpiLCs were further induced under the conditions of BMP4, LIF, SCF, and EGF for 4-6 days to generate mouse PGCLCs [49]. These mouse PGCLCs exhibited analogous transcriptomic and epigenetic profiles comparable to those of E9.5 migratory mouse PGCs *in vivo*. The epigenetic profiles of PGCLCs were evaluated by H3K9me2 and H3K27me3 which represent histone modification and 5mC levels and compared with their

Table 1: Mouse female GCs differentiation from PSCs *in vitro*.

Cell types	Main induction methods	Achievements: Generated cells	Achievements: Characterization of generated cells	Journal, year (reference)
ESCs	Spontaneous differentiation Suspension culture	FLCs Oocyte-like cells	Morphology Marker expressions (ZP2, ZP3, and FIGα) Estradiol secretion Estrogen biosynthesis	Science, 2003 [40]
ESCs	CM from testicular cell Suspension culture	Oocyte-like cells	Morphology Marker expressions (SCP3, ZP3, and FIGα)	Stem cells, 2006 [42]
ESCs	(i) Spontaneous differentiation Suspension culture (ii) Coculture with gonadal cells Adherent and suspension culture	PGCs Oocyte like cells	Marker expressions (ZP3, FIGα, and GDF9)	Differentiation, 2007 [43]
ESCs	DAZL overexpression Suspension culture	FLCs Oocyte-like cells	Morphology Marker expressions (ZP1, ZP2, ZP3, and GDF9) Parthenogenesis activation	J Mol Cell Biol2009 [51]
ESCs iPSCs	(i) 2i (MAPK and GSK3 inhibitors), LIF, ActA, and bFGF Adherent culture (ii) LIF, SCF, BMP, and EGF Suspension culture	EpiLCs PGCLCs	Morphology Marker expressions (Blimp1 and STELLA) Global transcription profiles Epigenetic analysis	Cell, 2011 [49]
ESCs iPSCs	(i) Coculture with gonadal cells (ii) *In vivo* transplantation into mouse (iii) IVM and IVF	PGCLCs GV oocytes Fertile GCs	Morphology Marker expressions (BLIMP1 and PRDM14) Live offspring delivery	Science, 2012 [4]
ESCs iPSCs	(i) bFGF and ActA Adherent culture (ii) Overexpression of PRDM14 or PRDM1, PRDM14, and TFAP2C Suspension culture	EpiLCs PGCLCs	Morphology Marker expressions (BLIMP1 and STELLA) Global transcription profiles Epigenetic analysis	Nature, 2013 [52]
ESCs iPSCs	(i) bFGF and ActA Adherent culture (ii) NANOG overexpression Suspension culture	EpiLCs PGCLCs	Morphology Marker expressions (BLIMP1 and NANOS3) Global transcription profiles Epigenetic analysis	Nature, 2016 [53]
ESCs iPSCs	(i) Coculture with gonadal cells (ii) IVD, IVG, IVM, and IVF	PGCLCs MII oocytes Fertile GCs	Morphology Marker expressions (DAZL and STELLA) Global transcription profiles Polar body extrusion Live offspring delivery	Nature, 2016 [5]
ESCs iPSCs	(i) 2i (MAPK and GSK3 inhibitors), LIF, ActA, and bFGF Adherent culture (ii) LIF, SCF, BMP, and EGF Suspension culture (ii) BMP2 and RA Adherent culture	PGCLCs Primary oocytes	Morphology Marker expressions (STRA8, SCP3, and NOBOX) Transcriptome dynamics Premeiotic DNA replication	The EMBO Journal, 2017 [55]
iPSCs	(i) iPSCs from granulosa cells (ii) PGCLCs coculture with gonadal cells (iii) IVD, IVG, IVM, and IVF	PGCLCs MII oocytes Fertile GCs	Morphology Marker expressions (BLIMP1, DAZL, and VASA) Telomere elongation Endocrine activity of FSH, E2, and AMH Live offspring delivery	Cell Rep, 2019 [58]

ESCs: embryonic stem cells; iPSCs: induced pluripotent stem cells; GCs: germ cells; EpiLCs: epiblast-like cells; PGCs: primordial germ cells; PGCLCs: primordial germ cell-like cells; FLCs: follicle-like cells; GV oocytes: germinal vesicle oocytes; MII oocytes: meiosis II oocytes; CM: conditioned medium; IVD: *in vitro* differentiation; IVG: *in vitro* growth; IVM: *in vitro* maturation; IVF: *in vitro* fertilization.

expressions during PGCs formation *in vivo*. The results showed that the H3K9me2 and 5mC levels were increased during ESCs differetiating into EpiLCs whereas they decreased significantly during EpiLCs differentiating into PGCLCs. However, the H3K27me3 level was decreased during ESCs differetiating into EpiLCs and was increased during EpiLCs differetiating into PGCLCs. These dynamic regulations are analogous to that of *in vivo* PGC differentiation. Afterward, mouse PGCLCs formed a "reconstituted ovary" through being aggregated with gonadal somatic cells; then, the "reconstituted ovary" was transplanted to the infertile mouse ovarian bursa [4]. The "reconstituted ovary" simulated the female GCs internal milieu *in vivo* and underwent first meiotic division and generated fully grown GV oocytes. These GV oocytes have multiple layers of granulosa and theca cells similar to the fully grown recipient follicles *in vivo*. GV oocytes then underwent *in vitro* maturation (IVM) to be matured into MII oocytes, which could be fertilized through *in vitro* fertilization (IVF) and obtain healthy fertile offspring that bring normal imprinting pattern [4]. Therefore, this was a remarkable achievement in female GCs development from PSCs *in vitro*. However, in this study, reconstituted PGCLCs were transplanted to the infertile mouse ovary bursa, which meant the ensuing oogenesis was not entirely completed *in vitro*. Therefore, researchers tried the first complete *in vitro* reconstitution of mammalian oogenesis from mouse PGCs in a culture system containing estrogen receptor antagonist [50]. Estrogen receptor antagonists improved normal secondary follicles that contain one single primary oocyte inside. *In vitro* oogenesis was completed following three processes including differentiation of primary oocytes through *in vitro* differentiation (IVD), growth of fully grown GV oocytes through *in vitro* growth (IVG), and maturation of MII oocytes through IVM. MII oocytes delivered healthy fertile offspring through IVF [50]. Afterward, mouse PGCLC induction and *in vitro* oogenesis from mouse PGCs referred to in the above studies were combined to reconstitute the whole process of mouse oocyte formation *in vitro*. PSCs were first differentiated to EpiLCs and generated PGCLCs in BMP4, LIF, SCF, and EGF conditions. Then, PGCLCs were aggregated into "reconstituted ovary" with E12.5 gonadal somatic cells and further generated MII oocytes through the IVD, IVG, and IVM process. These MII oocytes were fertilized with wild sperms *in vitro* and delivered healthy fertile offspring that have comparable weights, survival rates, fertility, and gene expression dynamics to wild types (Figure 3) [5]. Besides, the blastocyst from the fertilized PSCs-derived oocytes was evidenced to generate ESCs that could accomplish the whole female GCs generation. Thus, the mouse female germline cycle was established entirely *in vitro* from PSCs.

