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Advancements in cardiac regenerative therapy: Scalable human iPSC-derived cardiomyocyte differentiation and maturation

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Abstract

The demand for mass production of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) is escalating, driven by their potential to revolutionize cardiac regenerative therapies. The development of large-scale production protocols for iPSC-CMs is crucial to attain their therapeutic potential, enabling the treatment of millions of patients' worldwide suffering from cardiovascular diseases. However, the scalable production of iPSC-CMs hinges on overcoming several critical challenges, including cellular differentiation and maturation. In pursue of tackling these challenges, researchers are investigating novel strategies such as prolonged culture periods, mechanical stimulation, and co-culture with supporting cell types. Overcoming these challenges is essential for unlocking the full potential of iPSC-CMs and paving the way for a new era in cardiac regenerative medicine. In this review, following topics are covered: (1) improvement of differentiation and maturation of iPSC-CMs and (2) scalable production of iPSC-CMs.

Keywords: Induced pluripotent stem cell; Cardiac regenerative therapy; Cardiomyocyte; Stem cell maturation; Clinical translation

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1. Introduction

The human heart possesses a limited capacity for regeneration, characterized by a modest annual turnover rate of cardiomyocytes (CMs), which decreases with age, from approximately 1% at 25 years to 0.45% by 75 years, indicating a decline in regenerative potential over the lifespan.[1] The remarkable longevity of adult human CMs results in almost 50% being replaced over a 75-year lifespan, in stark contrast to the high mitotic and cytokinetic activity observed in infant CMs reaching 40%,[1] which drives cardiac growth and development, indicating a pronounced regenerative capacity in pediatric heart. Research investigations which incorporate findings based on animal model findings have consistently demonstrated that CMs undergo a developmental shift from a mononucleate to a binucleate state, concomitant with cell cycle withdrawal and

the loss of postnatal proliferative capacity.[2] In the context of prevalent cardiovascular diseases, such in myocardial infarction (MI), which results in the sudden loss of approximately one billion CMs, the inherent regenerative capacity is markedly insufficient. This limitation leads to structural repair through scar formation rather than functional repair through contractile restoration, potentially culminating in adverse ventricular remodeling and heart failure post-MI. Nevertheless, the discovery of adult CM repopulation suggests that native cell populations, both cardiac progenitor cells (CPCs) and existing CMs, may serve as viable sources for myocardial regeneration following injuries.[1]

Regenerative medicine requires a constant supply of cell sources, such as induced pluripotent stem cells (iPSCs), including those differentiated into CMs and other functional progenies. First founded in 2006 through reprogramming through the "Yamanaka Factors" (octamer-binding transcription factor 4 [OCT $^{4]}$, sex-determining region Y-box 2 [Sox $^{2]}$, Kruppel-like factor 4 [KLF$^{4]}$, and c-Myc protein; all abbreviated as OSKM), these somatic cells are collectively referred to as iPSCs, which can differentiate into all three embryonic germ layers.[3] As the research has been relying on the advancement of these iPSCs, their production should be resilient, financially feasible, and ultimately standardized by lab-scale protocols for iPSC expansion and cardiomyogenic differentiation toward more controlled processing in industry-compatible culture platforms. Here, advanced strategies for the cultivation and differentiation of iPSC will be reviewed by focusing on stirred bioreactor-based techniques for process-upscaling. The generation of CM mass from iPSCs could be enhanced by cardiovascular progenitor state, with equally important processes of cell fate control (differentiation and maturation). Finally, remaining challenges will be highlighted specifically regarding the adoption of three-dimensional (3D) iPSC suspension culture techniques for process-upscaling and critical safety issues ahead of clinical translation.

2. Differentiation and maturation of iPSC-CMs

2.1. Cardiac differentiation of iPSCs

The long-held notion that CMs are terminally differentiated, post-mitotic cells with negligible regenerative capacity after birth has recently been challenged. Bergmann *et al.* have provided evidence that CMs do indeed undergo renewal, although at a remarkably low rate. Their findings suggest that approximately 50% of CMs are replaced throughout an individual's lifetime, substantiating the concept of lifelong CM repopulation and overturning the previous understanding of CMs as a static cell population.[4]

