Chapter 4
In Vivo Lineage Reprogramming of Fibroblasts to Cardiomyocytes for Heart Regeneration

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4.1 Introduction

Heart disease is the leading cause of adult mortality in the developed world and continues to be a heavy burden to health care systems [1]. Resulting from the limited regenerative capacity of adult cardiomyocytes, it's difficult for heart to functionally recover after lesions such as myocardium infarction (MI). The lost cardiomyocytes in the injured area are replaced by activated cardiac fibroblasts (CFs) that proliferate and secrete excessive extracellular matrix to form scar tissues and pathologically remodel the myocardium. Although recently studies showed that mammalian hearts possess modest self-renewal and turnover under certain scenarios [2–5], it is still insufficient to regenerate a damaged heart.

Recent development of direct reprogramming, which directly converts cells from one differentiated phenotype to another without transitioning through the intermediate pluripotent state, offers a promising alternative approach for regenerative medicine. A single or multiple transcription factors have been shown to drive cell fata conversion from fibroblast into neuron like cells, hepatocyte like cells and many other somatic cell types [6]. As for heart, the activated residential CFs upon injury could serve as an endogenous source of new CMs for regenerative purpose if they could be directly reprogrammed into functional CMs. Several groups have successfully converted fibroblasts in to induced CM-like cells (iCMs) using a cocktail of transcription factors that reside at top of developmental regulatory hierarchy for cardiogenesis, both in vitro and in vivo. Alternatively, combinations of small molecules and microRNAs have been developed to either directly reprogram or enhance

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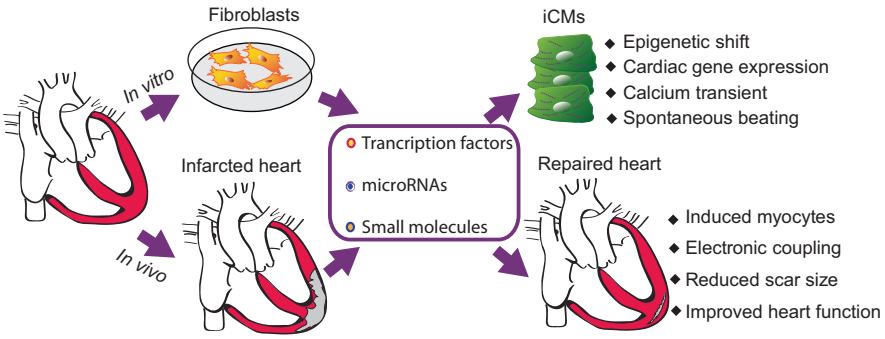

Fig. 4.1 Schematic of direct cardiac reprogramming in vitro and in vivo. Reprogramming factors including transcription factors, microRNAs and small molecules can be to delivered to cardiac or dermal fibroblasts in vitro, and can be transferred into infarcted heart to induce cell fate conversion

reprogramming of iCMs (Fig. 4.1). Now more effort has been taken into studying the mechanisms underlying this process. Here we will summarize current advances in direct iCM reprogramming and discuss its challenges and further applications for regenerative medicine.

4.2 Direct Reprogramming of Mouse Fibroblasts into iCMs

4.2.1 Transcription Factors

Starting from 14 transcription factors, Ieda et al. discovered that a specific combination of three factors, Gata 4 (G), Mef2c (M) and Tbx5 (T) (collectively referred to as GMT) was sufficient to transform mouse Thy1+ dermal or cardiac fibroblasts into iCMs [7]. The iCMs exhibit similar global gene expression and epigenetic imprinting as endogenous CMs, whereas the fibroblasts program is significantly repressed. Functionally, iCMs show calcium oscillation and spontaneous beating. Importantly, iCMs do not pass through a cardiac progenitor stage (in particular Mesp1+ and Isl1+ lineages), suggesting iCM generation is a direct conversion from one somatic cell type to another. In accordance with this observation, the fully reprogrammed iCMs morphologically and functionally resemble neonatal cardiomyocytes.