In the abovementioned milestone studies, "reconstituted ovary" containing gonadal somatic cells played a critical role in promoting differentiation into further stages [4, 5]. However, studies that induce female GCs without gonadal somatic cells are still useful in that they could reveal female GCs developmental mechanisms. Overexpression of germline-related genes also provided a distinct approach for female GCs induction. Transient overexpression of DAZL, which is essential for germ cell development and differentiation, could inhibit pluripotency genes NANOG expression and promote meiotic progression to oocyte-like cell formation [51]. Simultaneous overexpression of PRDM14 alone or of three germline genes BLIMP1, PRDM14, and TFAP2C could induce germline induction [52]. Overexpression of NANOG alone was also found to induce PGCs formation. In this study, NANOG was found to bind to PRDM14 and BLIMP1 enhancers, indicating NANOG functions upstream of both PRDM14 and BLIMP1 [53]. These TF-based inductions of the germline opened up new possibilities to generate female GCs without cytokines and elucidated the transcription networks more elaborately. Some other researchers expanded PGCLCs through cAMP signal stimulation [54]. Expanded PGCLCs maintained the characteristics of sexually uncommitted PGCs, after which sex differentiation was initiated with the presence of gonadal somatic cells *in vivo*. Then, BMP2 and RA synergistically further induced expanded PGCLCs into primary oocyte-like cells that expressed VASA and SCP3 comparable to E15.5 primary oocytes *in vivo* [54]. Thus, BMP and RA were demonstrated to synergically initiate sex determination without gonadal somatic cells [55]. It might be possible to extend the meiosis even further with extra cytokine exposure. These findings have provided a framework for sex differentiation and meiosis initiation.

As described above, mouse female GCs were recapitulated *in vitro* from PSCs using different approaches [51–53]. This demonstrated mouse PSCs act as an effective source for female GCs regeneration. The correct reconstruction of epigenetic reprogramming that occurred during female GCs formation has drawn attention recently. Considering both oocytes and gonadal somatic cells are originated from the fetal ovary, researchers assumed iPSCs derived from gonadal somatic cells may have germline epigenetic memory more analogous to oocytes than other somatic cell-derived iPSCs [56]. Previously, researchers assumed mouse iPSCs from mouse ovarian granulosa cells could spontaneously differentiate into cells expressing oocyte markers in a higher incidence [57]. Recently, researchers achieved granulosa cell-derived iPSCs with a high germline competency through a chemical approach containing crotonic sodium. These iPSCs were induced into PGCLCs following EpiLCs formation; then, PGCLCs formed the "reconstituted ovary" with E12.5 gonadal somatic cells. PGCLCs underwent normal meiosis and formed GV oocytes that could produce healthy fertile offspring after IVM and IVF treatment. Additionally, the "reconstituted ovary" exhibited endocrine functions, including FSH, E2, and AMH secretion. Thus, this study generated oocytes from germline-derived iPSCs [58]. These improvements provided new iPSCs sources and induction methods for stem cell-derived oocytes.

Collectively, through two decades of efforts, researchers have achieved healthy fertile offspring from MII oocytes induced from mouse PSCs. Both genetic manipulations through overexpressing related genes and environment modification strategies using gonadal somatic cells were successful in generating mouse female GCs from PSCs. The environment modification strategy was mostly welcomed since it could simulate *in vivo* environment [57]. Generating

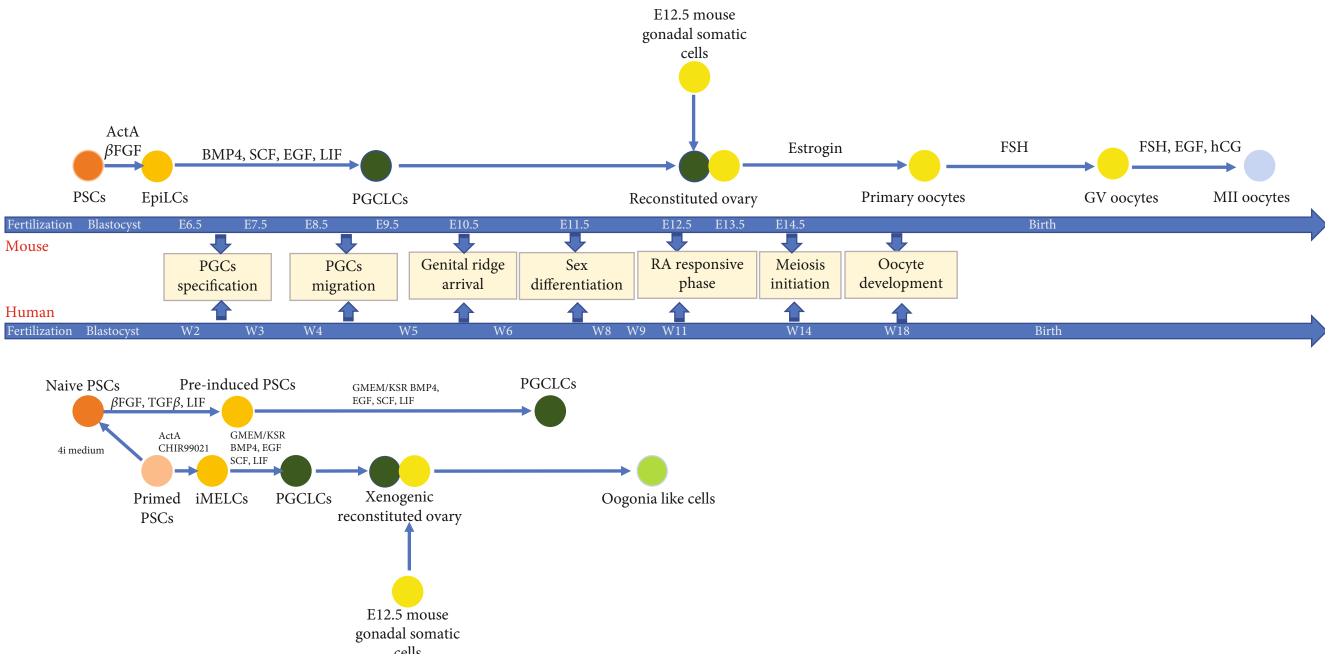

FIGURE 3: Schematic of the reconstitution of mouse and human female germ cells *in vitro*. Mouse and human female germ cell inductions are described in the upper and lower panel. *In vitro*-induced cells are represented with coloured circles.