Cardiac development is a complex, multidimensional process orchestrated by the sophisticated interplay of signaling pathways and gene transcriptional regulation. The quest for generating CMs has undergone a significant transformation. Formerly, embryonic stem cells were employed, but this approach was beset by ethical concerns. However, in 2006, Yamanaka pioneered an innovative method, harnessing iPSCs to reprogram terminally differentiated human fibroblasts,[5,6] obtained from skin or connective tissue biopsies, or human hair follicle keratinocyte, using four transcription factors (OSKM).[7-9] This reprogramming process yields iPSCs that can be further directed toward a cardiac fate through cardiac differentiation/induction, involving the activation of cardiac-specific transcription factors (T-box transcription factor 5 [TBX$^{5]}$, heart and neural crest derivatives expressed 1 [HAND1), GATA binding protein 4 [GATA$^{4]}$, and NK2 homeobox 5 [NKX2-$^{5]}$).[10,11] This process generates reprogrammed cardiac precursors, including CPCs, which have the ability to differentiate into multiple cardiac cell types, such as CMs, smooth muscle cells, and endothelial cells.

In iPSC-CM development, differentiation and maturation are two distinct processes that occur sequentially: (1) the process of differentiation refers to the process by which iPSCs are coaxed to undergo a series of molecular and cellular changes, acquiring a CM-like phenotype and expressing cardiac-specific markers and genes. This process is characterized by the activation of cardiac-specific transcriptional programs and the suppression of pluripotency genes.[12,13] It results in the formation of immature CMs with primitive structural and functional properties, which then will need to undergo (2) maturation process to acquire advanced structural and functional properties. This second process is characterized by the further development of sarcomeres, contractile apparatus, and mitochondrial density, leading to enhanced contractility and electrophysiological function. This results in the formation of mature CMs with improved functionality and resemblance to native adult CMs.

The capacity of allowing stem cells to differentiate into specific human cell types of interest facilitates the discovery of compounds that selectively influence the activity of these cells while exhibiting minimal effects on alternative cell types, thereby facilitating elucidation of cell-type-specific responses.[14] The differentiation process involves the orchestrated activation of transcriptional programs,

signaling pathways, and epigenetic modifications that ultimately lead to the formation of functional CMs with the ability to contract, propagate action potentials (APs), and respond to physiological stimuli. Differentiation protocols also enhance the expression of cardiac-specific markers and ion channels, improving their electrophysiological properties.[15,16] This process also promotes the development of sarcomeres, contractile apparatus, and mitochondrial density, leading to enhanced contractility and calcium handling.[17,18] In addition, differentiation promotes the formation of functional gap junctions, enabling proper cell-cell coupling and synchronous beating.[19,20] Moreover, differentiated iPSC-CMs exhibit an improved response to beta-adrenergic stimulation and increased sensitivity to pharmacological agents.[21,22] This process also reduces the activation of pluripotency markers and tumorigenic potential.[23,24]

The maintenance and differentiation of cardiac stem cells from *in vitro* iPSCs rely on the recreation of the heart's intrinsic signaling microenvironment, achieving through the targeted use of growth factors (GFs), neurotransmitters, epigenetic modulators, and extracellular matrix proteins, which necessitates the selective activation or suppression of distinct signaling cascades.[25] Various differentiation protocols have been developed, including modulation of Wnt/β-catenin signaling, ACTIVIN/NODAL activation, BMP signaling activation, and the application of small molecules that trigger mesodermal specification. This is followed by the inhibition of Wnt signaling to form cardiac mesoderm and, consequently, cardiac-specific progenitors, culminating in the expression of cardiac markers, including structure genes which encode sarcomeric proteins characteristic of terminally differentiated CMs.[26-29]

Inadequate differentiation of iPSCs into CMs can result in immature or partially differentiated cells, leading to reduced contractility, abnormal electrophysiology, increased susceptibility to disease modeling, limited response to pharmacological agents, reduced engraftment and survival, and tumorigenic potential. These immature CMs may exhibit decreased contractile force and velocity (less effective for contractility to pump blood), aberrant electrical activity (leading to arrhythmia), and decreased response to drugs targeting mature CM functions. Furthermore, they may be more prone to disease-related phenotypes, such as hypertrophy or fibrosis, and have lower survival rates and engraftment efficiency when transplanted into the heart. The use of iPSC-CMs with immature properties may also result in the development of teratomas or other kinds of tumor.[30-32]

2.2. iPSC differentiation into cardiac subtypes

Differentiating iPSCs into specific cardiac subtypes: atrial, ventricular, Purkinje fibers, nodal cells, and endothelial cells involves manipulating distinct signaling pathways and environmental conditions, which leads to the development of cells with specialized structural and functional properties. Below is an elaboration of how differentiation occurs for each subtype:

For atrial CM (iPSC-aCMs), a key factor is the addition of retinoic acid (RA), which is pivotal for atrial differentiation. RA helps to guide progenitor cells toward the atrial phenotype by influencing atrial-specific gene expression, such as atrial natriuretic peptide.[33] Compared to ventricular CMs (vCMs), aCM's exhibit functional differences, including a shorter AP duration and faster beating rates. iPSC-aCMs also show distinctive triangular-shaped APs, reflecting faster repolarization and higher beating frequencies, which are essential for their role in controlling the atrial contraction rhythm.[34,35]

In attaining vCMs, differentiation involves the inhibition of RA and the enhancement of Wnt signaling. The absence of RA guides cells away from an atrial fate and toward the ventricular lineage. The iPSC-vCMs display longer APs with a prominent plateau phase, reflecting the sustained contraction necessary for ventricular pumping as its functional differences. These cells also have a slower beating frequency and demonstrate different calcium transient kinetics, which are crucial for the forceful and sustained contractions of the ventricles.[36,37]

Nodal (Pacemaker) cells are induced by high levels of cyclic adenosine monophosphate (cAMP) and the transcription factor T-box 3 (TBX3), which suppresses typical chamber-specific genes, thus enabling pacemaker characteristics. Bone morphogenetic protein (BMP) pathway is also involved in their specification. Nodal cells exhibit automaticity, meaning they generate spontaneous APs to regulate the heartbeat. This pacemaker activity is a defining feature, where nodal cells exhibit slower AP upstrokes and lack the fast sodium current seen in working CMs.[38,39]

Differentiation into Purkinje fibers is less commonly studied in iPSC research. However, these cells can be derived by modulating Notch signaling and transforming GF-beta (TGF-β) pathways, which influence the specialized conduction system. Purkinje fibers are specialized for fast transmission of electrical signals in the heart. They have unique AP profiles with a quick rise velocity, essential for coordinating timely ventricular contraction. Their primary function is fast conduction

rather than contraction, distinguishing them from atrial and ventricular cells.[40]

Endothelial cells, crucial for blood vessel formation, can be differentiated from iPSCs by inducing basic fibroblast GF and vascular endothelial GF (VEGF). These GFs stimulate the cells to develop endothelial-specific characteristics. Unlike CMs, endothelial cells do not contract but are essential for forming the lining of blood vessels and maintaining vascular integrity. They produce nitric oxide, which helps to regulate blood vessel dilation and blood pressure, and contributes to angiogenesis, the formation of new blood vessels.[41]

Each of these subtypes, though derived from a common progenitor (iPSC-CPCs), displays unique molecular markers, ion channel properties, and electrophysiological characteristics that define their specific roles within the heart.

To further fine-tune the differentiation process, purification is essential. Post-differentiation, CM purification ensures a homogeneous cell population for therapeutic applications. Techniques such as metabolic selection (e.g., using lactate-based media) or immunomagnetic sorting (targeting cTnT or SIRPA) are commonly employed, with purities reaching up to 95%. This purification step is critical, as undifferentiated cells or non-cardiac lineages can pose risks such as tumor formation or other complications *in vivo*. During differentiation, purification helps to enrich specific cardiac subtypes with two ways: (1) surface marker-based selection: cells are sorted based on specific surface markers expressed during differentiation, such as c-kit, fetal liver kinase 1, or vascular cell adhesion molecule 1 and (2) intracellular marker-based selection: cells are sorted based on intracellular markers, such as troponin or myosin, using techniques like fluorescence-activated cell sorting.

2.3. Maturation of iPSC-CPCs

The natural maturation process of CMs is intricate and extends into human development, culminating around 6 years of age when CMs attain the specialized characteristics necessary for effective cardiac contraction and relaxation.[42] This maturation begins during the early postnatal period, marked by the cessation of cell division and the initiation of physiological hypertrophy, which enhances cell size and functional capacity, enabling adaptation to the dynamic hemodynamic environment. This process is driven by the orchestrated releases of specific transcription factors, structural protein isoforms, and ion channels, which collectively delineate the functional identity of each CM subtype. Understanding the mechanisms governing these processes is vital for elucidating human heart development and function, while also leveraging the clinical potential of iPSC-CMs in cardiac disease modeling, drug discovery, and regenerative medicine.[42]

The maturation of iPSCs into CPCs involves several overlapping pathways and factors. Critical signaling pathways, including BMP, Wnt and FGF, alongside pivotal transcription factors such as NKX2-5, TBX5, and GATA4, play significant roles in this differentiation process. In addition, epigenetic modifications refine these cellular transitions, emphasizing their shared relevance in both the differentiation of iPSCs into CPCs and the subsequent maturation of these progenitors into fully functional CMs.