The following in vivo studies using genetic linage tracing strategies demonstrated the regenerative capacity of iCM reprogramming. It has been demonstrated that retroviral delivery of GMT after coronary ligation produced iCMs characterized with mature CM features including bi-nucleation, well-organized sarcomere structures as well as similar gene expression and electrophysiological properties [8]. Importantly, the in vivo reprogramming efficiency is much higher than the in vitro one, suggesting the environmental factors may contribute to the enhancement

of this conversion process. In vivo delivery of GMT also improved heart function, decreased infarction size and reduced fibrosis in mice with myocardial infarction [8]. Similarly, injection of GMT retroviruses into immunosuppressed mouse heart suffering MI resulted in newly emerged iCMs 2 weeks after surgery [9]. To overcome the disadvantages of the retro- and lenti-viral infection (integration and chronic expression), Mathison et al. generated replication-deficient adenovirus expressing GMT (Ad-GMT) [10]. These Ad-GMTs appeared to be as efficient as lentiviral GMT for rat iCM reprogramming both in vitro and in vivo [10].

In addition, other combinations of transcription factors have been reported to succeed in converting fibroblasts into iCMs. It has been shown that another transcription factor, Hand2, can function together with GMT (referred to as HGMT) to improve iCM reprogramming efficiency of adult fibroblasts in vitro and produce iCMs in vivo to attenuate heart dysfunction after myocardial injury [11]. In comparison with GMT, HGMT appears to generate diverse cell types including atrial, ventricular and pacemaker cardiomyocytes in vitro [12]. Protze et al. screened a pool of 10 transcription factors in MEFs and found another three factor combination (Mef2c, Tbx5 and Myocd) could induce iCMs with cardiac program and functionally these cells are more mature than GMT derived iCMs [13].

4.2.2 MicroRNAs

MicroRNAs (miRNAs) are a class of small noncoding RNAs of 21–25 nt in length that in general repress gene expression at the posttranscriptional level by degrading their target mRNAs and/or inhibiting their translation [14, 15]. MiRNAs play pivotal roles in governing gene expression during cardiovascular development and disease [16, 17]. For example, miR-1 was the first reported miRNA to be involved in regulation of heart development through targeting Hand2 [18, 19]. Recent studies imply additional import roles of miRNA in controlling cell fate conversion. Using combination of miRNAs, both mouse and human fibroblasts could be directly converted into induced pluripotent cells and neurons [20–22]. Based on the potential roles of miRNAs, Jayawardena et al. identified a combination of miRNAs 1, 133, 208, and 499 (referred to as miR combo) that are capable of inducing iCMs both in vitro and in vivo [23–25]. iCMs generated by miR combo are characterized with similar gene expression as endogenous CMs, spontaneous calcium flux and contraction. Mice harboring miRNA combo after MI showed newly derived iCMs originated from fibroblasts, and resulted improvement in cardiac function. JAK inhibitor I treatment further increased miR combo mediated iCM reprogramming efficiency [23]. Mechanistically, removal of tri-methylation of the lysine 27 of histone H3 (H3K27me3) is essential for miR combo to initiate the reprogramming [26]. Most recently, with the development of three-dimensional (3D) tissue-engineered cardiac hydrogel patches, miR combo directed iCM reprogramming was further enhanced with observation of strong environmental matrix metalloproteinases expression [27].

4.2.3 Small Molecules

Canonical reprogramming utilizes retroviral or lentiviral based strategies to deliver transcription factors in vitro and in vivo. Application of virus inevitably brings up the challenge of viral integration into the host genome and thus limits the clinical translation. An alternative approach is to use small chemical compounds, which are cell permeable, nonimmunogenic and could be easily handled for the delivery procedure to be standardized. Proof-of-concept studies have demonstrated that combinations of small molecules can replace master transcription genes to initiate iPS production [28]. With this concept, Want et al. demonstrated the transdifferentiation of mouse fibroblasts into cardiomyocytes with a single transcription factor Oct4 and a defined small molecule pool consisting SB431542 (ALK4/5/7 inhibitor), CHIR99021 (GSK3 inhibitor), parnate (LSD1/KDM1 inhibitor), and forskolin (adenylyl cyclase activator) [29]. Fu et al. developed a full chemical approach to generate chemical induced cardiomyocyte-like cells (CiCMs). Using compounds (CRFVPTZ (C, CHIR99021; R, RepSox; F, Forskolin; V, VPA; P, Parnate; and T, TTNPB) together with optimized culture medium, MEFs were amenable to become contractile cardiomyocytes [30]. Of note, different from GMT induced direct reprogramming, CiCMs pass through cardiac progenitor stage with high expression of progenitor markers Msp1 and Isl1 [29, 30].