PGCLCs from PSCs through EpiLCs and subsequently combining the PGCLCs with gonadal somatic cells have been accepted as the most effective protocol for mouse female GCs induction [5, 41, 59]. More expanded studies about induction details had been investigated using this induction protocol and revealed further understandings about the genesis mechanism of female GCs, which in turn contributed to improving the culture system and induction efficiency [54, 55, 58, 60].

3.2. Female GCs Induction from Human PSCs In Vitro. Mouse PSCs-based female GCs induction lay the foundation for human female GCs generation *in vitro* (Table 2). After female GCs induction from mouse ESCs in 2003 [40], researchers detected that human ESCs also could be spontaneously differentiated into EBs in suspension culture and generate putative female GCs that express VASA and SCP3 as well as oocyte marker GDF9. This indicated that human ESCs could spontaneously enter the female germline and undergo meiosis [61]. To promote differentiation, researchers added BMP4 in the differentiation culture and found it could increase the induction efficiency and expressions of VASA and SCP3 compared with spontaneous differentiation [62]. RA supplementation also could enhance human ESCs induced into the oocyte and primordial FLCs that possess similar cellular morphology with *in vivo* counterparts [63]. However, the zona pellucida matrix was not detected in these original studies. The addition of gonadal somatic cells was assumed to promote female GCs induction. When human iPSCs and ESCs were induced with gonadal somatic cells at the initial phase, PGCs expressed increased C-KIT, SSEA1, and VASA [12]. Even though these abovementioned researches generated female GCs that express GCs markers, these studies also displayed lower induction efficiency and insufficient characterization of the generated cells [64, 65].

In mice, overexpression of GCs-specific genes without cytokines provided a new approach for mouse female GCs induction [51, 52]. Therefore, researchers also overexpressed GCs-specific genes in human PSCs to enhance induction efficiency [66–69]. Overexpression of DAZ, DAZL, and BOULE promoted meiosis initiation and formed later stage female GCs that express SCP3 [66–68]. Additionally, the STELLA overexpression with RA induction led to VASA upregulation [69]. However, human PSCs induction efficiency is closely correlated with their pluripotency state [70]. Researchers found conventional human PSCs exhibit primed pluripotency [70] and bear properties more similar to mouse postimplantation and epiblast-derived stem cells (EpiSCs) [71, 72], which essentially lack competence for female GCs fate. Naive human PSCs are prone to response for germline specification signals and possess higher induction efficiency compared to primed human PSCs [70]. If human primed PSCs could be transformed into naive PSCs, mouse PGCLCs induction methods could be directly applied to human PSCs. 4i medium (MAPK, GSK3, p38, and JNK inhibitors) facilitate primed state human PSCs transferred into the naive state [73]. Following mouse PGCLCs methods, naive state human PSCs were preinduced with TGFβ, βFGF, and LIF for 2 days, then achieved human PGCLCs under BMP2/4, LIF, SCF, and EGF conditions for 8 days (Figure 3) [7]. Thus, a robust approach for human PGCLCs was established. In another study, primed human iPSCs were cultured under a feeder-free condition with βFGF, then stimulated by ActA and a WNT signaling agonist (CHIR99021) for 2 days. The obtained cells expressed pluripotency and mesoderm genes,

Table 2: Human female GCs differentiation from PSCs *in vitro*.

Cell types	Main induction methods	Achievements: Generated cells	Achievements: Characterization of generated cells	Journal, year (reference)
ESCs	Spontaneous differentiation Suspension culture	Oocyte-like cells	Morphology Marker expressions (SCP1, SCP3, and GDF9)	Hum Mol Genet, 2004 [61]
ESCs	BMP4 Suspension culture	PGCs	Morphology Marker expressions (VASA and SCP3)	Stem cells dev, 2006 [62]
VSELs	Spontaneous differentiation Suspension culture	Oocyte-like cells	Morphology Marker expressions (C-KIT, VASA, and ZP2)	Differentiation, 2008 [78]
ESCs iPSCs	Coculture with fetal gonadal cells Adherent culture	PGCs	Morphology Marker expressions (DAZL, VASA, and SSEA1)	Stem Cells, 2009 [12]
ESCs	VASA overexpression BMP4, BMP7, and BMP8b Adherent culture	PGCs	Morphology Marker expressions (DAZL, VASA, and SCP3) Epigenetic analysis	Nature, 2009 [66]
ESCs	RA Suspension culture	Oocyte-like cells	Morphology Marker expressions (SSEA1, DAZL, and VASA)	Hum Repro, 2009 [63]
iPSCs	Overexpression of DAZL and BOULE BMP4, BMP7, and BMP8b Adherent culture	PGCs	Morphology Marker expressions (STELLA and DMC1) Elongated SC formation	Human Mol Genet, 2011 [68]
VSELs	Spontaneous differentiation Adherent culture	Oocyte-like cells	Morphology Marker expressions (DAZL, ZP4, and GDF9)	Stem Cells Dev, 2011 [79]
ESCs iPSCs	VASA overexpression Adherent culture	PGCs Postmeiotic GCs	Morphology Marker expressions (GCNF, LHR, and ZP2) SCP formation analysis Epigenetic analysis	Stem Cells, 2012 [67]
ESCs	STELLA overexpression RA Adherent culture	PGCs	Marker expressions (VASA, SCP3, and SOX17)	PloS one, 2013 [69]
ESCs iPSCs	(i) 4i (MAPK, GSK3, P38, and JNK inhibitors), LIF, TGFβ, and bFGF Adherent culture (ii) BMP2/4, LIF, SCF, and EGF Suspension culture	PGCLCs	Morphology Marker expressions (BLIMP1 and STELLA) Global transcription profiles Epigenetic analysis	Cell, 2015 [7]
iPSCs	(i) ActA and GSK3b inhibitor Adherent culture (ii) GMEM/KSR, BMP4, LIF, SCF, and EGF Suspension culture	iMeLCs PGCLCs	Morphology Marker expressions (PRDM14 andSOX17) Global transcription profiles Epigenetic analysis	Cell stem cell, 2015 [6]
ESCs iPSCs	(i) ActA, bFGF, and BMP4 (5 ng/ml) Adherent culture (ii) Lif and BMP4 (100 ng/ml) Suspension culture	Mesodermal-like cells PGCLCs	Morphology Marker expressions (BLIMP1 and STELLA) global transcription profiles Epigenetic analysis	EMBO J, 2015 [36]
ESCs	Overexpression of DAZL and BOULE GDF9 and BMP15 Adherent culture	FLCs	Morphology Marker expressions (ZP2, NOBOX, and AMH) Global transcription profiles Estradiol secretion	Nat commun, 2017 [74]
VSELs	Follicular fluid "serum" medium Adherent culture	Oocyte-like cells	Morphology Marker expression (ZP1-3)	Stem Cell Rev Rep, 2018 [80]
iPSCs	Coculture with mouse gonadal cells Suspension culture	PGCLCs Oogonia-like cells	Morphology Marker expressions (SCP3, REC8 and STRA8) Transcriptome dynamics Epigenetic analysis X chromosome activity	Science, 2018 [8]

