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From potential to practice: Overcoming the immaturity of iPSC-derived cardiomyocytes for regenerative medicine

Short title: Advanced maturation of iPSC-derived cardiomyocytes is a prerequisite for heart bioengineering

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ABSTRACT

Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) hold great promise for revolutionizing regenerative medicine. Preclinical studies indicate their potential to repair damaged myocardial tissue in animal models of heart disease. Despite ongoing advances in the field, the incomplete maturation of iPSC-CMs remains a critical barrier that significantly

hinders their translation into clinical applications. The maturation of cardiomyocytes is crucial for the successful integration of iPSC-CMs into damaged heart tissue. Compared to adult cells, immature CMs have impaired structural characteristics, contractile function and electrophysiological properties. Recent studies have focused on identifying key factors, such as altered cell metabolic pathways or mechanical and electrical stimulation, that may promote iPSC-CM maturation. Progress in this area have profound implications for the development of personalized disease models and cell therapies that promote the regeneration and repair of damaged heart tissue. This review describes the current achievements in the application of regenerative medicine using iPSC-CM and tissue engineering, highlighting the molecular mechanisms, culture strategies, and biophysical approaches that have contributed to improved maturation of these cells. Numerous studies are currently being carried out using both *in vitro* and *in vivo* models to better understand the complex mechanism of regeneration of the damaged heart. The combination of stem cell therapy and 3D cardiac cell cultures aims to repair and regenerate damaged cardiac tissue more effectively.

Key words: cardiac tissue engineering, cardiomyocytes maturation, heart disease, iPSC-derived cardiomyocytes, regenerative medicine

INTRODUCTION

Ischemic heart disease (IHD) is the most common cardiovascular disease which is caused by reduced blood flow to the cardiac muscle, leading to chronic oxygen deficiency, permanent damage and apoptosis of cardiomyocytes (CMs) [1]. Due to the limited proliferative capacity of CMs, damaged cells are replaced by fibroblasts, prompting ongoing research into effective methods for regenerating cardiac muscle and reversing pathological fibrosis [2, 3]. In recent years, numerous studies have investigated heart tissue regeneration using stem cells [4]. Notably, induced pluripotent stem cells (iPSCs) offer a number of advantages that make them particularly promising tool for both basic and applied cardiac research [5]. Since iPSCs are reprogrammed from readily available patient-derived somatic cells, such as peripheral blood [6] or urine cells [7], they eliminate the need for invasive biopsies, address ethical concerns, and minimize the risk of immune rejection.

Despite the intensive development of protocols for differentiating iPSCs into cardiac cells [8–11], the incomplete maturation of iPSC-derived cardiomyocytes (iPSC-CMs) remains a persistent challenge. iPSC-CMs often resemble fetal rather than adult CMs in terms of

structural, metabolic, and electrophysiological characteristics. iPSC-CMs are smaller, less elongated, and predominantly mononuclear compared to adult CMs [12]. They display disorganized and incomplete sarcomeres, lack a mature T-tubule system, and contain irregularly arranged myofibrils with immature Z- and I-bands [13, 14]. The iPSC-CMs rely more on glycolysis than fatty acid oxidation — the primary energy pathway in mature CMs [15]. Additionally, their mitochondria are diminutive, perinuclear and lack well-developed cristae [16]. Unlike adult CMs, iPSC-CMs have lower contractile force, less synchronized dynamics and higher spontaneous beating rates [17]. They generate cardiac currents but are deficient in the IK1 current, which is necessary for the stabilization of the resting potential [18]. Furthermore, iPSC-CMs display mixed atrial, nodal and ventricular-like action potentials [19]. Immature iPSC-CMs also show higher MYH6 and lower myosin heavy chain 7 (MYH7) gene expression compared to adult CMs, and express both atrial (MYL7) and ventricular (MYL2) isoforms, emphasizing their heterogeneity [20].

The maturation of CMs is a complex process governed by key signaling pathways, including Wnt [10], Notch [21], and Hippo [22], and tightly regulated by transcription factors such as GATA4, MEF2, NKX2.5, and Tbx5 [23]. In recent years, extensive efforts have been dedicated to refining the multifaceted genetic and epigenetic mechanisms involved in CMs development, alongside optimizing environmental and mechanical factors such as extracellular matrix (ECM), mechanical stretching, and culture conditions, to enhance similarity of iPSC-CMs to native heart cells [24]. This review discusses current advances in maturation strategies aimed at improving iPSC-CMs for therapeutic purposes (Figure 1).

