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Review

Induced Pluripotent Stem Cells Technology and Cardiomyocyte generation: progress, uncertainties and challenges in the biological features and clinical applications.

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Abstract: Human induced pluripotent stem cells (hiPSCs) are reprogrammed cells that have hallmarks similar to embryonic stem cells including the capacity of self-renewal and differentiation into cardiac myocytes. The improvements in reprogramming and differentiating methods achieved in the past 10 years widened the use of hiPSCs, especially in cardiac research. hiPSC-derived cardiac myocytes (CMs) recapitulate phenotypic differences caused by genetic variations, making them human attractive disease models and useful tools for drug discovery and toxicology testing. In addition, hiPSCs can be used as source cells for cardiac regeneration in animal models. Here, we review the advances in the genetic and epigenetic control of cardiomyogenesis that underlies the significant improvement of the induced reprogramming of somatic cells to CMs. We also cover the phenotypic characteristics of the hiPSCs derived CMs, their ability to rescue injured CMs through paracrine effects, the novel approaches in tissue engineering for hiPSC-derived cardiac tissue generation, and finally, their potential use in biomedical applications.

Keywords: regenerative medicine; reprogramming; cardiac differentiation; secretoma; tissue engineering

0. How to Use This Template

1. Introduction

Cardiovascular disease (CVD) and heart failure (HF) still represent the major causes of mortality and morbidity in the Western world [1]. CVD and HF can arise from myocardial infarction (MI) [2], chemotherapy-derived cardiotoxicity [3], and congenital defects [4] affecting cardiac function. The pathological basis is mainly related to the very limited ability of the heart to withstand injury and aging, which is due to insufficient cardioprotection combined with almost the complete lack of myocardial renewal. In such scenarios, cardiac transplantation still represents the ultimate therapeutic option for HF, although it is severely

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hindered by the short supply of available donor hearts. This also translates into an economic burden for national health institutions, as more than a million hospitalizations due to HF are annually reported in the EU alone [4]. Cell-based cardiac tissue engineering strategies could provide regenerative therapeutic options and if these strategies utilize autologous cells, the limitations derived from biocompatibility and immune response would be surmounted. Recently, the development of reprogramming technology in 2006 in Yamanka' lab. [5] and the knowledge acquired in the cardiac specification and differentiation makes the potential replacement of the lost cardiomyocytes (CMs) more feasible. Indeed, the ability of human induced pluripotent stem cells (hiPSCs) to differentiate into autologous tissue-specific cells, similar to embryonic stem cells (ESC), but without the need to destroy a human embryo, is an important breakthrough in human stem cell biology [6]. A number of pre-clinical studies have explored the effects of intramyocardial injection of hiPSCs derived cardiomyocytes into murine and porcine models of MI (a complete recent list of pre-clinical studies is provided in Lalit et al. [7]). Nelson et al. [8] showed that the intramyocardial injection of iPSC-derived CMs into a murine model of acute MI determined an improvement in the clinical outcomes four weeks after permanent coronary artery ligation. Thereby, hiPSCs have demonstrated significant potential as a tool in regenerative medicine.

Here, we review the recent advances in our understanding of the induced reprogramming of somatic cells to CMs. Starting from the growing understanding of heart development and from new insight in the epigenetic control of cardiac differentiation, we covered the progressions obtained in the cell culture approach and in the differentiation methods, the analysis of the secretoma of the hiPSCs differentiated cell, and the new advances in hiPSC-derived bioengineered cardiac tissues.

2. Regulatory Pathways and Epigenetic Control of Cardiomyogenesis

Since hiPSCs can play a role in the therapeutic approach of CVD, a comprehensive understanding of the regulatory pathways that expand and functionally differentiate cardiac cells from their multipotent mesoderm precursors is required. Advances in cardiac progenitor

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cell biology are relevant, indeed, for the development of translation studies employing hiPSCs derived cells, since the possibility to obtain a near homogenous population of cardiac cells should help to minimize teratoma formation following cell transplantation.

The major steps of heart development are conserved between humans and other mammalians. This step by step complex consists of a conserved regulatory network of transcription factors and signaling pathways that control specification, maturation, and maintenance of each of the multiple highly specialized myocardial lineages (ventricular, atrial, and conduction system cells) (Figure 1).

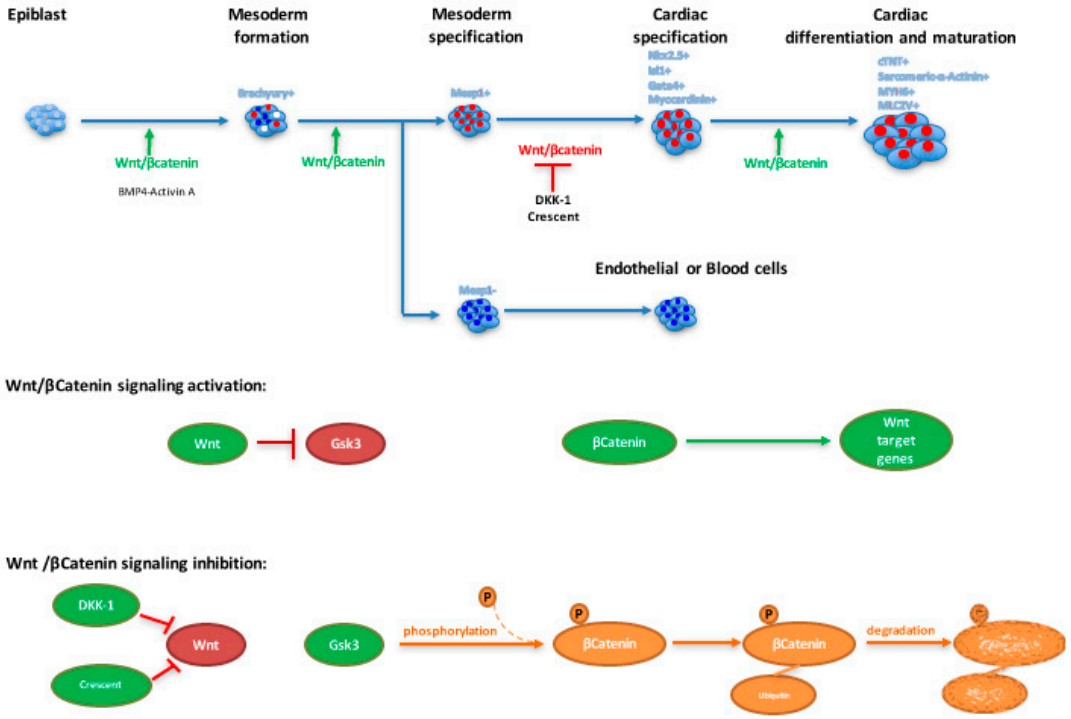

Figure 1

Figure 1: Scheme of current knowledge about the Wnt signaling involvement in cardiac differentiation of hiPSCs. The Wnt signaling has a biphasic role: it must be active in the induction of the mesoderm formation and in the proliferation of the committed cells; than there is a critical switch with the inhibition of the Wnt pathway that is essential for the cardiac development. The inhibition of the Wnt/beta catenin signaling is required to instruct committed progenitor cells to leave the proliferative state and to start differentiating.