ESCs: embryonic stem cells; iPSCs: induced pluripotent stem cells; VSELs: very small embryonic-like stem cells; iMeLCs: incipient mesoderm-like cells; PGCs: primordial germ cells; PGCLCs: primordial germ cell-like cells; FLCs: follicle-like cells; SCP: synaptonemal complex protein.

indicating that they were corresponding to incipient mesoderm-like cells (iMeLCs). Then, iMeLCs were cultured under the GMEM/KSR, BMP4, LIF, SCF, and EGF conditions for 4 days and generated human PGCLCs that correspond to Wk7 human PGCs *in vivo* (Figure 3) [6]. Another team also achieved human PGCLCs differentiation from human PSCs almost at the same time in a concentration-dependent manner. They induced human PSCs into mesodermal-like cells with ActA, βFGF, and a low concentration (5 ng/ml) of BMP4, then generated mesodermal-like cells differentiated to human PGCLCs with a high concentration (100 ng/ml) of BMP4 [36]. Thus, the successful derivation of human PGCLCs *in vitro* enabled researchers to reveal more female GCs differentiation mechanism to reestablish them *in vitro*.

Further induction of mouse PGCLCs was continued with the presence of E12.5 gonadal somatic cells; however, human gonadal somatic cells are hard to be acquired from early embryos. Therefore, an alternative approach that does not need the human embryonic gonadal somatic cells was required to enhance *in vitro* differentiation. Overexpression of DAZL and BOULE enabled human ESCs to exit the pluripotent state and enter meiosis. Then, the subsequent addition of GDF9 and BMP15 enhanced the FLCs induction that expresses ZP2 and NOBOX [74]. Thus, they provided a significant new model for generating FLCs from human ESCs without gonadal somatic cells. However, to establish human female GCs *in vitro*, gonadal somatic cells are indispensable considering *in vivo* female GCs development. Considering the restrictions on human embryonic gonadal somatic cell acquisition, in a recent study, researchers substituted human embryonic gonadal somatic cells with that of mice [75]. Human PGCLCs were aggregated with mouse gonadal somatic cells to form a "xenogenic reconstituted ovary." In the "xenogenic reconstituted ovary," human PGCLCs were induced for 121 days (Figure 3) [8]. In the generated cells, early PGC genes BLIMP1, TFAP2C, SOX17, and NANOS3 were downregulated; DAZL, VASA, and RA responsive genes STR8 and SCP3 were further upregulated, whereas key meiosis genes DMC1, γH2AX, or SCP1 were not adequately upregulated. Therefore, these generated cells in the "xenogenic reconstituted ovary" were corresponding to RA-responsive female GCs and oogonia, indicating that these cells were in a state corresponding to meiotic entry signals but not yet initiated meiotic recombination. Additionally, these oogonia-like cells expressed similar DNA demethylation and imprint erasure characteristics with oogonia at Wk10 *in vivo*. These results indicated that mouse gonadal somatic cells had provided a suitable environment for human PGCLCs to enter sex differentiation. However, human PGCLCs did not enter meiosis after cultivation up to 121 days, during which human PGCs would have completed meiosis I *in vivo* [1]. This might be because the signals generated from mouse gonadal somatic cells are inadequate to initiate meiosis. Theoretically, human PSC-induced human gonadal somatic cells would be an alternative to human fetal gonadal somatic cells and could further enhance human PGCLCs to postmeiotic phase. In previous studies, human granulosa cells that induce from human iPSCs were transplanted into POF mouse ovaries. They were found to improve ovarian maturation and enhance follicular growth through hormone secretion [76]. Recently, other researchers also derived granulosa cells from human iPSCs through EB formation, and these granulosa cells also contribute to estradiol synthesis *in vitro* [77]. Next, whether these human iPSCs-derived granulosa cells could serve as human gonadal somatic cells and aggregate with PGCLCs to prompt further differentiation and support oocyte formation needs to be investigated.

Remarkably, in recent years, ovarian-related pluripotent stem cells have been discovered in the ovary surface epithelium. Initially, small round cells with diameters from 2 to 4 μm were derived from the ovary surface epithelium of women who had no natural oocytes and follicles. These cells expressed early embryonic markers SSEA4, OCT4, NANOG, SOX2, and C-KIT and possessed a robust proliferation ability. Therefore, they were named as very small embryonic-like stem cells (VSELs) and considered as new stem cell sources for oocytes. These VSELs could be differentiated into oocyte-like cells with diameters of 80–95 μm at day 20, which is comparable to human oocytes that could be used to fertilize. They also expressed VASA and ZP2 and even formed a zona pellucida-like structure. However, meiotic marker SCP3 was not detected in these cells, indicating that they were immature compared with their *in vivo* counterparts [78]. Afterward, another study also established the VSELs in menopausal women ovaries, and these VSELs were evidenced to spontaneously differentiate into oocyte-like cells with zona pellucida-like structures and protrude polar body-like structures. However, the fertilization functionality of these oocyte-like cells had not been tested [79]. Recently, a study showed oocyte-like cells from premature ovarian failure patients' VSELs. These cells exhibited zona pellucida-like structures and could react to sperm. In turn, the sperm could recognize the oocyte-like cells and bound to them strongly. However, these oocyte-like cells did not express ZP1 and ZP2 in spite of the presence of zona pellucida-like structures. Therefore, regardless of the reaction to sperms, these oocyte-like cells could not be a substitute for fully functional oocytes *in vivo* yet [80]. Further precise investigations are still needed to achieve more matured functional oocytes from VSELs.