4.3 Enhancement of Mouse iCM Generation

4.3.1 Optimization of Transcription Factors

Three transcription factors GMT are sufficient to induce cell fate conversion from fibroblasts to iCMs. Suffering from the relatively low efficiency and incomplete reprogramming, several studies aimed at improving reprogramming efficiency through harnessing the transcription factor pool. Addition of MYOCD and SRF alone or in combination with Mesp1 and SMARCD3 enhanced GMT activated basal cardiac gene expression, though no significant difference was observed in terms of myocyte functionality [31]. Taking advantage of a transgenic calcium florescent reporter system driven by cardiac specific Troponin T promoter, Addis et al. evaluated several transcription factor combinations for their capacity to produce functional iCMs [32]. Interestingly, they found that addition of Nkx2-5 to HGMT cocktail (referred to as HNGMT) resulted in highest reprogramming efficiency [32]. There are also studies attempting at modifying activity of one reprogramming factor Mef2c. MyoD was one of the skeletal muscle master genes which has been identified to transform fibroblasts into myoblasts [33]. Hirai et al. fused MyoD transactivation domain to Mef2c and demonstrated that chimeric Mef2c together with Gata4, Tbx5 and Hand2 (referred to as MM3-GHT) yields larger contractile

iCM clusters with shortened time window in comparison with traditional HGMT [34]. Alternatively, Abad et al. enhanced binding of Mef2c to the promoter region of cardiac genes, which also resulted in higher reprogramming efficiency [35].

4.3.2 Stoichiometry of Transcription Factors

The use of transcription factor cocktails raised a critical question on relationship between expression level of each exogenous factor and outcome of iCM conversion. During heart development, delicate regulation and dose-spatial-temporal balance of these transcription factors are required to initiate and maintain cardiac specification and differentiation properly [36–39]. To address this question, Wang et al. manipulated expression level of GMT using polycistronic constructs and showed distinctive protein expression based on splicing orders among identical self-cleaving 2A sequences. They further demonstrated that relative ratio of G, M, T protein was crucial for efficient iCM reprogramming. An optimal expression of GMT with relative high level of M and low levels of G and T achieved by using polycistronic MGT vector (hereafter refer to as MGT) significantly increased reprogramming efficiency and improved iCM quantity and quality in vitro [40, 41]. Moreover, in vivo MGT delivery generated more iCMs and further improved heart functions than traditional delivery of GMT separate viruses [42, 43]. Another two polycistronic constructs encoding GMT were also reported to improve in vivo reprogramming [9, 44]. These reports emphasized the importance of stoichiometric expression of transcription factors and established a single vector platform to facilitate consistent and reproducible iCM reprogramming and further moved the basic and translational research on iCMs.

4.3.3 Addition of Small Molecules

Enhancement of iCM reprogramming could be achieved through addition of small molecules and microRNAs to base transcription factor cocktails (Table 4.1). In brief, there are three main groups of the supplements. First group consists of proteins and peptides like Thymosin β4, Akt and growth factors such as VEGF and FGFs. Thymosin β4 is a natural peptide that has been implied to be critical for cardiac development and play cardio-protective roles upon heart injury [45–48]. Co-administration of thymosin β4 resulted in a better delivery of GMT to reactivated fibroblasts, hence increased iCM numbers and resulted in further functional improvement in ejection fraction and cardiac output in vivo [8, 49]. Another in vivo research demonstrated that preconditioning rats with VEGF promoted iCM generation and improved cardiac function after MI [50], suggesting the possible important role of angiogenesis for heart repair. In vitro, treatment of serum free