2.1. Mesoderm Induction, Cardiac Specification, and Differentiation

During embryonic development, the formation of the nascent mesoderm requires the spatially and temporally regulated expression of Wnt, BMP, and Nodal/Activin pathway

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molecules. These factors regulate the entrance and the migration of the epithelial cells in the gastrulating epiblast, resulting in the generation of mesodermal cells marked by the expression of Brachiury T (Bry) [9].

Subsequent fate restriction of mesodermal precursors toward CV and hemopoietic progenitors can be identified by the expression of other specific factors. Indeed, the heart forms soon after gastrulation in the anterior mesoderm adjacent to the endoderm, whereas blood cells arise from the posterior mesoderm. Since bone morphogenetic proteins (BMPs) 2 and 4 are expressed in the lateral endoderm along the entire anterior-posterior axis, whereas heart induction is restricted to the anterior part, this implies that additional factors are required for the cardiac commitment of the undifferentiated mesodermal cells. One key gene in heart development is the mesoderm posterior 1 (Mesp1), which is considered the "master regulator" of cardiac progenitor specification [10–12]: in fact, it drives cardiac differentiation via the DKK1-mediated inhibition of Wnt signaling [12]. Mesp1 has been correlated with the definitive cardiac commitment by activating the expression of CV lineage defining transcription factors such as Nkx2.5, Isll, and myocardin.

Once these myocardial precursors have fully committed, it is necessary to activate the Wnt pathway to facilitate the full maturation into differentiated CMs [9,10,12].

2.2. The Wnt Signaling

The neuronal tube and the adjacent notochord are potent sources of signals that repress cardiogenesis in the neighboring mesoderm. In particular, *Wnt* genes are highly expressed by the neuronal tube. Wnt proteins bind the frizzled receptors that block glycogen synthase kinase-3 (GSK3). This enzyme, when active, phosphorylates the β-catenin, resulting in its degradation by ubiquitin-mediated proteolysis. Thereby, Wnt signaling blocking GSK3 activity prevents the degradation of β-catenin that is able to move from the cytoplasm to the nucleus where it activates the Wnt target genes. In this way, Wnt signaling blocks cardiogenesis in the posterior mesoderm. On the other hand, Wnt signaling must be blocked to permit the heart development from the Mesp-1^{+} cells in the anterior mesoderm. Crescent is a family of proteins that share homology with the extracellular part of the Wnt receptor.

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Crescent is present in the anterior part of the mesoderm where another Wnt antagonist, DKK-1 is also expressed: in this way, the anterior mesoderm becomes permissive for heart formation interfering with the signal of Wnt [13,14].

2.3. Epigenetic Regulation of Human Cardiac Differentiation

During cardiac differentiation, cells express specific genes in a temporal and spatially accurate manner. The development of the mammalian heart is indeed dependent on the activation of a gene program regulated by specific histone modifications, nucleosome remodeling, and DNA methylations [15]. The epigenetic modifications that occur across the genome induce a chromatin pattern that is coordinated with the stage-specific expression of cardiac genes. This temporal evolution of histone modifications is a chromatin "signature" [16,17].

Table 1 summarizes the recent advances in epigenetic control of human cardiogenesis and cardiac differentiation. The histone modifications mainly include methylation or acetylation/deacetylation (by histone acetyltrasferases or HAT and deacetylates or HDAC), whereas the DNA methylation involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring by DNA methyltransferases (DNMTs). Short stretches of CG are often found at the gene promoter and their hypermethylation can facilitate the methyl binding domain association and the recruitment of chromatin remodelers for gene silencing and repressive histone modifiers [18]. Gilsback et al. investigated DNA methylation in murine CMs and ES cells as a model for undifferentiated cell type. They showed that CMs have a short region of low DNA methylation in comparison with ES and these demethylated regions contained binding motifs for tissue-specific transcription factors. Some of the longest demethylated regions were identified in the cardiac ryanodine receptor (RYR2), titin (TTN), and in the α_{1C}-subunit of the L-type Ca^{2+} channel [19]. The role of epigenetic factors in controlling the cardiac lineage differentiation and specification is widely described and exploited to improve the cardiac differentiation of hiPSCs. 5-Azacytidine, an inhibitor of DNA methylation, promotes cardiac differentiation in ES and adult mesenchymal stem cells [20].

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Recently, the long noncoding RNAs (lncRNAs), which are transcripts longer than 200 nucleotides that are not translated into protein, have gained widespread attention as potentially new and crucial regulators of cardiac differentiation [21]. In particular, lncRNA Braveheart seems to play a central role in cardiac differentiation, stimulating and maintaining CMs lineage commitment [21–24], while the lncRNA Fendrr, expressed specifically in embryonic lateral mesoderm, regulates heart development, most likely by modifying the chromatin signature of genes encoding transcription factors that direct cardiomyocyte differentiation [25].

The post-transcriptional regulation of the cardiac gene program also involves the microRNAs (miRNAs), non-coding RNAs of about 22 nucleotides in length that generally interact with the 3' untranslated region (3'UTR) of mRNAs target. This mode of pairing usually negatively regulates the translation of the target through the repression of the initial ribosome binding to the mRNA or the ribosome drop-off [21]. The analysis of miRNA expression in human cardiomyocyte progenitor cells (CMPCs) showed that 188 miRNAs were detectable in proliferating CMPCs and 195 in differentiated CMPCs such as miR1, miR1-2, miR499, miR322, miR503, miR208, miR133, and miR26b [26–30]. MiR-208, together with miR-1, miR-133, and miR-206, are called myomiRs as they are expressed specifically in the heart and skeletal muscles. While miR-208 is expressed only in the heart, mir-206 is skeletal muscle specific [31]. Recently, the role of the miRNAs (in particular the let-7 family) during CM maturation has been also described [32].

Table 1: Recent advances in epigenetic control of human cardiogenesis and cardiac differentiation.