In summary, similar to mouse PGCLCs, human oogonia-like cells have been successfully achieved through "xenogenic reconstituted ovary" from iPSCs [8]. The multistage systemic protocols for human PGCLCs generation are the remarkable methods in this field over these years [6, 7]. VSELs that contributed to sperm reactive oocyte-like cells have provided a new prospect for functional oocyte formation. Even though fully functional oocytes for clinical researches are still at a distance, these attempts and improvements have provided accessible approaches to study female GCs-specific genes, PGCs migration pathway, sex differentiation, and meiotic initiation. Now, highly efficient and reproducible protocols for PGCLCs differentiation into genetically and epigenetically healthy, patient-specific oocytes are in demand.

4. Current Challenges and Future Perspectives

Mouse and human female GCs induction *in vitro* from PSCs achieved significant improvements. It gave us perspectives when they also aroused some challenges in PSCs sources, female GCs development progression, induction culture conditions, and ethical issues.

Firstly, a key issue to be investigated is the stem cell characteristics which are associated with the robustness of induction. ESCs and iPSCs both have the competence for female GCs reconstitutions *in vitro*. Especially, iPSCs are more welcomed because of less harmful access and less immune rejection [10]. Researchers demonstrated that different iPSC lines derived from distinct cell types possess different female GCs fate competency [81]. After the researchers demonstrated mouse oocytes from granulosa cell-derived iPSCs possess a higher germline competency than other cell lines, the certain human granulosa cells discarded after IVF were also considered as a more permissive cell source for iPSCs to generate oocytes [58]. Human iPSCs could provide patient-specific PSCs which could be used to investigate disease-specific pathogenesis *in vitro* [26, 82, 83]. Recently, a study established human iPSCs in 4i medium from patients with premature ovarian insufficiency. Patient-specific iPSCs were preinduced with βFGF and TGF for 4 days; then, unlike previous studies [7], the DNA methyltransferase inhibitor was added on day 5. Then, generated cells were further induced into human PGCLCs with BMP2/4, LIF, SCF, EGF, and GMEM/KSR supplement. Compared to the previous human PGCLCs induction methods, the addition of DNA methyltransferase inhibitor enhanced human PGCLCs induction. Thus, they provided a complementary way for human PGC differentiation from patient-specific iPSCs [82]. Furthermore, the PSCs pluripotent state was also considered as an important factor during induction. Previously, human-primed PSCs were maintained in a 4i medium for 2 weeks to obtain naive pluripotency, but studies showed naive human PSCs maintained in the 4i medium for a prolonged time had chromosomal instability and structural anomalies [84]. When researchers cultured human naive PSCs in 4i medium for 3 days instead of 2 weeks, they gained more stable human naive PSCs that could be induced into PGCLCs through EB formation with a high yield in 13 days [85]. Therefore, the efforts on coordinating human PSCs pluripotency state to establish more stable PGCLCs are also an important issue on GCs induction.

Secondly, female GCs development progression has not been clearly revealed yet. The mechanisms underlying female GCs differentiation after Wk3 have been acquired largely; however, the investigations of early embryos before Wk2 remained inadequate for a long time. Recently, a genome-wide DNA methylation map during human preimplantation development was revealed by single-cell chromatin overall omic-scale landscape sequencing in human preimplantation embryos [86]. This gives us a hint about the human PGCs origin before Wk2. Single-cell RNA-seq technology which was recently used to analyze transcriptomic mechanisms among different stage spermatids could be used on PGCs to further analyze PGCs migration, proliferation, and differentiation [87]. Furthermore, researchers used single-cell transcriptome and epigenome sequencing technologies and divided female fetal GCs into three sequential differentiation stages, including the RA responsive stage, the meiotic prophase stage, and the primordial follicle stage. Different stages correspond to distinct gene expressions and epigenetic regulations [26]. These distinct epigenetic regulatory networks of female GCs at sequential developmental phases could be studied through the genome-wide DNA methylation and chromatin accessibility using single-cell resolution [88]. These efforts on female GCs development mechanisms would contribute to a more efficient and stable female GC induction *in vitro*.

Thirdly, the culture condition would also affect the survival of female GCs. Although human PGCLCs had been recapitulated *in vitro*, U-bottom 96 plates or other similar plates used in these studies limited the scale production of human PGCLCs production [6–8]. Recently, a new modified system of methylcellulose-based 3D induction system combined with low-cell attachment plates was reported to produce human PGCLCs from human PSCs at a large scale, with similar gene expression and epigenetic modification profiles to human PGCs [88]. Besides the 3D induction system, 3D bioprosthetic ovaries were also confirmed to provide 3D support for oocyte cultivation. Pore geometry of 3D-printed microporous hydrogel scaffold affected the mouse ovarian follicle survival through the intrafollicular signaling and the ovarian microenvironment [89]. When a 3D-printed scaffold with ovarian follicles was transplanted to a surgically sterilized mouse, they could give birth to healthy fertile offspring. In the next step, whether a 3D-printed ovary could provide an environment more analogous to *in vivo* ovarian microenvironment for PSCs induction needs to be investigated in the future.

Finally, the ethical issues of reproductive medicine have always attracted attention from the scientific community and the public. The establishment of iPSCs has eliminated the concerns about embryo destruction [10], and there are no serious abnormalities in the offspring from mouse PSCs [5]. However, when it comes to the human female GCs induction, concerns about stem cell sources, technology safety, the clinical application of generated cells, and the epigenetic regulation of offspring still exist widely.

Although complete oocytes from human PSCs have not been achieved in a dish yet, it may theoretically possible to integrate the existing methods such as human PGCLCs induction, granulosa cell induction from iPSCs, GV oocyte formation, IVM treatments to form MII oocytes *in vitro*. If it is possible, this would create a great promise for understanding the complex biological process of oocyte development, also would provide a unique cell model for infertility-related drug testing, and even become a more plausible prospect for treating infertility.