POWER OF PERSUASION — BIOCHEMICAL COMPOUNDS AND SMALL MOLECULES TRIGGER IPSC-CM MATURITY

Recent studies report an important role for a variety of biochemical factors and small molecules in facilitating the differentiation and maturation of CMs from iPSCs [25]. CHIR99021, a potent inhibitor of GSK3β kinase, is one of the key molecules involved in the differentiation of iPSCs into cardiomyocytes. This inhibitor works mainly by activating the Wnt/β-catenin signaling pathway, which is required to direct iPSCs towards the mesodermal lineage - the first step in differentiation into CMs [11]. Further analysis of CHIR99021 showed that incubation of iPSCs with this compound at early stages of differentiation induces mesodermal markers, which provide the basis for subsequent stages of differentiation towards the cardiac lineage [10, 25]. The effect of CHIR99021 is enhanced when combined with other agents that act on parallel pathways. The combination of CHIR99021 with rapamycin, an mTOR kinase inhibitor, has

been shown to increase differentiation efficiency by reducing p53-dependent apoptosis. By promoting cell survival, this combination significantly improved the efficiency of cardiomyocyte harvest and their functional quality [25, 26]. Another key step in achieving efficient differentiation of iPSC-CMs is the inhibition and activation of the Wnt pathway. After the initial activation of Wnt signaling by CHIR99021, inhibition by IWP-4 factor, promotes the expression of cardiac markers — cTnT and GATA-4 — which are essential for cardiac function [27].

CMs use fatty acids (FA) as a major source of metabolic energy, so enriching iPSC-CMs culture medium with FA at later stages of differentiation helps to mimic the energy profile of mature cardiomyocytes, supporting both their metabolism and the structural maturation. The culturing of CMs in glucose-deprived medium but supplemented with palmitate, oleate, linoleate, and 3,3',5-triiodothyronine (T3) increases the electrophysiological efficiency of cardiomyocytes and improves their overall metabolic profile [28–30]. In addition, treatment of iPSCs with fatty acids during differentiation into CMs resulted in increased mitochondrial oxidative capacity, characteristic of mature heart cells [29].

Growth factors and hormones also play a key role in promoting the differentiation of iPSCs into cardiomyocytes. Vascular endothelial growth factor enhances cardiomyocyte differentiation via Flk-1 receptor activation [31]. Furthermore, insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) synergistically upregulate cardiac markers such as GATA4, facilitating cardiomyocyte transdifferentiation [32]. Forskolin, by increasing cAMP levels through adenylate cyclase activation, enhances electrophysiological maturation during iPSC differentiation [33]. Additionally, glucocorticoids such as dexamethasone stabilize the differentiation environment and promote cardiac gene expression [34].

HEART-TO-HEART TALK IN TISSUE ENGINEERING: TRANSFORMING FROM 2D TO 3D PLATFORMS

Traditional two-dimensional (2D) *in vitro* cell culture models are unable to fully reproduce the physiological or pathological characteristics of adult CMs, mainly due to the lack of ECM connectivity to divergent cardiac cell types [35, 36]. In fact, 2D static co-cultures of iPSC-CMs and cardiac fibroblasts (CF) or endothelial cells (EC) co-subjected to bioelectromechanical cues have been reported to effectively overcome fetal-like CM properties as observed by organized ultrastructure, the presence of transverse tubules, oxidative metabolism, and functional calcium handling [23, 35–38]. In addition, studies of the EC-CM interaction model have shown that EC produce cardioprotective hypoxia-inducible factor 1α- (HIF1α) and NO-dependent mechanism

against myocardial ischaemia and reperfusion injury [39]. More importantly, co-culture of human iPSC-CMs with bone marrow-derived mesenchymal stem cells improved the maturation and functionality of hiPSC-CMs *in vitro* and enhanced the survival and therapeutic potential of iPSC-CM for failing myocardial tissues *in vivo* [40].