Epigenetic modifications	Name	Action	Reference
Histone acetylation	Histone acetyltransferase (HAT)	**P300** is essential for cardiac development. It contributes to Gata4, Srf, Mef5c expression. P300 knockout mice are embryonically lethal	[33]
	Histone deacetylase (HDAC)	Mice lacking both **HDAC1** and **HDAC2** show neonatal lethality due to arrhythmias and dilated cardiomyopathy	[34]

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	Inhibitors of HDAC	**Trichostatin A** promotes cardiac differentiation increasing expression of Gata4, Mef2c and Nkx2.5	[20]
Histone methylation	Histone methyltransferases (HTMs)	Loss of HMT **Smyd1** is embryonic lethal, because mice show right ventricular hypoplasia and impaired cardiomyocyte maturation.	[21]
		HTM **WHSC1** is involved in Nkx2.5 repression via H3K3me37.	
	Histone demethylase (HDMs)	The HDM **UTX** removes H3K29me3 activating the cardiac transcription factors Gata4, Nkx2.5, Srf, Tbx5. Mice lacking UTX show severe heart malformation.	[35]
	H3K4me and H3K27	**H3K4 methylation** levels are fundamental in murine CMs. A loss of H3K4 methylation can result in intracellular calcium modifications and increased contractility	[17]
		FGF19 and NODAL genes show high levels of **H3K4me3** and **H3K27me3** in undifferenziated ESC and low levels during the differentiation	[16]
		Cardiac transcription factorsGata4, Wnt2, Tbx2, Nkx2.5 show high levels **H3K27me3** during the pluripotency that decrease during differentiation, in the same time there is a gradual increase in **H3K36me3** and **H3K4me3**	
		Wnt, Hedgehog, TGFβ family, VEGF, FGF family, PDGF(pathways involved in cardiac differentiation) show a stage-specific repression by **H3K27me3** and activation by **H3K36me3** and **H3K4me3**	
DNA methylation	DNA methyl transferase (DNM)	**DNMT1** expression decreases from mesoderm to CM stage while **DNMT3A** icreases from ESC to primitive mesoderm stage. WNT and TGF-β genes undergo promoter methylation changes, the latter pathway became hypomethylated and upregulated in CM stage, whereas generally WNT genes acquire promoter methylation	[36]
	Inhibitors of DNA methylation	**5-Azacytidine** promotes cardiac differentiation in ES and adult mesenchymal stem cells	[20]
Long non coding RNA	Braveheart	**Braveheart** is an activator of Mesp1, Gata4, Nkx2,5, TBx5, Hand1. Braveheart acts upstream Mesp1 and regulates the temporal activation of cardiac genes through modulation of Mesp1 itself	[23]
		Braveheart interacts with SUZ12 that acts as a histone methyltransferase.	
		Braveheart induces the differentiation of murine bone-marrow-derived mesenchymal cells into cells with a cardiogenic phenotype. It increases sarcomeric α-actin and cardiac troponin T expression and the upregulation of Gata4, Nkx2.5, Isl-1 and Mesp1.	[24]
	Fendrr	**Fendrr** Interacts with PRC2 and Trg/MLL complex to modulate the chromatin signature of pitX2 and Foxf1.	[25]

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		Loss of **Fendrr** affects the expression of Nkx2.5 and Gata4. Fendrr knockout is embryonic lethal in mice due to defect on the heart septum .	[25]
MicroRNAs	miR-1, miR-499	**miR-1** controls myogenic differentiation in mouse heart	[26]
		miR-499 is a cardiac specific miRNA	
		miR-1 and **miR-499** enhance the cardiac differentiation of cardiomyocyte progenitor cells, probably targeting Sox6 with a consequent increasing of α-cardiac actinin and cardiac troponin T	
		Inhibition of **miR-1** and **miR-499** blocks cardiac differentiation.	
	miR-322/-503 cluster	**miR-322/-503 cluster** encodes in an intergenic region on the X-chromosome and increases Nkx2.5, Mef2c, Tbx5, α-MHC inducing CM differentiation, probably targeting Celf1, whereas their deletion reduces the expression of cardiac markers	[29]
		miR-322/-503 cluster acts by the repression of their target Celf1, that lead the ESC to the neuronal differentiation: it is likely that the miR-322/-503 cluster promotes the cardiac differentiation impairing the neuronal through Celf1 inhibition	
	miR-208	**miR-208** is involved in the regulation of myosin heavy chain isoform switch during developmental and pathophysiological condition.	[31]
	miR-1-2	**miR-1-2** induces cardiac differenziation of murine bone marrow-derived mesenchymal stem cells by Wnt signalling pathway	[30]
		Trasfection with **miR-1-2** increases expression of Nkx2.5, Gata4, cTnI	
	miR-133	**miR-133** together with Gata4, Tbx5 and Mef2c improves cardiac reprogramming from human or murine fibroblast, by repressing Snai1	[26,28]
	miR-26b	**miR-26b** promotes cardiac differentiation of P19 cells, by regulating canonical and non-canonical Wnt parhway. It represses the expression of Wnt5a and Gsk3β	[27]
	let-7	**let-7** family is upregulated during in vitro human cardiac differenition.	[32]

		The overexpression of members of let-7 family for 2 weeks in hESC derived CMs increases contractile force, cell size, sarcomere length and action potential duration. Knockdown of let-7 results in a reduction of sarcomere length and expression of cardiac maturation markers. Let-7 family probably acts downregulation two of its targets, IRS2 (a member of insulin signaling pathway) and EZH2 (a histone methytrasferase that can regulate gene expression)	

2.4. The "Epigenetic Memory" in hiPSCs Differentiation Potential

Although hiPSCs can be generated from different somatic cells (fibroblasts, peripheral blood cells, keratinocytes), they maintain a residual DNA methylation signature transmitted from the parental cells, known as "epigenetic memory", leading them to differentiate preferably into their original cell line [37–39]. Sanchex-Freire studied the contribution of epigenetic memory on the differentiation potential and maturity of hiPSCs derived from cardiac progenitor cells (CPC-hiPSCs) and dermal fibroblasts (Fib-hiPSCs). They found that Fib-hiPSCs had higher methylation levels of a region immediately upstream of the first coding exon of Nkx2.5 when compared to CPC-hiPSC. This evidence seems to suggest that the incomplete resetting of the pre-existent epigenetic state contributes to increased differentiation efficiencies and to the enriched cardiac gene expression observed in CPC-hiPSCs [40].