During the differentiation phase, the primary focus lies in inducing cardiac lineage commitment and suppressing alternative cell fates. In contrast, the maturation phase of CPCs into CMs emphasizes refining cardiac subtypes,[43] enhancing electrophysiological properties, and improving contractile functionality.[44] This transition illustrates a nuanced interplay between signaling pathways and epigenetic regulation, where factors pivotal to CPC differentiation are subsequently modulated to facilitate CM maturation. For instance, the Wnt/β-catenin pathways instrumental in promoting formation of CPCs undergoes suppression as maturation progresses.[45] Conversely, the BMP/Smad pathway, initially inhibitory to CPC differentiation, becomes activated to further define cardiac subtype identity.[46] In addition, microRNAs (miRNAs) such as miR-1 and miR-133 are dynamically regulated during the maturation process, meticulously tuning the expression of essential ion channels and contractile proteins.[47,48] These nuanced shifts in gene regulation and signaling are crucial for achieving mature CM phenotypes, highlighting the necessity for precise control over these processes *in vitro*.

Recent advancements have led researchers to develop maturation strategies that simulate *in vivo* cardiac conditions, utilizing a variety of biochemical and biophysical techniques. These strategies aim to enhance the maturation of iPSC-CMs, as summarized in Table 1, which highlights progress in recapitulating the complex processes associated with cardiac maturation, evaluated through the presentation of developed sarcomeric and ion/electrolyte channel proteins in high-throughput screening assays.

Table 1. iPSC-CM maturation and characteristics

Method	Approach	Maturation duration (day)	Mature CM features	Recent Findings (2018 – 2024)
Physical[50]	Prolonged culture time	360	(Day 30 as comparison) Expanded cellular dimension Sarcomere elongation Reduced rate of beating Decreased dual MCL22α/v expression Greater sarcomere-alignment	Prolonged culture leads to improved sarcomere alignment and functionality.[51]
Metabolic (hormone treatment)	Media +Triiodothyronine (T3) +Glucocorticoid +Dexamethasone	7 – 14	Expanded cellular dimension Reduced roundness Enhanced MHC-α and SERCA2a expression Suppressed β-MHC expression Decline of proliferative activity Boost in contractile strength and dynamics Accelerated calcium signaling Heightened mitochondrial activity for energy production	Hormone treatments significantly enhance cardiac maturation markers.[52]
Metabolic (glucose replacement)	Media Remove Glucose +palmitate +oleic acid +carnitine	≥3 – ≤20	Expanded cellular dimension Reduced roundness Elongated contractile unit (sarcomere) Enhanced mitochondrial quantity and respiratory activity Boost in contractile strength and dynamics Rise of velocity rate and membrane electrostatic storage	Replacement of glucose with fatty acids improves metabolic activity.[53]
Physical (substrate modification)	Substrate stiffness increase in: polydimethylsiloxane (PDMS)/polyacrylic acid (PAA) (6~10 kPa)	3 – 9	Regulates sarcomere stress and contractile activity Enhanced force production More elongated form adoption	Stiffer substrates enhance contractility and structural maturity.[54]
Physical (electrical stimulation)	Continuous electrical stimulation (6.6 V/cm and 1 Hz in 2 ms duration)	4	Enhanced expression of HCN1, MLCV, SCN5A, SERCA, Kv4.3 and GATA4 Rise of $TNNT^{+}$ cells Cell extension Ventricular-like action potential Increased action potential duration Improved calcium regulation In conjunction with mechanical stimulation through photoactivated PDMS: amplified surface N-cadherin signaling and sarcomere contraction	Electrical stimulation promotes maturation and improves action potential characteristics.[55]
Biochemical/physical	CM-fibroblast/MSC co-culture and static-cyclic stretching	2 × 7	Enhanced contraction speed Larger twitch amplitude Elevated tensile modulus More developed sarcomere architecture Connexin 43 aggregation Surface localization of n-cadherin Reduced cell proliferation capacity Improved upregulation of MYL2/7 and MYH7/6 including MLC2, CACNA1C, NPPA, and SERCA2 Stretching further amplified these effects, especially with cyclic stress	Co-culture with FB/MSC enhances functional properties of iPSC-CMs.[56]

(Cont'd...)