Notwithstanding numerous efforts to improve the intrinsic limitations of routine 2D iPSC-CM cultures combined with microelectrode arrays, they still operate in a physiological and structural context far removed from the complexity of the native cardiac tissue in spatial architecture, contractility and metabolism [36]. As a result, three-dimensional (3D) cardiac culture models — the most willingly vascularised — were better suited to more accurately mimic cell-to-cell interactions and arrangements [41, 42]. Two primary platforms were used in this area of tissue engineering - scaffold-based and scaffold-free 3D models, which allowed the analysis of iPSC-CM in both artificial and neutral ECM microenvironments, respectively [43]. Multicellular microtissues and multilineage cardiac organoids serve as simplified organ structures tailored to monitor cardiogenesis and model disease [44]. Treatment of cardiac organoids consisting of fibroblasts, human iPSC-derived cardiomyocytes and endothelial cells (iPSC-EC) with the synthetic adenosine analogue 2-Cl-C.OXT, which has neovascularising effects, enhanced platform's contraction combined with CM maturation [45]. Nanowired human cardiac organoids composed of iPSC-CMs, CFs and established vascular stem cell lines significantly improved inner organoid maturation and therapeutic efficacy in treating infarcted hearts [43].

On the other hand, the use of hydrogels, decellularised ECM and bioprinting enables generation of 3D scaffolds with different geometries [41]. The Heart-on-a-Chip microfluidic device provides electrical, mechanical (e.g. shear stress, stretch forces) and biochemical (vascular endothelial/fibroblast growth factors) stimuli to mature CMs and integrates sensors for continuously controlled monitoring of tissue contractile functions and oxygen concentration [41, 46]. Using fluidic micro-modalities enables more complex, three-dimensional cardiac tissue to be created [42]. The AngioChip system from a synthetic polymeric elastomer allowed for an incorporation of self-organised human iPSC-CMs with HUVEC cells and supported a perfusable vascular system through a central microchannel [47]. Patient-derived atrial and ventricular iPSC-CMs or human iPSC line BJ1D were co-embedded with CFs in hydrogel within the Biowire II Platform and subjected to the slow electrical conditioning. Electric stimulus effectively supported CM differentiation and promoted sarcomeric organisation, the expression of chamber-specific proteins, calcium handling, and the reliance of differentiated CMs on glycolysis [47–49]. Such approach allowed for up to 8 months enabled modelling of

polygenic left ventricular hypertrophy starting from iPSC patient cells [49]. Additionally, a filtration Layer-by-Layer technique of 3D-bioprinting was applied to develop a vascularised human iPSC-CM tissue combined with fabricated ECM-fibronectin-gelatin nanofilms [50]. Moreover, introduction of normal human cardiac fibroblasts or cardiac microvascular endothelial cells into the iPSC-CM tissues supported contraction and provided blood capillary-like networks in 3D generated-iPSC-CM construct [50, 51]. A bi-layer patch composed of human iPSC-CMs and endothelial cells/pericytes of patient origin was combined with a personalised hydrogel bioink and implanted on the epicardial surface of a nude rat infarct model [52]. The two-layer configuration improved the survival and maturation of the human iPSC-CM and led to a rapid inosculation of microvessels on the injured myocardium [52].

WAITING FOR THE CUES: PHYSICAL STIMULATION

In recent years, many studies have used nanofibrous mats as scaffolds for iPSC-CM differentiation, mimicking the architecture of the ECM and providing them with an optimal growth environment [52–54]. The above-mentioned mats are mainly made of biodegradable polymers such as poly(lactic-co-glycolic acid), poly(ε-caprolactone), polyurethane and natural polymers, such as collagen, fibrin, and gelatin [55]. Present data demonstrated that iPSC-CMs cultured on nanofiber scaffolds exhibited improved maturation, manifested by increased expression of contractile proteins (cardiac actin and troponin T) and mature cardiac proteins, such as β-MHC and MLC2v [55–57]. Moreover, orientation of cardiomyocytes along the nanofibers is associated with augmented contractility and more synchronized beating [57, 58].

In native heart tissue, maturing cardiomyocytes are exposed to mechanical forces — including stretch and shear — that continuously stimulate the cells during cardiac contraction, promoting their maturation and mimicking *in vivo* conditions. Therefore, the use of elastic substrates and casts for iPSC-CMs culture supported cell alignment, increased expression of maturity genes (connexin-43), and improved sarcomere organization [59, 60]. In addition, mechanical stress ameliorates the contractile force and electrophysiological properties of CMs, including action potential duration and calcium handling [61–63].