3. Generation of CMs from hiPSCs Culture

The number of protocols that derive CMs from hiPSCs have increased exponentially over the past decade and the differentiation protocols were modulated to generate mainly atrial-, ventricular-, and nodal- like CM subtypes. Important advances have been achieved in chemical-based cardiac differentiation, cardiac subtype specification, large-scale suspension culture differentiation, and the development of chemically defined culture conditions. These protocols of hiPSCs require key steps for the differentiation progression that have already been thoroughly reviewed [9,41,42].

In vitro differentiation of hiPSCs into CMs, regardless of the methodological approach, should mimic the sequential steps of *in vivo* embryonic cardiac development

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providing temporal administration of molecules that regulate specific signaling cascades: the activation of the canonical Wnt signaling induces the early primitive streak/mesendoderm stage and the following inhibition of the same pathway at a later stage allows it to achieve the cardiac mesoderm specification [9,41,42].

Three main culture approaches have been described for small scale hiPSCs-CMs generation: (i) the co-culture of the hiPSCs with the inducing visceral endodermal cell line END-2. This was the first system used, but was also the least efficient one [43]; (ii) the embryoid body formation assay (EB) based on a three dimensional (3D) aggregation system; and finally (iii), the monolayer culture system used in many labs even if with different protocols. Large-scale cell cultures rely on culturing cells in dynamic suspension systems such as spinner flasks and bioreactors.

3.1. EB Formation-Based Differentiation Protocol

EBs are round, multi-cellular, 3D aggregates formed by hiPSCs and are able to differentiate into cells of all three germ layers including beating cardiomyocytes with low efficiency in the presence of fetal bovine serum [44,45]. In 2008, Yang et al. established a three-step serum-free protocol characterized by subsequent supplementation of several cytokines (Activin A, BMP4, VEGF, DKK1, and bFGF) that resulted in a more efficient differentiation of the EBs into CMs. It became evident, however, that each hiPSC line needed optimal concentrations and timing of the administration of Activin A and BMP4, which are the factors responsible for the crucial step of mesodermal induction.

Recently, the discovery of the biological effects induced by the small molecules pushed scientists to apply them in stem cell biology. Karakikes et al. increased the efficiency of beating iPSCs-CMs production by modifying Yang's protocol [46] with the addition of the small molecules. Interestingly, the small molecule IWR-1, an inhibitor of the Wnt signaling pathway, caused all CMs to exhibit a typical ventricular-like phenotype, while the application of recombinant protein DKK1 generated a heterogeneous population that consisted of atrial-, ventricular-, and nodal-like phenotypes [47].

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However, the lack of uniformity in EB size, resulting in nonhomogeneous and asynchronous differentiation of the residing cells may hamper their effective employment in regenerative medicine. In 2007, Burridge et al. modified the CMs differentiation protocol based on EB formation by introducing the forced aggregation technique [48], which was later improved using different engineered 2D and 3D technology to obtain controlled-size EBs [49,50].

More recently, Zhang et al. combined the various specific advantages of existing protocols: EBs formation was performed by forced aggregation in serum-free medium supplemented with growth factors and small molecules. Interestingly, cardiac differentiation in the serum and serum/albumin-free basal media was improved by insulin supplementation during EBs formation, that resulted in 100% beating EBs, which were mostly ventricular or early ventricular-like cells. This differentiation method was easily translated to large scale CM differentiation by the generation of EBs and subsequent differentiation in static or dynamic suspensions [51].

3.2. Monolayer Culture-Based Differentiation Protocol

Although the 3D EBs format reproduces some aspects of the *in vivo* tissue architecture, the monolayer format is generally considered more reproducible and, in principle, a more suitable approach for the scale-up for clinical purposes. A 2D system guarantees, indeed, a more homogeneous exposure of the cultured cells to the soluble environment and might thus contribute to reducing the differences in the quality and quantity of CM differentiation between different cell lines. The first monolayer culture–based CMs differentiation of hESCs was performed by Laflamme et al. [20]. They cultured high-density undifferentiated hESCs as a confluent monolayer on Matrigel-coated plates and cells were sequentially treated with Activin A and BMP4; however, the differentiation efficiency was low and not successfully applicable to numerous other hESC or hiPSC lines. Improvements were then obtained with a Matrigel sandwich that facilitated epithelial–mesenchymal transition: layering on the cells a Matrigel overlay one day before the addition of the differentiation medium and maintaining it during induction with activin A, bFGF, and BMP4

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247 resulted in CM differentiation efficiencies of up to 98% of cTNT positive cells [52].

248 Recently, Lian et al. [53] and Burridge et al. [54] looked into CMs with high
249 efficiency (>95% cTnT Positive CMs). Both protocols are based on the application of two
250 small molecules, a Gsk3- and a Wnt-inhibitor, that sequentially promote mesoderm
251 formation and CM specification at precise developmental stages. However, unlike Lian's
252 observations, Burridge et al. reported that albumin was necessary for CMs differentiation
253 with high yield and purity. Differences between the two studies included the cell density
254 at the beginning of the differentiation protocol and the concentration and the exposure
255 windows for small molecules. Indeed, Lian et al. reported that the optimal window for a
256 Gsk3-and Wnt-inhibitor was from days 0 to 1 and from days 3 to 5, respectively, whereas
257 Burridge et al. applied the Gsk3 inhibitor from days 0 to 2 and the Wnt inhibitor from days
258 2 to 4. Lian et al. also found that reducing the Gsk3-inhibitor concentration and/or
259 treatment time in the absence of albumin permitted efficient mesendoderm induction
260 without cytotoxicity [21–23].

261 More recently, Parikh et al. [56] found that combining thyroid and glucocorticoid
262 hormones during the cardiac differentiation process on a Matrigel mattress resulted in
263 hiPSCs-CMs exhibiting T-tubule development, enhanced Ca-induced Ca release, and more
264 ventricular-like excitation-contraction coupling.

265 Cao et al. evidenced that hiPSCs cultured with small molecules in combination with
266 growth factors induced the formation of multipotent cardiovascular progenitors that were
267 able to stably self-renew and expand as a monolayer under feeder- and serum-free conditions.
268 Most importantly, these CV progenitor cells retained the potential to efficiently generate
269 CMs, smooth muscle cells, and endothelial cells *in vitro* [57]. The identification and isolation
270 of a cardiac precursor cell population is expected to provide a source of cells for tissue
271 regeneration, while also providing valuable insight into cardiac development.

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4. Morphological and Functional Properties of hiPSCs Derived CMs

Although it has been reported that in general, iPSCs-CMs structurally resemble embryonic or fetal CMs [43,58], it is well known that hiPSCs-CMs' maturity depends on the time in culture. The hiPSCs-CMs phenotype, indeed, is strongly influenced by the timing of the culture and several groups have classified them as "early" and "late" hiPSCs-CMs. The early-phase characteristics are typical of the first month after starting the spontaneous beating whereas the late-phase characteristics develop afterwards [58–60].