Table 4.1 Small molecules enhance transcription factors induced murine iCM reprogramming

Reprogramming factors	Supplements	Targets/function		Reference
GMT	Thymosin β4	Pro-angiogenic and fibroblast-activating peptide	In vivo	[8, 49]
GMT	VEGF	Increased angiogenesis	In vivo	[50]
GMT/MT	FGF2, FGF10 and VEGF	Activation of p38MAPK and PI3K/AKT pathways	In vitro	[51]
GHMT	Akt/protein kinase B		In vitro	[52]
MM3-GHT	GSK126	Ezh2 inhibitor	In vitro	[56]
MM3-GHT	UNC0638	G9a and GLP inhibitor	In vitro	[56]
HNGMT	SB431542	TGFβ inhibitor	In vitro	[57]
MGT	MM408, MI503	Mll1 complex specific inhibitor	In vitro	[58]
GHMT	DAPT	Notch inhibitor	In vitro	[35]
GMT	SB431542; XAV939	TGFβ inhibitor; WNT inhibitor	In vitro and In vivo	[53]
GMT	miR-133	Repress snail1	In vitro	[54]
GHMT	miR-1 and miR-133		In vitro	[55]
GHMT2m	Y-27632/Thiazovivin/ SR-3677; A8301	ROCK inhibitors; TGFβ inhibitor	In vitro	[55]

medium containing VEGF with FGFs leads to faster maturation of iCMs induced by GMT, possibly through activation of Akt [51]. Overexpression of Akt also led to more efficient generation of contractile iCMs [52]. Moreover, VEGF and FGFs can substitute Gata4 and contribute to direct iCM generation with M and T [51].

The second group consists of chemical compounds targeting epigenetic modifiers and signaling pathways that will be discussed in later section. Noticeably, almost all the drugs were tested in dish, only a very recent study from Srivastava group showed the applicability of small molecules to enhance in vivo reprogramming [53]. They first depicted the reinforcement of in vitro reprogramming by using TGFβ inhibitor and WNT inhibitor with GMT (referred to as GMTc) characterized with shortened duration and enhanced iCM quantity and quality. Mice exposed to GMTc developed smaller scar size, thicker re-muscularized myocardium and further improvement in heart function than GMT alone. At cellular level, the number of ex vivo isolated iCMs from GMTc group was five-fold higher than that from GMT group, and GMTc-iCMs are functionally closer to adult cardiomyocytes in terms of their electrophysiological properties [53].

The third group includes microRNAs with or without chemical compounds. Although miRNA itself could generate iCMs in vitro and in vivo [23, 24], addition of miRNAs to cardiac transcription factors enables higher iCM reprogramming

efficiency and better cellular quality. Ectopic expression of miR-133 alone with GMT increased beating iCMs by sevenfold and noticeably enhanced the speed (from 30 to 10 days) for iCM maturation [54]. Overexpression of miR-1 and miR-133 together with GHMT (referred to as GHMT2m) induced more matured iCMs that started to beat by day 8 [55]. In combination with these two miRNAs, ROCK inhibitor and/or TGFβ inhibitor converted fibroblasts into functional iCMs with the efficiency over 60% when quantifying the percentage of beating cells [55].