Table 1. ***(Continued)***

Method	Approach	Maturation duration (day)	Mature CM features	Recent Findings (2018 – 2024)
Metabolic/ biochemical/physical	Media+ Thyroid hormone-dexamethasone-IGF-1 (TDI) formed in microtissue	7	Enhanced cellular alignment Lengthened sarcomere structure Elevated expression of ATP2A2, MYH7, SLCBA1, PLN, PPARG, ADRB1, CHRM2, and p-AKT Reduced level of MYH6, TNNI1 Boosted contractile strength Prolonged time to peak Extended relaxation phase	Microtissue formats improve cellular alignment and contractility.[57]
Metabolic/biochemical	EHT-based media culture	3 – 5 × 7	Elevated fatty acid oxidation Greater sensitivity to tetrodotoxin Enhanced aerobic metabolic activity Mitochondria aligned in sarcomere structure Improved calcium regulation Increased density of I_{k1} Reduced diastolic membrane potential	EHT Formats enhance metabolic activity and functional maturation.[58]
3D Bioprinting	3D printing of cardiac tissue	2 – 4 × 7	Vascularization of tissues Enhanced cell alignment Mimics native heart structure and function	3D bioprinting advances functional cardiac tissue engineering.[59]
Genetic Modification	CRISPR/Cas9 and other gene editing technologies	Editing: 2 × 7 Further iPSC maturation: 2 – 4 × 7	Enhanced expression of cardiac markers Improved metabolic functions Enhanced cell survival	Genetic modifications using CRISPR enhance cardiac differentiation.[60,61]
ECM Engineering	Modifications to ECM properties	~30	Improved cell-matrix interactions Enhanced mechanical properties Mimics native ECM composition	ECM engineering improves cardiac patch integration[62]
Integration with Devices	Connection with electronic and sensing devices	As early as 48 h	Real-time monitoring of cardiac activity Enhanced integration with engineered tissues Feedback mechanisms for stimulation	Device integration enhances functionality of engineered cardiac tissues[63,64]

Note: This table is adapted and updated from Thomas *et al.*[49]
Abbreviations: MHC-α: Alpha-myosin heavy chain; SERCA2a: Sarcoplasmic reticulum $Ca^{[2]+}$ ATPase; β-MHC: Beta-myosin heavy chain; HCN1: Hyperpolarization activated cyclic nucleotide-gate potassium channel 1; SCN5A: Sodium voltage-gated channel alpha subunit 5; GATA4: GATA binding protein 4; TNNT: Troponin T; CM: Cardiomyocyte; FB: Fibroblast; MSC: Mesenchymal stem cell; MLCV: iPSC: Induced pluripotent stem cell; MYL: Myosin light chain; MYH: Myosin heavy chain; CACNA1C: Calcium voltage-gated channel subunit alpha 1C; NPPA: Natriuretic peptide precursor A; ATP2A2: ATPase sarcoplasmic/endoplasmic reticulum $Ca^{[2]+}$ transporting 2; PLN: Phospholamban; PPARG: Peroxisome proliferator-activated receptor gamma; ADRB1: Adrenoceptor beta 1; CHRM2: Cholinergic receptor muscarinic 2; EHT: Engineered heart tissue; ECM: Extracellular matrix.

One of the methods used to further mature CPCs is electrical stimulation (ES). ES promotes cardiac repair by recruiting stem cells to injury sites through cardioprotective extracellular vesicles (EVs) and exosomes released from CPCs. These EVs carry bioactive molecules, including VEGF, cytokines (such as interleukin [IL]-6, IL-8, and tumor necrosis factor-alpha), matrix metalloproteinases, tissue inhibitors of metalloproteinases, stromal cell-derived factor 1, and heat shock proteins, which support angiogenesis, remodeling, and inflammation resolution, including in Q-waves denervation.[64,65] EVs also contain peptides, mRNA, and miRNAs that modulate recipient cell function and protect CMs from hypoxia-induced cell death, aiding tissue repair and regeneration.[64,65] Neutral sphingomyelinase 2 (nSMase2) assists in ceramide formation, further promoting EV biogenesis under ES and enhancing cell survival. In addition, cAMP is involved in signaling to support stem cell survival.[66,67]