Early phase hiPSCs-CMs (within 30–40 days post-induction) resemble embryonic or fetal mammalian CMs appearing as small (cell area: 400–500 μm^2), rounded cells with some proliferative capacity lacking any discernible organized cardiac structure; immunocytochemical staining for α-actinin, a cardiac Z-disk protein, revealed poorly organized contractile machinery, characterized by low myofibril density and orientation, and variable Z-disc alignment [58–60]. They exhibit spontaneous contractile activity [61,62] and are characterized by a small negative membrane potential and small action potential amplitude [63,64]. Over the course of the next two months, hiPSCs-CMs lose their proliferative capacity [65] and change their morphology by becoming larger (cell area: 600–1700 μm^2), more elongated, and with a lower circularity index. Like hESC-CMs, these late hiPSCs-CMs (between days 80 and 120 of in vitro development) demonstrate dramatic increases in the density and alignment of myofibrils throughout the cytoplasm and show repetitive banding patterns characteristic of organized sarcomeres with good registration across the entire width of the cell [58–60]. Different elements of maturity appear to be affected by hiPSCs line [66,67] or culture conditions [61,68]. However, late hiPSCs-CMs never reach either the dimension (cell area: around 1500 μm^2) or the morphology of adult CMs, instead becoming closer to embryonic CMs. Indeed, adult CMs have elongated anisotropic shapes [69] and are aligned in the context of cardiac tissue. *In vivo*, immature CMs are rod-shaped, similar to the adult ones, but when cultured in vitro, the immature CMs flatten and spread in all directions while the adult ones maintain their cylindrical morphology in short term culture [70]. Thus far, hiPSCs-CMs have irregular shapes and they do not

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typically show alignment in two-dimensional cultures. These morphological differences are also reflected by a lower expression when compared to adult CMs of maturation-related sarcomeric genes such as *MYL2, MYH7, TCAP*, and *MYOM2,* and ion transport– related genes such as *KCNJ2* and *RYR2* [43,67,71]. Another aspect that confirms the immaturity of hiPSCs-CMs regards the localization of gap junction components. In adult CMs, these proteins accumulate at the intercalated disks, while in iPSCs-CMs, they are mainly localized at the circumference of the cell, recalling the structure of embryonic CMs [72].

The relative immaturity of hiPSCs-CM also involves the development of the T-tubule network, a key component of excitation contraction coupling: extensive in adult CMs, it is absent in both iPSCs-CMs and embryonic CMs [73]. Since T-tubules allow an adult CM to have rapid electric excitation, initiation, and synchronous triggering of sarcoplasmic reticulum calcium release and, therefore, coordinated contraction throughout the entire cytoplasm, their lack of hiPSCs-CMs results in a lower excitation-contraction coupling, and in unsynchronized Ca^{2+} transients, as reflected by the non-uniform calcium dynamics across the cell and greater calcium peak amplitude in the sarcolemma than in the sarcoplasmic reticulum [74–76]. Thus, early iPSCs-CMs structurally resemble embryonic CMs, while late iPSCs-CMs develop a more adult-like morphology but do not appear to develop T-tubules.

Parikh et al. [56] broke the T-tubule barrier by discovering the appropriate combination of matrix and hormones to generate hiPSCs-CMs with a functional network of T-tubules producing more adult-like Ca^{2+} cycling. Their discovery of T-tubules in hiPSCs-CMs was a step forward, but the promise of adult-like hiPSCs-CMs in a dish has yet to be reached. The T-tubule network, in fact, lacked the abundance and detailed organization found in adult ventricular cardiomyocytes and, although hiPSCs-CMs treated with T3 and Dex on the Matrigel mattress were larger cells, they were still smaller when compared to adult CMs.

Although the rate of contraction may be affected by cell line or culture conditions, the spontaneous and synchronous contraction of hiPSCs-CMs can be maintained over time in culture [77,78]. Interestingly, hiPSCs-CMs differentiated from hiPSCs obtained from

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patients with long QT syndrome showed slower repolarization, thus recapitulating the *in vivo* behavior [79–81].

Common cardiac differentiation protocols produce predominantly ventricular cells with ~15–20% atrial cells and few nodal cells [82] as determined by electrophysiological analysis of action potential [9]. In clinical application, an enriched population of nodal-like cells could potentially be used in the formation of a biological pacemaker, whereas ventricular types may be used for recovery from myocardial infarction. It has been demonstrated that the pharmacological inhibition of NRG-1β/ErbB signaling enhanced the population of nodal-like CMs [83] and that retinoic acid could increase the proportion of atrial-like CMs whereas its inhibition could increase the proportion of ventricular-like cells [84]. Furthermore, it was possible to strongly increase the nodal population by inhibiting the neuregulin signaling using small molecules [85].

It has been demonstrated the hiPSCs-CMs present cardiac specific inotropic and chronotropic receptors, other than the β1 and β2 adrenoceptor response [59,86–88]. Similar to adult CMs, isoprenaline increases both the contraction rate and the amplitude of the calcium transient, and decreases the relaxation time [87]; on the other hand, the observation that, unlike adult CMs, isoprenaline does not affect the contraction force [89] supports the functional immaturity of this cell type.

5. hiPSC Paracrine Effects for Cardiac Repair and Regeneration

Recent work has demonstrated an endogenous restorative program within the heart based on the restoration of the biological modulatory activity of cardiac progenitor cells [85,89] along with resident cardiomyocyte proliferation [64], although these mechanisms become unexpressed soon after birth, leaving the heart with limited repair potential in pathological situations. Therefore, a working strategy is urgently needed to restore in full the potential for both cardiac repair and regeneration.