Another valuable method for promoting the maturation of iPSC-CMs is electrical stimulation. Such biophysical cues imitate natural electrical signals, significantly increasing the expression levels of TNNT2, ACTC1, TNNT2, MYH7, and MYL7 and improving calcium handling capacity [64]. In addition, iPSC-CMs subjected to electrical stimulation showed a positive force-frequency relationship in contractility and an increase in peak calcium flux, indicating advanced tissue maturation [65]. Notably, electrical stimulation of CMs resulted in

sarcomeric elongation, significant up-regulation of gap junction protein alpha 1 (GJA1) and potassium inwardly rectifying channel subfamily J member 2 (KCNJ2) [66]. Additionally, exposure of hiPSC-CMs to synchronized electrical and mechanical stimulation resulted in increased N-cadherin localization towards the plasma membrane, shortened sarcomeres, and reduced transmembrane calcium current, suggesting a more mature phenotype [67].

THE NEW RULES OF THE OLD GAME — GENETIC AND EPIGENETIC APPROACHES TO IMPROVE iPSC-CM MATURATION

Gene editing strategies have emerged as powerful tools to promote maturation of iPSC-CMs by targeting specific genes and pathways known to influence cardiomyocyte development, structural organization, and functionality. Latest research have demonstrated that cyclin D2 (CCND2) overexpression showed promising results in activating cell cycle progression in hiPSC-CMs [68, 69]. Transplantation of CCND2-overexpressing hiPSC-CMs into mouse or pig hearts with myocardial infarction significantly augmented myocardial repair [68, 69]. Furthermore, overexpression of N-cadherin (CDH2) in hiPSC-CMs increased their survival, engraftment and integration into infarcted mouse hearts, leading to better cardiac function and reduced infarct size [70]. Combinatorial overexpression of the transcription factors KLF15, ESRRA, and HOPX improved multiple aspects of hiPSC-CM maturation, including calcium handling, ATP generation, and morphological characteristics [71]. Similarly, enhanced electrophysiological maturation, Ca2+ signaling and structural development were observed in hiPSC-CMs overexpressing KCNJ2 or Kir2.1 [72, 73].

In the intervening years, epigenetic mechanisms such as DNA methylation, histone modifications, and non-coding RNAs, have gained considerable interest as they provide a promising approach to generate more mature iPSC-CMs by adjusting gene expression without altering the underlying DNA sequence [74]. Another current issue in the field is also residual epigenetic memory, which causes variation in the potential of iPSCs for lineage-specific differentiation and makes the origin of the cell source crucial for the final transplant outcome [75, 76]. A whole-genome screening identified 96 miRNAs capable of increasing the proliferation of hiPSC-derived cardiomyocytes, whereby most of them targeted components of the Hippo signaling pathway [77]. Further studies showed that overexpression of miR-199a or miR-302d activated cell cycle re-entry in hiPSC-CMs [77–79]. Upregulation of miR-1, let 7i, and miR-452 or knockout of miR-122 and miR-200a increased cell size, elevated fatty acid usage, and enhanced contractile force in hESC-CMs or hiPSC-CMs [80–82].

Although in-depth heart profiling has indicated the essential involvement of DNA methylation and histone modifications in regulating gene expression at different stages of myocardial development and regeneration [83, 84], the understanding of how these epigenetic marks contribute to the maturation and proliferation of hiPSC-CMs remains limited. The usage of polyinosinic-polycytidylic acid, a mimic of viral RNA, primed cardiac progenitor cells for more efficient and robust maturation into cardiomyocytes by altering the acetylation of lysine 9 on histone H3 [85]. A recent study showed that inhibition of lysine demethylase 5 (KDM5), which specifically demethylates lysine 4 of histone H3 (H3K4me3), enhanced the maturation of iPSC-CMs by promoting fatty acid oxidation, oxidative phosphorylation, and myofibrillar organization [86]. Additionally, hiPSC-CMs overexpressing CXXC zinc finger protein 1, binding to the Set1/COMPASS complex responsible for deposition of the histone modification H3K4me3, displayed more mature phenotype [87]. Of particular interest is also ALKBH5, which indirectly promotes the expression of genes involved in cell cycle progression and proliferation in iPSC-CMs by demethylating the mRNA of YTHDF1 [88].