In cell culture, ES promotes the differentiation of CPCs into mature CMs by driving calcium ion (Ca^{2+}) influx through voltage-gated channels. This influx activates calmodulin and calcineurin, leading to the nuclear translocation of transcription factors. Key pathways, such as AKT and GSK3β phosphorylation, along with focal

adhesion kinase and MAPK/ERK signaling, enhance cell adhesion, growth, and migration. Connexin-43 gap junctions also form under ES, promoting electromechanical coupling and cell alignment for synchronized contraction, which is essential for mature cardiac tissue formation.[66,67]

In addition to promoting stem cell recruitment, ES helps prevent atrophy caused by denervation, which occurs when nerve supply to tissues is lost. By maintaining heart contractility and enhancing cellular responsiveness to electrical signals, ES can counteract the effects of denervation. ES activates pathways involved in muscle protein synthesis and cell survival, including the mechanistic target of rapamycin pathway, which is crucial for maintaining muscle mass.[67,68] Furthermore, ES has a neurogenic effect, influencing the autonomic nervous system to regulate neurotransmitter release and modulate cardiac function.[65,68] This interaction supports tissue repair by improving the heart's electrical activity in response to injury. Findings highlight the potential of ES in enhancing stem cell homing to cardiac injury sites, supporting tissue repair, and preventing atrophy due to denervation.

In large MIs with Q-waves and denervation, pulsed electromagnetic fields (PEMF) simulate EM forces,[69] potentially aiding sympathetic re-innervation and autonomic regulation. PEMF has shown benefits in experimental studies, improving metrics such as ejection fraction and supporting myocardial healing. At the biomolecular level, PEMF promotes VEGFR-mediated angiogenesis, nerve GF for re-innervation, and IL-10 to reduce chronic inflammation, all of which are essential for controlled tissue repair. In addition, piezoelectric materials are being investigated to localize electrical signals, improving stem cell migration to the infarct site without invasive power sources. Further studies employing 3D visualization methods, such as CUBIC and CLARITY,[70] have made strides in clarifying sympathetic nerve remodeling in post-MI contexts. This technology has enabled detailed mapping of cardiac sympathetic networks, which could help in monitoring EM treatment effectiveness and improving nerve regeneration post-MI. Together, these biomolecular mechanisms position ES and EM therapies as potent tools for cardiac repair

3. Scalable production of iPSC-CMs

The discovery of iPSCs has revolutionized the field of regenerative medicine, enabling the generation of diverse cell types for therapeutic applications. However, the high costs associated with generating and validating iPSC lines pose a significant obstacle to their widespread adoption. Consequently, there is an urgent need for the establishment of cell banks that provide access to high-quality, validated iPSC lines, serving as repositories to cater the various requirements of clinical research communities. This would facilitate the widespread adoption of iPSCs in therapeutic research, ultimately advancing the regenerative medicine field. Clinical trials have begun utilizing human iPSCs to address debilitating diseases. For instance, iPSC-derived retinal-pigment epithelial cells have been transplanted to tackle macular degeneration (MD),[71] while β-like cells are being differentiated to overcome insulin dependence in type 1 diabetes mellitus patients.[72] These advancements highlight the need for large-scale, clinically compliant iPSC production to support these innovations. Several key factors must be optimized, including bioreactor design, culture conditions, and cell differentiation protocols. The goal is to achieve rejection-free cell transplants without needing lifelong immunosuppressive therapy (that comes with direct organ transplantation as earlier therapy finding).

From a bioprocess perspective, the autologous method necessitates modestly sized, condition-targeted treatment doses for each individual patient. This approach may also require extra cells for quality assurance, efficacy assays, and reserved dosages for subsequent transplantation, if applicable. For instance, estimates suggest that retinal-pigment epithelium cells from approximately 50,000 iPSCs would suffice to treat MD in one patient, as demonstrated in a successful case using human iPSCs.[73] To accommodate therapeutic cell production on such a limited scale, automation, facility space with minimal good manufacturing practice (GMP) standards, and the simultaneous production of various patient samples in single-use culture systems are crucial to the continuing of this production.[74]

These facts collectively strongly advocate for an allogeneic approach, wherein a "universal cell source" is intended for transplantation into non-related recipients, likely requiring immunosuppressive therapy similar to traditional organ transplants. However, the degree of immunosuppression needed will depend on human leukocyte antigen compatibility, which can be achieved through the strategic deployment of multiple iPSC lines to cater to specific patient populations within a given region or country, as discussed in greater detail elsewhere.[75] This allogeneic "off-the-shelf" strategy also ensures timely cell availability to meet pressing clinical demands. This highlights the need for robust, cost-effective, and reliable GMP-compliant protocols for scaling up iPSC cultivation and differentiation into specialized cell types (including iPSC-CMs), ensuring consistency and reproducibility in research applications, without displaying heterogeneous behaviors.