In this scenario, growing interest has been driven to the so called “stem cell-derived paracrine effect” as a putative working strategy to restore such dormant mechanisms of cardiac restoration. Indeed, it is now well accepted that either autologous or allogeneic

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transplantation of different populations of stem cells into the injured heart results in quite limited differentiation, while providing overall significant improvement in heart function [90]. Thus, the beneficial effects obtained following an injection of stem cells seem to be mainly due to their modulatory paracrine effects [91]. As a matter of fact, several studies have supported the paracrine hypothesis by reporting successful reduction of infarct size and improvement of angiogenesis and cardiac output that are most likely attributable to the release of soluble factors, rather than de novo cardiomyogenesis by the engrafted cells. Hence, the detailed analysis of the stem cell "*secretome*"—as the whole of growth factors and chemo-attractant molecules produced by paracrine secretion—has gained growing attention and the quest is now to identify the most suitable cell candidate to obtain the ideal paracrine cocktail of factors to be delivered to the injured myocardium. Several populations of stem cells have been evaluated, with adult somatic mesenchymal stromal stem cells (MSC) isolated from different tissues being the most investigated. However, while adult MSC may represent a feasible option, given the ease of their collection from clinical waste material, (i.e., adipose tissue harvested during surgical procedure) they present several limitations such as low yield, invasive sampling, and controversial self-renewal, all aspects that may limit their therapeutic application. In contrast, hiPSCs may offer a valuable choice to overcome these limits given their pluripotency, high self-renewal, and embryonic stem cell-like properties. Most studies involving the use of hiPSCs for *in vivo* cardiac regeneration have focused on the exploitation of their cardiomyogenic differentiation potential. Nonetheless, hiPSCs have also been recently described as playing a significant role in modulating the cardiac microenvironment by mediating pro-survival effects while improving cardiac function and homeostasis via secretory mechanisms of action, thus suggesting a remarkable paracrine potential. Indeed, recent work from Singla et al. has shown that hiPSCs systemically transplanted into a preclinical mouse model of diabete-induced cardiomyopathy contributed to the increase in antioxidant levels, and counteracted adverse cardiac remodeling while improving cardiac function by acting on the Akt, ERK1/2, and MMP-9 signaling pathways via multiple paracrine mechanisms [92]. Likewise, when considering a chemotherapy drug-derived murine model of cardiomyopathy, as the one obtained by regular

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administration of the oncological drug doxorubicin, a well-known cardiotoxic agent, hiPSCs transplantation following ischemic injury resulted in the decrease of cardiac apoptosis and interstitial fibrosis via paracrine modulation of Notch-1 signaling [93]. Further studies have highlighted the potent paracrine effect of the secretome of hiPSC-derived MSC as specifically enriched with macrophage migration inhibitory factor (MIF) and growth differentiation factor-15 (GDF-15) citokines; indeed, the iPSC-derived-MSC-conditioned medium was shown to exert remarkable cardioprotective effects on neonatal rat CMs and murine cardiac tissue against anthracycline-induced cardiomyopathy [94].

The hiPSCs secretome has also been used to prime endogenous progenitor cells such as cardiac mesenchymal stromal cells (cMSCs) to enhance their proliferative, survival, and CV differentiation potential. In particular, human hiPSCs-secreted extracellular vesicles/microvesicles mediated the horizontal transfer of genetic cargo (mRNA and microRNA) resulting in transcriptomic and proteomic reprogramming of the target cells, suggesting the promising therapeutic potential of this approach [95].

In the last few years, we have witnessed the dramatic and rapid expansion of hiPSC biology and preclinical application of hiPSCs-cell derivatives for future therapy. Nonetheless, several reports have indicated limited engraftment of hiPSCs-CMs when transplanted *in vivo*; yet, there is evidence of improvement of resident cell survival and local angiogenesis along with remarkable decrease of fibrosis and inflammation, following injury. These results are likely to be due to paracrine modulatory effects exerted by the transplanted hiPSCs-CMs on the neighboring resident cells via the secretion of biologically active extracellular vesicles including exosomes [96]. Indeed, immunosuppressed mice experiencing a myocardial infarction showed better outcomes when transplanted with hiPSCs-CMs when compared to undifferentiated cells, despite the poor cell engraftment in both treated groups, suggesting differential paracrine effects, with differentiated cardiac lineage cells contributing more significantly via the secretion of promigratory, proangiogenic, and antiapoptotic mediators [97].

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In light of such evidence, hiPSCs can offer an appealing therapeutic tool for future cardiac regenerative medicine via the combined advantage of their pluripotency and peculiar paracrine potential.

6. Advanced Technologies and Tissue Engineering: Novel Approaches for hiPSC-Derived Cardiac Tissue Generation.

In parallel with the revolution brought by the use of hiPSCs, the advances in cell culture techniques and methods have led to more innovative approaches to personalized medicine. Engineering approaches to stem cell cultures can open doors into previously inaccessible scenarios and poorly understood biological phenomena. Although still key for biological discoveries, standard culture techniques in Petri dishes cannot be fully representative of the mammalian *in vivo* complexity. This is due to a series of limitations such as: (i) 2-dimensional (2D) growth; (ii) poor mimic of *in vivo* structure and substrate compliance; and (iii) batch-wise operations (media change, addition of drugs or other factors, etc.) that result in unpredictable kinetics, poorly defined timescales, and the lack of precise patterns of stimulation [98]. In contrast, advanced "bioreactor-based" culture techniques have overcome such limitations and offer a series of main undeniable advantages [99]. Focusing on the microscale, on devices with reduced size and features ranging from a few microns to a few centimeters, we gain: (i) feasibility of working in 3D; (ii) better mimic of the *in vivo* microenvironment (we are closer to the characteristic sizes of cells and extracellular structures); (iii) steady state conditions, translating into operating parameters that are constant in time and are kept at well-defined values, and in the possibility of introducing precise spatial and temporal patterns of stimulation, and (iv) increased throughput [100,101]. Focusing on transport phenomena, when using dynamic systems (where culture media flows through the device) we can control both convection and diffusion, thus enabling the generation of complex patterns of stimulation on the cellular microenvironment. The microscale here plays again to our advantage, as the typical length scales and flow rates determine the establishment of a laminar regime, one where velocity and concentration profiles are more easily controlled and modeled/predicted [102]. Finally, a downsizing of

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culture systems results in reduced costs and the number of cells and reagents used, an important advantage especially for experiments involving hiPSCs and hiPSCs-CMs.

Nowadays, heart-on-chip technologies are developing at a fast pace thanks to the strong drive towards improving the drug development process and reducing multi-billion USD losses due to late stage failures (reviewed in Conant [103]). Novel technological approaches involving heart cells/tissues are fundamental for two main reasons: (i) the need for new and improved drugs for heart conditions and, (ii) the need for earlier and more effective evaluations of cardiotoxicity of other drugs.

Engineering the culture systems also allows for the introduction of physiologically relevant stimulations such as electrical, mechanical, topographical, and so on. Since hiPSC-derived cardiac tissues tend to have immature phenotypes, electrical stimulation—a known strong effector for cardiomyocytes maturation—has often been used in conjunction with advanced technological solutions to push cell maturation [104,105]. Biowires™, as an example, is derived from the combination of hiPSCs-CMs, 3D cell cultivation systems, and electrical stimulation specifically tailored to generate tissues with more mature structural and electrophysiological properties [106].