ALL IN GOOD TIME: LONG TERM CULTURING OF iPSC-CMS

The incubation time of iPSC-CMs *in vitro* cultures positively influences cell morphology, structural organization and functional properties. Long-term cardiac cell maintenance results in cell hypertrophy and anisotropy, and a significant increase in multinucleated cardiomyocytes [88–90]. However, there is some controversy about the tendency of the beating rate during *in vitro* cell maturation. Although spontaneous beating becomes apparent as early as 7–8 days post differentiation, in some studies the beating rate increased gradually [91, 92], while in others it decreased with longer incubation times [93]. Moreover, transcript levels of cardiac contractile genes were reported to increase with the length of culture [94].

Long time of CMs culture maintenance led to increased myofibril density and more organized sarcomeric arrangement [89, 90, 93–95]. Initially, iPSC-CMs contain a small number of narrow, diffusely distributed, poorly aligned myofibrils and immature high-density Z-bands, which develop into sarcomeres with a clear band pattern including the Z-, I-, and A-bands at between 30 and 90 days. Around 140 day M-bands started to form and became clearly detectable at 231–360 days [89, 90, 93–95]. On the other hand, the ventricular-like type of maturation was confirmed by an increase in MYH7 transcript levels [92–94] and a decrease in the percentage of cTnT-positive cells over time [89].

The mitochondrial network also changed over the course of the *in vitro* culture. As the cells grew, the mitochondria became larger in size, more elongated, and more evenly and

densely distributed throughout the entire volume of the cell. They formed a system of interconnected channels with generated membrane potential, arranged along the contractile myofilaments [92]. Mitochondrial integrity, functional viability, and membrane potential also increased with culture time [91]. Moreover, metabolic function per mitochondrion increased at the late time point, and there was a partial shift in iPSC-CMs from a predominantly glycolytic metabolism to an intensified use of OXPHOS and β-oxidation.

Prolonged iPSC-CMs maintenance also revealed dynamic developmental behavior in calcium handling properties [91, 92, 96]. As the cells aged, they displayed significantly greater L-type Ca^{2+}current density ($I_{Ca,L}$) and increased $I_{Ca,L}$-evoked Ca^{2+} transient amplitude, together with a functional increase in key Ca^{2+} removal mechanisms, NCX and SERCA, which contributed to increased sarcoplasmic reticulum Ca^{2+} release and faster diastolic Ca^{2+}removal from the cytosol in late versus early cultures [96].

In terms of electrophysiological maturation during long-term culture of CMs, the maximum rate of depolarization (dV/dt max) increased dramatically between CMs early and late in the culture, although the values were lower than those reported for adult human CMs [95].

Reduced basal inward-rectifier K^+ current (I_{K1}) is typical for iPSC-CMs, but during prolonged culture,I_{K1} density increased, which was shown by an *in silico* model to be a key determinant of action potential shortening in older cells. Moreover, electrophysiological maturation of hiPSC-CMs is associated with increased peak Na^+ current (I_{Na}), which contributes to increased action potential upstroke velocity, although it is still low compared to adult ventricular cardiomyocytes [96].

CURRENT ACHIEVEMENTS AND CLINICAL PERSPECTIVES OF iPSC-CMS: NOT JUST A MATTER OF SCALE AND HOSPITALITY

Although the use of iPSC-CMs has overcome the limitations of tissue availability for *in vitro* studies, their application in the therapy of heart diseases is associated with several challenges, including cell quality, low cell survival after transplantation, arrhythmogenicity or immune rejection [97]. Due to the aforementioned obstacles that still need to be resolved, the near future application of iPSC-derived cardiomyocytes will remain linked to the unparalleled possibilities of patient-specific cardiac disease modelling, their use in cytotoxicity studies and drug discovery.

The iPSC-CMs have been used, among others, to model cardiac channelopathies, caused by mutations in genes encoding cardiac ion channels, mainly for sodium (Na^+),

potassium (K^+) and calcium (Ca^{2+}) channels, respectively [98]. Models of LQTS1 and LQTS2 disease have been successfully developed by several research groups [99–106]. In addition, iPSC-CM cells were employed in studies investigating the pathophysiological phenotypes and mechanisms of catecholaminergic polymorphic ventricular tachycardia [107–109] and Brugada syndrome [110–112].