Conflicts of Interest

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

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References

[1] K. Makar and K. Sasaki, "Roadmap of germline development and in vitro gametogenesis from pluripotent stem cells," *Andrology*, vol. 8, no. 4, pp. 842–851, 2019.

[2] M. Vermeulen, M.-G. Giudice, F. Del Vento, and C. Wyns, "Role of stem cells in fertility preservation: current insights," *Stem Cells Cloning*, vol. 12, pp. 27–48, 2019.

[3] D. Rodriguez Gutierrez and A. Biason-Lauber, "Pluripotent cell models for gonadal research," *International Journal of Molecular Sciences*, vol. 20, no. 21, p. 5495, 2019.

[4] K. Hayashi, S. Ogushi, K. Kurimoto, S. Shimamoto, H. Ohta, and M. Saitou, "Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice," *Science*, vol. 338, no. 6109, pp. 971–975, 2012.

[5] O. Hikabe, N. Hamazaki, G. Nagamatsu et al., "Reconstitution _in vitro_ of the entire cycle of the mouse female germ line," *Nature*, vol. 539, no. 7628, pp. 299–303, 2016.

[6] K. Sasaki, S. Yokobayashi, T. Nakamura et al., "Robust in vitro induction of human germ cell fate from pluripotent stem cells," *Cell Stem Cell*, vol. 17, no. 2, pp. 178–194, 2015.

[7] N. Irie, L. Weinberger, W. W. C. Tang et al., "SOX17 is a critical specifier of human primordial germ cell fate," *Cell*, vol. 160, no. 1-2, pp. 253–268, 2015.

[8] C. Yamashiro, K. Sasaki, Y. Yabuta et al., "Generation of human oogonia from induced pluripotent stem cells in vitro," *Science*, vol. 362, no. 6412, pp. 356–360, 2018.

[9] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[10] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[11] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells," *Nature*, vol. 448, no. 7151, pp. 313–317, 2007.

[12] T. S. Park, Z. Galic, A. E. Conway et al., "Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells," *Stem Cells*, vol. 27, no. 4, pp. 783–795, 2009.

[13] M. Ginsburg, M. H. Snow, and A. McLaren, "Primordial germ cells in the mouse embryo during gastrulation," *Development*, vol. 110, no. 2, pp. 521–528, 1990.

[14] C. M. Spiller and J. Bowles, "Sex determination in mammalian germ cells," *Asian Journal of Andrology*, vol. 17, no. 3, pp. 427–432, 2015.

[15] M. Saitou and H. Miyauchi, "Gametogenesis from pluripotent stem cells," *Cell Stem Cell*, vol. 18, no. 6, pp. 721–735, 2016.

[16] U. C. Sarma, J. K. Findlay, and K. J. Hutt, "Oocytes from stem cells," *Best Practice & Research Clinical Obstetrics & Gynaecology*, vol. 55, pp. 14–22, 2019.

[17] D. Chen, J. J. Gell, Y. Tao, E. Sosa, and A. T. Clark, "Modeling human infertility with pluripotent stem cells," *Stem Cell Research*, vol. 21, pp. 187–192, 2017.

[18] T. Kobayashi and M. A. Surani, "On the origin of the human germline," *Development*, vol. 145, no. 16, article dev150433, 2018.

[19] K. Kurimoto and M. Saitou, "Mechanism and reconstitution in vitro of germ cell development in mammals," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 80, pp. 147–154, 2016.

[20] K. Tilgner, S. P. Atkinson, A. Golebiewska, M. Stojković, M. Lako, and L. Armstrong, "Isolation of primordial germ cells from differentiating human embryonic stem cells," *Stem cells (Dayton, Ohio)*, vol. 26, no. 12, pp. 3075–3085, 2008.

[21] F. Campolo, M. Gori, R. Favaro et al., "Essential role of Sox2 for the establishment and maintenance of the germ cell line," *Stem Cells*, vol. 31, no. 7, pp. 1408–1421, 2013.

[22] T. Endo, M. M. Mikedis, P. K. Nicholls, D. C. Page, and D. G. de Rooij, "Retinoic acid and germ cell development in the ovary and testis," *Biomolecules*, vol. 9, no. 12, p. 775, 2019.

[23] W. Felix, *Die entwicklung der harn- und geschlechtsorgane*, Hirzel, 1911.

[24] A. Fuss, "Ueber extraregionare geschlechtszellen bei einem menshlichen embryo von 4 wochen," *Anatomischer Anzeiger*, vol. 39, pp. 407–409, 1911.

[25] W. W. C. Tang, S. Dietmann, N. Irie et al., "A unique gene regulatory network resets the human germline epigenome for development," *Cell*, vol. 161, no. 6, pp. 1453–1467, 2015.

[26] L. Wen and F. Tang, "Human germline cell development: from the perspective of single-cell sequencing," *Molecular Cell*, vol. 76, no. 2, pp. 320–328, 2019.

[27] G. Politzer, "Über einen menschlichen embryo mit sieben Urwirbelpaaren," *Zeitschrift für Anatomie und Entwicklungsgeschichte*, vol. 93, no. 3-4, pp. 386–428, 1930.

[28] E. Witschi, "Migration of the germ cells of human embryos from the yolk sac to the primitive gonadal folds," *Contributions to Embryology*, vol. 32, pp. 67–80, 1948.

[29] W. W. C. Tang, T. Kobayashi, N. Irie, S. Dietmann, and M. A. Surani, "Specification and epigenetic programming of the human germ line," *Nature Reviews Genetics*, vol. 17, no. 10, pp. 585–600, 2016.

[30] S. Tomaselli, F. Megiorni, L. Lin et al., "Human RSPO1/R-spondin1 is expressed during early ovary development and augments β-Catenin signaling," *PLoS One*, vol. 6, no. 1, article e16366, 2011.

[31] L. Li, J. Dong, L. Yan et al., "Single-cell RNA-Seq analysis maps development of human germline cells and gonadal niche interactions," *Cell Stem Cell*, vol. 20, no. 6, pp. 858–873.e4, 2017, e4.

[32] J. M. Stringer and P. S. Western, "A step toward making human oocytes," *Nat Biotechnol*, vol. 37, no. 1, pp. 24-25, 2019.

[33] J. K. Findlay, K. J. Hutt, M. Hickey, and R. A. Anderson, "What is the "ovarian reserve"?," *Fertility and Sterility*, vol. 103, no. 3, pp. 628–630, 2015.