Among others, Healy's group developed an interesting microphysiological system (MPS) that integrated the use of hiPSC-derived CMs with advanced microfluidic platforms that has proved useful in pharmacological studies [107]. Their design ensured that cells self-organized into an aligned 3D micro-tissue and enabled the generation of tissue-like gradients of drugs in a shear-protective environment (Figure 2A), mimicking that provided by the endothelial barrier. hiPSCs-derived CMs organized and started to spontaneously contract within seven days of culture, and their movement could be tracked and measured online with non-destructive imaging techniques (Figure 2B). The authors successfully used their MPS to test the cardiac response of four model drugs and obtained data showing half maximal inhibitory/effective concentration values that were more representative of whole organ responses than those typically obtained at the cellular scale. Overall, their results suggested how these approaches could significantly improve the outcomes of *in vitro* screening studies of drug efficacy and cardiotoxicity.

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Great efforts are also being devoted towards the development of heart-on-chip technologies to model human disease. In a relevant example of this matter, Wang et al. used advanced technologies to gain insight into the pathophysiology underlying the frequent cardiomyopathy experienced by patients affected by Barth syndrome (BTHS), a mitochondrial disorder [108]. Their bioengineered microchips were based on thin elastomer films that functionalized with micropatterned thin strips of fibronectin (rectangles ~100 x 15 μm length x width, and lines 15 x 2 μm width x spacing). BTHS hiPSCs- CMs, when seeded on top of these films were organized following the pattern dictated by the adsorbed protein, generating a laminar anisotropic myocardium. Their dual approach allowed: (i) the quantification of the contractile properties of the engineered myocardial tissues (Figure 2C); and (ii) the evaluation of the phenotypic maturation of the constructs based on the analysis of sarcomeres and fibrous structures organization (Figure 2D). The main results proved that: (i) engineered BTHS hiPSCs-CMs micro-tissues exhibited impaired sarcomere assembly, and (ii) they correctly recapitulated the pathophysiology of BTHS cardiomyopathy by developing significantly lower twitch and peak systolic stress, both when compared to the controls. Finally, and most importantly, the authors proved that engineered tissues effectively modeled disease correction showing restored contractile function after treatment with TAZ modRNA.

In a concerted effort, a consortium led by top scientists across the US is actively working to develop an integrated microphysiological platform, HeLiVa, capable of reproducing the complexity of the “whole body” [109]. HeLiVa is an integrated heart–liver–vascular system for drug testing in human health and diseased settings. The micro-tissues are produced starting from a single line of human pluripotent stem cells (and are thus patient-specific), and the platforms are compatible with real-time biological readouts. Once again, the technology-enabled production of functional human tissue units and their use in studies seeking to measure physiological responses to known or pipeline drugs, greatly benefit from their higher biological fidelity and can be transformative to drug screening and the modeling of disease.

In recent years, there has also been a great drive towards the birth of start-ups and university spin-offs devoted to the fast translation of laboratory-scale discoveries to their wider adoption and application. An example is uSTEM, a joint venture whose mission is to develop and provide leading-edge stem cells manufacturing and cellular reprogramming services. uSTEM is proprietary of an extremely efficient microfluidic cell reprogramming method [110] that has been successfully applied to hiPSCs differentiation to hepatocytes, cardiomyocytes, and neurons. Another example is TARA Biosystems, a company dedicated to pioneering predictive cardiac tissue models that enable faster, safer, and more reliable development of new medicines. TARA's technology is based on the Biowire™ platform [106], a next-generation technological solution capable of generating *in vitro* human cardiac models mimicking adult heart muscles.

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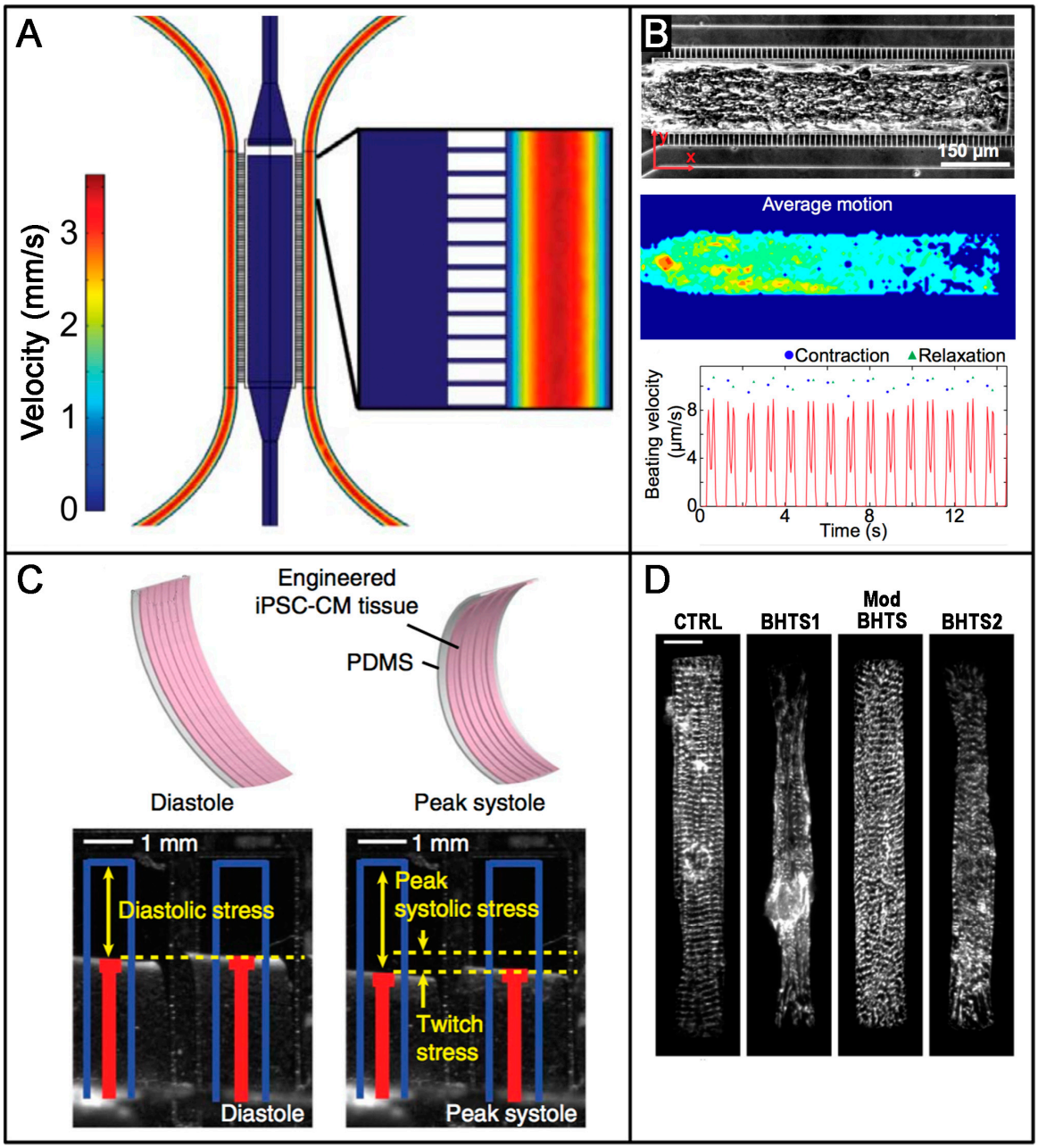