In addition, iPSC-CMs have also found application in another group of heart diseases, cardiomyopathies, which are associated with dysfunction of the heart's muscular and electrical functions, leading to heart failure or sudden cardiac death, and are linked to inherited mutations in genes [113]. Ultimately, iPSC-based modelling has changed the understanding of the variable clinical presentation and the combined impact of different pathogenic gene variants in this group of diseases, especially in hypertrophic cardiomyopathy [114, 115], dilated cardiomyopathy [116, 117] and arrhythmogenic right ventricular cardiomyopathy [118].

On the other hand, iPSC-CMs, as a promising cellular therapeutic platform, offer a potential route to induce cardiac regeneration by cell replacement approach only upon successful engineering of their adolescence. Recently, an efficient and reproducible generation of human iPSC-derived cardiomyocytes (bCMs) and cardiac organoids (bCO) has been proposed using stirred suspension cardiac differentiation and bioreactor formats, which only potentiate experimental reproducibility, disease modelling and clinical translation [119]. The resulting bCMs were highly viable after cryo-recovery, had improved contraction kinetics, greater metabolic and structural maturity and predominantly ventricular identity compared to standard monolayer-differentiated cardiomyocytes [119]. More importantly, the suspension-delivered bCOs were primarily composed of CMs and modelled the ventricular wall and the formation of the central chamber [119].

Pararelly, either autologous or potentially immune-shielded allogeneic transplants of iPSC-CMs remains a long-term priority for cardiac cell therapy to reach clinical application [120]. Autologous graft of rhesus macaques iPSC-derived sodium/iodide symporter-labelled cardiomyocytes (RhiPSC-NIS-CMs) demonstrated proper growth, maturation kinetics and electromechanical integration with host cells in chronically infarcted myocardium. This long-term engraftment was monitored by non-invasive serial positron emission tomography/computed tomography imaging and it remained stable for over 1 year, whereas allogeneic RhiPSC-CMs without immunosuppression underwent rejection within 8 weeks of transplantation [120]. If viable and integrable human iPSC-CMs were to engraft and mature in a similar way, it would be a remarkable milestone in the unlimited supply and far-reaching transition to large-scale format and further bedside use for cardiomyopathies.

CONCLUSIONS

The technology of patient-specific cardiomyocytes derived from iPSCs enables the mimicry of genetic heart disease, aspects of its metabolic deterioration, and evaluation of possible cardiotoxic response to drugs in high-throughput screenings [41, 44, 121]. Although iPSC-CMs hold great promise for recapitulating relevant organ features, their immaturity remains a major obstacle. Recent advances in techniques to mature cardiomyocytes derived from iPSC-CMs are bringing the applications of these cells in regenerative medicine and tissue engineering closer. Structural, functional and electrophysiological differences between immature iPSC-CMs and mature cardiomyocytes require the development of new strategies for their maturation. In the last few years, a broad repertoire of studies has identified key effectors of iPSC-CMs maturation, including biochemical factors, changes in metabolic pathways, mechanical and electrical stimulation, the role of the extracellular matrix, and three-dimensional culture systems. It is expected that the use of an integrated approach, combining multiple iPSC-CM maturation factors, will lead to a fully functional heart cells that can integrate into native tissue. Only a comprehensive and multidisciplinary path will be able to shape the field of cardiovascular research, deliver personalized disease models and regenerative therapies. However, further studies are required to improve the critical area of maturation techniques and to ensure the long-term functionality and safety of therapeutic iPSC-CM-based platforms. In order to advance these therapies clinically, we are still eagerly seeking more robust cell retention, stimulation of vascularisation, inclusion of novel scaffolding and electroconductive materials, or other bioengineering approaches [120, 122]. Furthermore, the ongoing refinement of methods to increase the maturity of these cells, together with their potential for drug screening and more appropriate disease modelling, suggests a future where iPSC-based therapies could become a routine part of clinical practice and truly revolutionize the treatment of heart diseases.