[34] Y. Kojima, K. Sasaki, S. Yokobayashi et al., "Evolutionarily distinctive transcriptional and signaling programs drive human germ cell lineage specification from pluripotent stem cells," *Cell Stem Cell*, vol. 21, no. 4, pp. 517–532.e5, 2017, e5.

[35] J. A. Hackett, T. Kobayashi, S. Dietmann, and M. A. Surani, "Activation of lineage regulators and transposable elements across a pluripotent spectrum," *Stem Cell Reports*, vol. 8, no. 6, pp. 1645–1658, 2017.

[36] F. Sugawa, M. J. Araúzo-Bravo, J. Yoon et al., "Human primordial germ cell commitment in vitro associates with a unique PRDM14 expression profile," *The EMBO Journal*, vol. 34, no. 8, pp. 1009–1024, 2015.

[37] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, no. 5819, pp. 154–156, 1981.

[38] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro et al., "Embryonic stem cell lines derived from human blastocysts," *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[39] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, I. I. Slukvin, and J. A. Thomson, Eds., "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[40] K. Hubner, "Derivation of oocytes from mouse embryonic stem cells," *Science*, vol. 300, no. 5623, pp. 1251–1256, 2003.

[41] K. Hayashi, "In vitro reconstitution of germ cell development," *Biology of Reproduction*, vol. 101, no. 3, pp. 567–578, 2019.

[42] O. Lacham-Kaplan, H. Chy, and A. Trounson, "Testicular cell conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes," *Stem Cells*, vol. 24, no. 2, pp. 266–273, 2006.

[43] T. Qing, Y. Shi, H. Qin et al., "Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells," *Differentiation*, vol. 75, no. 10, pp. 902–911, 2007.

[44] A. E. Baltus, D. B. Menke, Y.-C. Hu et al., "In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication," *Nature Genetics*, vol. 38, no. 12, pp. 1430–1434, 2006.

[45] A. Kerkis, S. A. S. Fonseca, R. C. Serafim et al., "In vitro differentiation of male mouse embryonic stem cells into both presumptive sperm cells and oocytes," *Cloning Stem Cells*, vol. 9, no. 4, pp. 535–548, 2007.

[46] X.-y. Zhao, W. Li, Z. Lv et al., "iPS cells produce viable mice through tetraploid complementation," *Nature*, vol. 461, no. 7260, pp. 86–90, 2009.

[47] M. Imamura, T. Aoi, A. Tokumasu et al., "Induction of primordial germ cells from mouse induced pluripotent stem cells derived from adult hepatocytes," *Molecular Reproduction and Development*, vol. 77, no. 9, pp. 802–811, 2010.

[48] Q.-L. Ying, J. Wray, J. Nichols et al., "The ground state of embryonic stem cell self-renewal," *Nature*, vol. 453, no. 7194, pp. 519–523, 2008.

[49] K. Hayashi, H. Ohta, K. Kurimoto, S. Aramaki, and M. Saitou, "Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells," *Cell*, vol. 146, no. 4, pp. 519–532, 2011.

[50] K. Morohaku, R. Tanimoto, K. Sasaki et al., "Complete in vitro generation of fertile oocytes from mouse primordial germ cells," *Proceedings of the National Academy of Sciences*, vol. 113, no. 32, pp. 9021–9026, 2016.

[51] Z. Yu, P. Ji, J. Cao et al., "Dazl promotes germ cell differentiation from embryonic stem cells," *Journal of Molecular Cell Biology*, vol. 1, no. 2, pp. 93–103, 2009.

[52] F. Nakaki, K. Hayashi, H. Ohta, K. Kurimoto, Y. Yabuta, and M. Saitou, "Induction of mouse germ-cell fate by transcription factors _in vitro_," *Nature*, vol. 501, no. 7466, pp. 222–226, 2013.

[53] K. Murakami, U. Günesdogan, J. J. Zylicz et al., "NANOG alone induces germ cells in primed epiblast in vitro by activation of enhancers," *Nature*, vol. 529, no. 7586, pp. 403–407, 2016.

[54] H. Ohta, K. Kurimoto, I. Okamoto et al., "In vitroexpansion of mouse primordial germ cell-like cells recapitulates an epigenetic blank slate," *The EMBO Journal*, vol. 36, no. 13, pp. 1888–1907, 2017.

[55] H. Miyauchi, H. Ohta, S. Nagaoka et al., "Bone morphogenetic protein and retinoic acid synergistically specify female germ-cell fate in mice," *The EMBO Journal*, vol. 36, no. 21, pp. 3100–3119, 2017.

[56] R. Anchan, B. Gerami-Naini, J. S. Lindsey et al., "Efficient differentiation of steroidogenic and germ-like cells from epigenetically-related iPSCs derived from ovarian granulosa cells," *PLoS One*, vol. 10, no. 3, article e0119275, 2015.

[57] A. Mouka, G. Tachdjian, J. Dupont, L. Drévillon, and L. Tosca, "In vitro gamete differentiation from pluripotent stem cells as a promising therapy for infertility," *Stem Cells and Development*, vol. 25, no. 7, pp. 509–521, 2016.

[58] C. Tian, L. Liu, X. Ye et al., "Functional oocytes derived from granulosa cells," *Cell Reports*, vol. 29, no. 13, pp. 4256–4267.e9, 2019.

[59] N. Irie, A. Sybirna, and M. A. Surani, "What can stem cell models tell us about human germ cell biology?," *Current Topics in Developmental Biology*, vol. 129, pp. 25–65, 2018.

[60] K. Kurimoto and M. Saitou, "Epigenome regulation during germ cell specification and development from pluripotent stem cells," *Current Opinion in Genetics & Development*, vol. 52, pp. 57–64, 2018.

[61] A. T. Clark, M. S. Bodnar, M. Fox et al., "Spontaneous differentiation of germ cells from human embryonic stem cells in vitro," *Human Molecular Genetics*, vol. 13, no. 7, pp. 727–739, 2004.

[62] K. Kee, J. M. Gonsalves, A. T. Clark, and R. A. Reijo Pera, "Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells," *Stem Cells And Development*, vol. 15, no. 6, pp. 831–837, 2006.

[63] B. Aflatoonian, L. Ruban, M. Jones, R. Aflatoonian, A. Fazeli, and H. D. Moore, "In vitro post-meiotic germ cell development from human embryonic stem cells," *Human Reproduction*, vol. 24, no. 12, pp. 3150–3159, 2009.