Figure 2 A. Schematic of the device: the central cell loading channel is connected to the lateral C-shaped medium-delivering channels by a "ladder" of thin microchannels, purposely design to protect cells from shear and switch to a diffusive mass transport regime. The colors are representative of the linear velocities on a chosen plane, and show decreasing values from red to blue. B. Characterization of the 3D cardiac tissue formed in the middle channel. Top: optical microscopy image showing tissue density and overall organization and alignment; middle: heat map of the average motion generated by the contractile activity and, bottom: corresponding average beating kinetics. Adapted with permission from Mathur et al.[111] C. Top: schematic representation of contracting constructs and approach to measurements; Bottom: iPSC- CMs seeded onto thin elastomers with patterned lines of fibronectin self-organized into microscaled myocardial tissues and exhibited contractile properties in response to electrical stimulation. D. Representative images showing actinin staining of

iPSC-CMs on micropatterned fibronectin rectangles. BTHS iPSC-CM micro-tissues show impaired sarcomere organization (BTHS1 and 2 in Galactose and Glucose medium, respectively), while cells transfected with TAZ modRNA (Mod BHTS) clearly demonstrate a rescued organization, comparable to that of the control cultures (CTRL). Adapted from Wang et al. [112]

7. The Promise of hiPSCs-CMs in Biomedical Applications.

hiPSCs demonstrate pluripotency, the ability to self-renew, and are patient-specific. Based on these features, hiPSCs are expected to be applicable in drug discovery, disease modeling, and cell therapy.

Screening for drug discovery and cardiotoxicity testing. Despite the immature phenotype of hiPSC-CMs, extensive pharmacological validation confirms the potential utility of hiPSC-CMs in drug evaluation [113]. Burridge et al. showed that patient specific hiPSCs-CMs can recapitulate individual propensities to doxorubicin induced cardiotoxicity [114]. Safety in pharmacology is important as the heart is sensitive to the side effects of drugs. In fact, drugs that cause heart damage as a side effect have been implicated in around 30% of drug withdrawals in the US over the past 30 years [43]. On the other hand, experimental drug candidates have been used for *in vitro* improvement of diseased CMs derived from hiPSCs models.

CV disease modeling. Since hiPSCs generated from individuals with genetic disorders maintain these anomalies [115], patient-derived hiPSCs are candidates to model the molecular basis of pathologies. Recently, hiPSCs have been modeled for several cardiac pathologies including familial dilated cardiomyopathy [116], Barth syndrome [112], arrhythmias (LQT1, LQT2, LQT3, and LQT8/Timothy syndrome) [38,79], catecholaminergic polymorphic ventricular tachycardia [38,117], and LEOPARD syndrome [118]. Given the rapid pace of developments in the hiPSCs field, it is likely that hiPSCs-patient derived could be useful for investigating the phenotypes and disease mechanisms in cells of variable mutations and other genetic conditions. These properties of hiPSCs make them a powerful tool for providing new clinical insights and for use as source cells for cardiac

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552 regeneration. However, scientists shall address several issues such as arrhythmias before their
553 clinical use can become a reality.
554 ***Cell therapy and myocardial infarction repair***. hiPSCS-CMs are of great interest for cell-
555 based heart regeneration. To avoid arrhythmia, which is the most severe side-effect of cell
556 replacement therapy, it is essential to implant relatively homogeneous, probably mature CMs
557 that have ventricular phenotypes. Several groups have reprogrammed murine embryonic
558 fibroblasts to iPSCs, that when injected intramyocardially in immunodeficient mice after
559 coronary artery ligation were able to differentiate into CMs, vascular smooth muscle cells,
560 and endothelial cells determining an improvement of the ventricular function. However, the
561 observation that transplanted iPSCs could generate tumors in the recipient mice strongly
562 limited the possibility of translating these research findings into clinical practice [7,119].
563 Following studies focused on transplanting iPSC-derived cardiac progenitors cells [57] or
564 iPSC-CMs [120] into the infarcted area of immunocompetent mice, the data showed an
565 improvement of ventricular contractility without tumor formation.
566 The main limitation of regenerative medicine is the poor cell retention into the organ
567 after injection, with 95–99% of grafted cells lost within a few days. Cell retention can be
568 enhanced by delivering cells on biomaterials such as hydrogels, tissue patches, or scaffolds
569 [121]. This approach can reduce mechanical stem loss and provide a protective environment
570 for cell survival [122].
571 Recently, Menasche and colleagues reported on the first clinical case report that showed the
572 feasibility of generating a clinical-grade population of human ESC-derived cardiac
573 progenitors and combining it within a tissue-engineered construct [123]. Considering the
574 similarity hESCs-CMs and hiPSCs-CMs, this result opens new opportunities for the clinical
575 application of hiPSCs.
576

577 **8. Conclusions**

578 hiPSCs- CMs are a promising tool in drug discovery, disease modeling, and cell
579 therapy, but despite the high hopes and expectancy, issues with the reprogramming

technology and the biology of reprogrammed cells still cast a shadow on the clinical application of hiPSCs. The technical hurdles in reprogramming have resulted in diversity in the quality of hiPSCs generated, the "epigenetic memory" influences the differentiation efficiency, and the reprogrammed cells present poorly controlled risks of unpredictable reactions in both the processes of dedifferentiation and subsequent differentiation of the cell strains employed for therapeutic or experimentation goals. However, although the reprogramming technology that creates hiPSCs-CMs is currently imperfect and much additional basic research will be required before their clinical application, these cells will likely impact future therapy, representing multi-purpose tools for medical research and illuminating many areas related to CV disease.

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