Culture medium is a critical component for large-scale production, impacting both cost and quality control. Standard iPSC culture media, such as mTeSR™1 or Essential 8™, are commonly used, but their high costs present a bottleneck for scalability. On average, these media cost \$500 – \$700/L, with daily consumption in large-scale bioreactors ranging from 1 – 2 L, depending on cell density and bioreactor volume.[76] Thus, media optimization, including potential shifts toward serum-free or chemically defined formulations, is a key strategy for cost reduction. Studies have shown that adjusting key components such as GFs (e.g., fibroblast growth factor 2 [FGF2] and TGF-β) can reduce media consumption by 20 – 30% while maintaining high cell viability and pluripotency.[77,78]

Scalable production of iPSC-CMs requires meticulous optimization of biomolecular and process factors to drive differentiation toward CPCs mimicking the complexity and multipotentiality of first heart field and second heart field.[79-81] Key factors include: (1) transcription factors: Essential cardiac-specific marker genes such as GATA4-6,[82,83] HAND1/2,[84] NKX2-5,[85] TBX5,[85,86] and cardiac troponin T (cTnT) must be expressed to initiate cardiac development;[10,11] (2) signaling pathways: activation of pathways such as activin A, Wnt/β-catenin, and BMP/TGF-β to regulate lineage specification;[87] (3) GFs and cytokines: supplementation with FGF,[88] VEGF,[89] BMP4,[90] and cytokines such as IL-6[91] and IL-11[92] supports directed differentiation; (4) extracellular matrix (ECM) components: collagen, fibronectin, and laminin in synthetic scaffolds or decellularized matrices create an ideal cellular environment, enhancing maturation;[93-95] (5) cell culture conditions: controlled oxygen levels, pH, and nutrient flow are crucial for maintaining cell viability;[96,97] (6) timing and duration: the schedule and length of exposure to these cues directly impact differentiation efficiency;[98] (7) iPSC line quality: the initial quality and pluripotency of the iPSC line affect differentiation outcomes;[99,100] (8) reprogramming method: the method of iPSC generation shapes the epigenetic profile and influences differentiation potential;[101,102] (9) epigenetic modifications: DNA methylation and histone modifications regulate cardiac gene expression throughout differentiation;[103] and (10) mechanical forces and biophysical cues: shear stress and ES enhance maturation and functional rhythm.[104-107]

The use of stirred tank bioreactors (STBs) remains a widely employed method for scalable iPSC-CM production, particularly for generating high-yield quantities. Parameters such as medium composition, ECM scaffold selection, and precise biochemical signaling are essential to ensure consistency and reproducibility. In current large-scale differentiation protocols, optimized conditions involving factors such as activin A (100 ng/mL) and BMP4 (10 ng/mL) have demonstrated the potential to generate 10^8 – 10^{10} iPSC-CMs (one to two folds of which are required per patient) in a single run, yielding sufficient quantities of iPSC-CMs for therapeutic applications in 5 – 10 L volumes within a 2 – 3 week timeframe. Differentiation efficiencies of 70 – 90% are achieved through these optimized protocols, meaning 7 – 9 out of every 10 iPSCs successfully differentiate into CMs, depending on the initial seeding density and bioreactor volume.[20,108] The estimated cost for generating these cells, including reagents, media, and labor, ranges from \$10,000 to \$20,000 per patient dose, with ongoing efforts aimed to reduce this cost through process optimization and economies of scale.[63,109,110]

As for process dimensions, estimates indicate that approximately 1 – 10 × 10^9 (1 – 10 billions) functional active cells for each patient are required for solid organ restoration, including substituting hepatocytes, pancreatic β-cells, or CMs. However, significantly larger quantities are required in producing "*in vitro* blood," with calculations suggesting that around 2 – 3 × 10^{13} human iPSC-derived red blood cells (iPSC-RBCs) are required to match 5 L of blood in adults, considering that 1 millimeter of blood contains approximately 5 billion erythrocytes.[43]

Calculations derived from experimental data indicate that current production of one billion human iPSC-CMs demands a bioprocess scale of 1 – 2.5 L.[110] This implies that even with substantial future advancements, mass production of iPSC progenies will demand industrial-level procedures, which have already been developed for producing recombinant proteins or vaccines in mammalian cell cultures at scales of approximately 1,000 L.[111] In contrast, bioprocess development for iPSCs remains in its nascent stage, requiring the further advancement to meet industrial demands.