[64] F. D. West, D. W. Machacek, N. L. Boyd, K. Pandiyan, K. R. Robbins, and S. L. Stice, "Enrichment and differentiation of human germ-like cells mediated by feeder cells and basic fibroblast growth factor signaling," *Stem Cells*, vol. 26, no. 11, pp. 2768–2776, 2008.

[65] F. D. West, M. I. Roche-Rios, S. Abraham et al., "KIT ligand and bone morphogenetic protein signaling enhances human embryonic stem cell to germ-like cell differentiation," *Human Reproduction*, vol. 25, no. 1, pp. 168–178, 2009.

[66] K. Kee, V. T. Angeles, M. Flores, H. N. Nguyen, and R. A. Reijo Pera, "Human _DAZL, DAZ_ and _BOULE_ genes modulate primordial germ-cell and haploid gamete formation," *Nature*, vol. 462, no. 7270, pp. 222–225, 2009.

[67] J. V. Medrano, C. Ramathal, H. N. Nguyen, C. Simon, and R. A. Reijo Pera, "Divergent RNA-binding proteins, DAZL

and VASA, induce meiotic progression in human germ cells derived in vitro," *Stem Cells*, vol. 30, no. 3, pp. 441–451, 2012.

[68] S. Panula, J. V. Medrano, K. Kee et al., "Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells," *Human Molecular Genetics*, vol. 20, no. 4, pp. 752–762, 2011.

[69] P. Wongtrakoongate, M. Jones, P. J. Gokhale, and P. W. Andrews, "STELLA facilitates differentiation of germ cell and endodermal lineages of human embryonic stem cells," *PloS one*, vol. 8, no. 2, article e56893, 2013.

[70] J. Nichols and A. Smith, "Naive and primed pluripotent states," *Cell Stem Cell*, vol. 4, no. 6, pp. 487–492, 2009.

[71] I. G. M. Brons, L. E. Smithers, M. W. B. Trotter et al., "Derivation of pluripotent epiblast stem cells from mammalian embryos," *Nature*, vol. 448, no. 7150, pp. 191–195, 2007.

[72] P. J. Tesar, J. G. Chenoweth, F. A. Brook et al., "New cell lines from mouse epiblast share defining features with human embryonic stem cells," *Nature*, vol. 448, no. 7150, pp. 196–199, 2007.

[73] O. Gafni, L. Weinberger, A. A. Mansour et al., "Derivation of novel human ground state naive pluripotent stem cells," *Nature*, vol. 504, no. 7479, pp. 282–286, 2013.

[74] D. Jung, J. Xiong, M. Ye et al., "_In vitro_ differentiation of human embryonic stem cells into ovarian follicle-like cells," *Nature Communications*, vol. 8, no. 1, article 15680, 2017.

[75] H.-F. Chen, P.-S. Jan, H.-C. Kuo et al., "Granulosa cells and retinoic acid co-treatment enrich potential germ cells from manually selected Oct4-EGFP expressing human embryonic stem cells," *Reproductive BioMedicine Online*, vol. 29, no. 3, pp. 319–332, 2014.

[76] Q. L. Te Liu, S. Wang, C. Chen, and J. Zheng, "Transplantation of ovarian granulosa-like cells derived from human induced pluripotent stem cells for the treatment of murine premature ovarian failure," *Molecular Medicine Reports*, vol. 13, no. 6, pp. 5053–5058, 2016.

[77] S. Lipskind, J. S. Lindsey, B. Gerami-Naini et al., "An embryonic and induced pluripotent stem cell model for ovarian granulosa cell development and steroidogenesis," *Reproductive Sciences*, vol. 25, no. 5, pp. 712–726, 2018.

[78] I. Virant-Klun, N. Zech, P. Rožman et al., "Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes," *Differentiation*, vol. 76, no. 8, pp. 843–856, 2008.

[79] S. Parte, D. Bhartiya, J. Telang et al., "Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary," *Stem Cells and Development*, vol. 20, no. 8, pp. 1451–1464, 2011.

[80] I. Virant-Klun, "Functional testing of primitive oocyte-like cells developed in ovarian surface epithelium cell culture from small VSEL-like stem cells: can they be fertilized one day?," *Stem Cell Reviews and Reports*, vol. 14, no. 5, pp. 715–721, 2018.

[81] S. Yokobayashi, K. Okita, M. Nakagawa et al., "Clonal variation of human induced pluripotent stem cells for induction into the germ cell fate," *Biology of Reproduction*, vol. 96, no. 6, pp. 1154–1166, 2017.

[82] S. Yang, S. Ding, S. He et al., "Differentiation of primordial germ cells from premature ovarian insufficiency-derived induced pluripotent stem cells," *Stem Cell Research & Therapy*, vol. 10, no. 1, p. 156, 2019.

[83] L. Leng, Y. Tan, F. Gong et al., "Differentiation of primordial germ cells from induced pluripotent stem cells of primary ovarian insufficiency," *Human Reproduction*, vol. 30, no. 3, pp. 737–748, 2015.

[84] L. Weinberger, M. Ayyash, N. Novershtern, and J. H. Hanna, "Dynamic stem cell states: naive to primed pluripotency in rodents and humans," *Nature Reviews Molecular Cell Biology*, vol. 17, no. 3, pp. 155–169, 2016.

[85] S. Mitsunaga, K. Shioda, K. J. Isselbacher, J. H. Hanna, and T. Shioda, "Generation of human primordial germ cell-like cells at the surface of embryoid bodies from primed-pluripotency induced pluripotent stem cells," *Journal of Visualized Experiments*, vol. 143, 2019.

[86] L. Li, F. Guo, Y. Gao et al., "Single-cell multi-omics sequencing of human early embryos," *Nature Cell Biology*, vol. 20, no. 7, pp. 847–858, 2018.

[87] Y. Chen, Y. Zheng, Y. Gao et al., "Single-cell RNA-seq uncovers dynamic processes and critical regulators in mouse spermatogenesis," *Cell Research*, vol. 28, no. 9, pp. 879–896, 2018.

[88] X. Wang, T. Liao, C. Wan et al., "Efficient generation of human primordial germ cell-like cells from pluripotent stem cells in a methylcellulose-based 3D system at large scale," *Peer J*, vol. 6, article e6143, 2019.

[89] M. M. Laronda, A. L. Rutz, S. Xiao et al., "A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice," *Nature Communications*, vol. 8, no. 1, article 15261, 2017.