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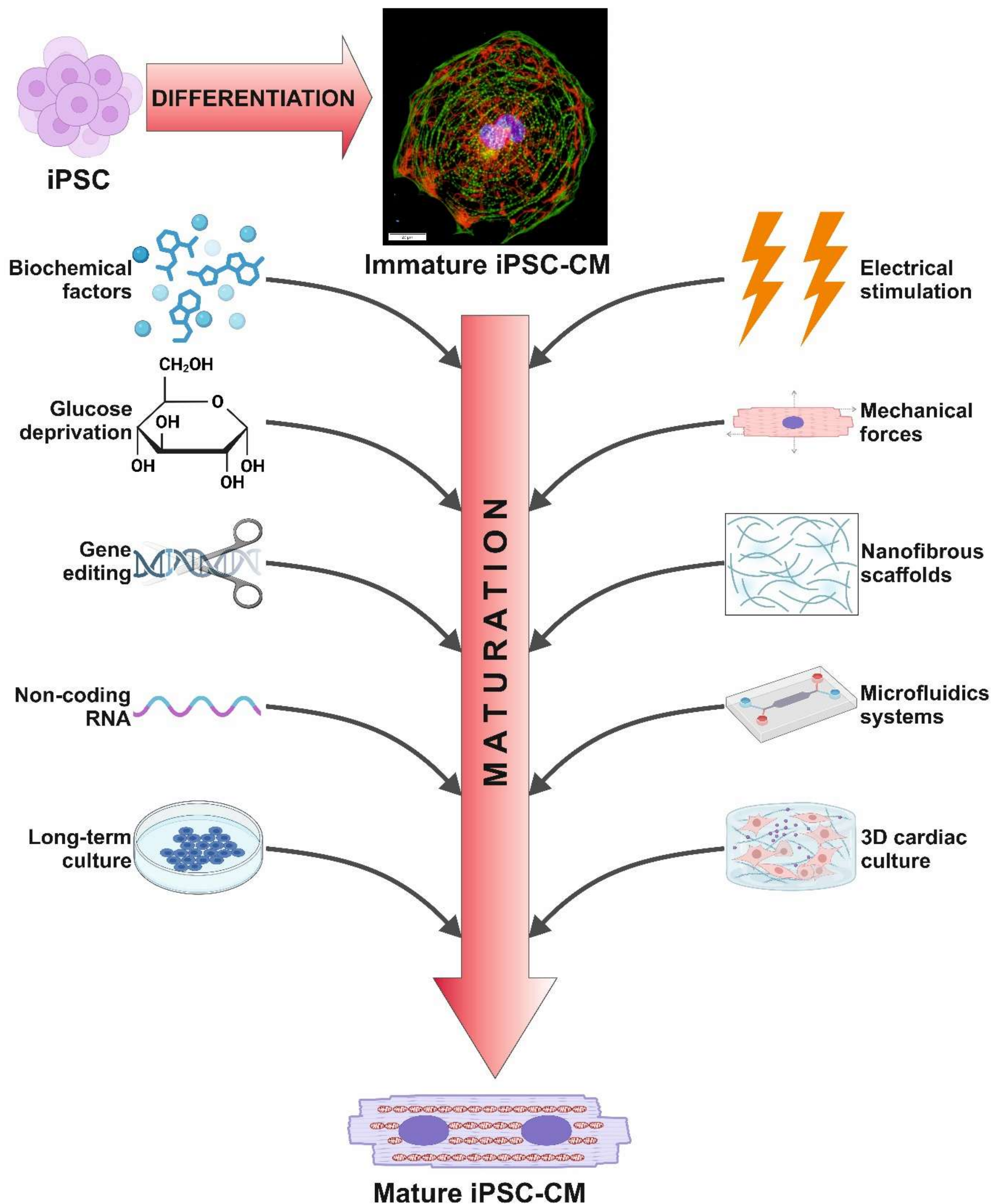

Figure 1. Latest developments in strategies to improve induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) maturation. Biophysical methods including electrical stimulation, microfluidics devices, nanofibrous scaffolds, mechanical forces, 3D cardiac culture. Biochemical methods including biochemical factors, glucose deprivation (replaced by fatty acids), gene editing, non-coding RNA, long-term culture. Black box shows immunostaining of iPSC-CM cells, blue color — cell nucleus with DAPI; red color — mitochondria with MitoTracker Red CMXRos; green color — F-actin with ActinGreen 488 ReadyProbes Reagent