However, it is worth noting that STBs are not 3D systems in the same way as other bioreactor types, such as 3D suspension bioreactors. While STBs remain useful for large-scale production, advancements in 3D cultures have been increasingly explored to enhance the differentiation and maturation of iPSC-derived cells. These systems allow for more complex interactions, potentially improving both the yields and functionalities of differentiated cells, though STBs still provide a reliable and scalable approach for achieving high-yield production. Some of the latest scalable methods include: (1) vertical-wheel bioreactors: designed to reduce shear stress using a gentler mixing mechanism compared to traditional impeller-based stirred tanks, potentially improving cell viability and differentiation consistency, (2) microcarrier-based systems: By allowing iPSCs to attach and grow on microcarriers, these systems

help increase cell density in the bioreactor, enhancing yield without significantly increasing reactor size, and (3) perfusion bioreactors: by continuously supplying fresh medium while removing waste, perfusion bioreactors maintain optimal conditions for cell growth, supporting longer culture durations and higher cell densities, and (4) 3D suspension culture systems: enabling cells to grow as aggregates or spheroids, which can better mimic *in vivo* environments, potentially leading to more mature cell phenotypes and scalable expansion.[111]

3D suspension culture systems have emerged as a powerful method for scalable iPSC-CM production, offering significant advantages over traditional 2D culture systems by better mimicking the *in vivo* environment. In these systems, iPSCs aggregate into spheroids, fostering enhanced cell-cell and cell-matrix interactions and creating a more uniformed cellular microenvironment, promoting CPCs differentiation into functional CMs. This setup supports key signaling pathways, such as gap junction maturation. 3D cultures generate CMs with better electrophysiological properties, contractile function, and drug response, making them ideal for drug screening and regenerative medicine. In addition, 3D cultures are more likely to exhibit a mature phenotype, including better synchronization of cell contractions and enhanced responses to pharmacological agents, making them more suitable for drug screening and regenerative medicine applications. Their scalability is crucial for therapeutic applications, supporting heart disease modeling, personalized medicine, and autologous therapies with improved consistency.

To optimize these systems for scalable production, bioreactor design plays a pivotal role. Maintaining continuous nutrient flow and removing waste through continuous perfusion are crucial for supporting large-scale cell cultures. For instance, spinner flasks, wave-induced motion systems, and perfusion-based platforms have been used to maintain optimal cell health and growth while ensuring uniform differentiation. These bioreactor systems allow the maintenance of large volumes of spheroids, streamlining the process of expanding iPSC-CMs. The success of these cultures also hinges on controlling key parameters, such as shear forces, agitation speed, and seeding density, all of which can influence cell viability and differentiation outcomes.

While scalable production is crucial for iPSC-based therapies, it is equally important to ensure cell fate control of the mature cell phenotype and its functional rhythm control. By combining optimized bioreactor conditions with precise biochemical cues, these systems are advancing the field of iPSC-derived cardiac models, enabling the development of more effective, scalable, and clinically relevant platforms for heart disease modeling, drug discovery, and regenerative medicine. The development of economically and methodologically efficient large-scale iPSC-CM culture is closely tied to understanding and promoting cell maturation, whereas optimization of rhythm control in CMs requires a comprehensive approach which incorporates advances in scalable production and cellular maturation, underscoring the importance of these three pillars.[112]

4. Conclusion

Substantial advancements have been made in leveraging iPSC-CMs for cardiac regenerative medicine, with current bioreactor systems capable of producing up to 10^{11} cells per batch. To enhance predictive power and recapitulate population-level cardiovascular risks, further refinements are required, such as implementing robust quality control endpoints, scalable differentiation protocols with minimized variability, and nutrient-driven maturation strategies. Notably, 3D bioprinting and microphysiological systems enable the replication of complex tissue structures and interactions, facilitating more accurate disease modeling and drug screening. Such bidirectional scalability is crucial for translating iPSC-CMs into large-scale regenerative applications. Efforts to harmonize patient iPSC banking and generation of cardiovascular cell types with reduced batch-to-batch variability will support personalized treatment approaches. These advancements set the foundation for population-based studies and pharmacogenetic tools, and, importantly, potential autologous transplant therapies to address myocardial repair in clinical settings. Continued progress on cost reduction, iPSC-CM maturation, and safety protocols will be pivotal for making iPSC-CM-based therapies a viable option in clinical practice.

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Conflict of interest

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