4.4 Molecular Mechanisms Underlying iCM Generation

4.4.1 Epigenetic Regulation of iCM

Epigenetic regulation plays fundamental roles in cellular specification and lineage commitment during development. Emerging evidence indicates that dysregulated epigenetic landscape contributes to cardiomyopathy and heart failure [59, 60]. Recent studies on cellular reprogramming also demonstrated the dynamic alternation of epigenetic modifications [61–64]. In the first iCM paper, Ieda et al. discovered that trimethylation of histone H3 at lysine 27 (H3K27me3), a commonly used marker to mark transcriptionally inactive chromatin, was significantly reduced at the promoter region of several cardiac specific genes in iCMs 4 weeks after GMT induction [7]. Whereas, trimethylation of histone H3 at lysine 4 (H3K4me3), which labels an open chromatin, was increased at the same promoter region in iCMs compared to fibroblasts. Liu et al. further analyzed the repatterning of H3K27me3, H3K4me3 at cardiac and fibroblast loci at the MGT mediated reprogramming day3 and day10 [65]. Loss of H3K27me3 at cardiac gene loci appeared as early as day3, suggesting the rapid suppression of fibroblast signatures and early activation of cardiac program. Furthermore, data from ChIP-Seq revealed that upon transduction of GHMT, H3K4 dimethylation (H3K4me2, a general marker of both promoter and enhancer regions [66, 67]) peak shifted from fibroblast toward myocyte status at reprogramming day 7, indicating the existence of epigenetic orchestration at gene regulatory regions during early phase of iCM reprogramming [55].

To explore the underlying mechanism and identify potential epigenetic barriers to iCM reprogramming, our lab performed the first loss of function screen with a shRNA pool consisting 35 components that were involved in chromatin remodeling and modification and identified several factors that could either facilitate or blunt iCM reprogramming [68]. In particular, Bmi1, an important component of the Polycomb repressive complex 1 (PRC1) [69, 70], functioned as a major epigenetic barrier at the early stage of iCM reprogramming. Bmi1 suppressed iCM reprogramming through direct binding to a battery of cardiogenic loci including Gata4, Nkx2.5, Isl1, Pitx2, Tbx20, and Hand2. Furthermore, we demonstrated that Bmi1 depletion could replace Gata4 and convert fibroblasts into iCMs together with a single vector encoding Mef2c and Tbx5.

Liu et al. adopted a gain-of-function approach and identified Men1 and Suv39h1 as epigenetic inhibitors of iCM reprogramming [58]. Men1 is an essential component of a MLL/SET1 histone methyltransferase (HMT) complex responsible for H3K4 methylation and H3K9 methylation [71–73]. Suv39h1 also mediates H3K9 methylation [71, 74]. Chemical inhibitors targeting MLL1 complex to repress H3K4 methyltransferase activity significantly enhanced reprogramming efficiency, indicating that Men1 regulate iCM generation through modifying H3K4m3 instead of H3K9m3.

Enhancer of Zeste Homolog 2 (Ezh2), a catalytic subunit of PRC2 complex for H3K27me2 and H3K27me3, behaved as one of the epigenetic barriers for MM3-GHT mediated reprogramming [56]. Exposure to Ezh2 inhibitor GSK126 resulted in a decrease of H3K27me3 and an increase of beating iCM clusters. Similarly, UNC0638, an inhibitor to G9a and GLP that mainly controls H3K9me and H3K9me2, led to a higher iCM reprogramming efficiency in association with lower level of H3K9me2 in iCMs [56].

Taken together, iCM reprogramming is largely guided by specific cardiac transcription factors and the associated chromatin modifiers to establish authentic myocyte cell fate in another distinct cell type.

4.4.2 Suppression of Fibroblast Program

During reprograming, transcription factors drive fibroblast toward a differentiated cardiomyocyte lineage. Genome wide transcriptome research demonstrated that iCM reprogramming requires depletion of the original fibroblast signatures and de novo establishment of myocyte programs such as the contractile machinery, sarcomere structures, high mass of mitochondria and the metabolic switches [35, 51–55]. Suppression of fibroblast program has been shown to fundamentally affect iCM reprogramming. Overexpression of miR133 with GMT repressed Snail1 to silence fibroblast signatures and activates cardiac programs [54]. Snail1 is one of the major mediators of epithelial-mesenchymal transition (EMT) that contributes to cardiac fibrosis [75, 76]. MiR133 directly targeted Snail1 for degradation and overexpression of Snail1 inhibited iCM reprogramming.

Accumulating studies revealed the pivotal role of TGFβ signaling pathway during iCM conversion [53, 55, 57]. TGFβ signals activate cardiac microvascular endothelial cells to undergo endothelial-to-mesenchymal transformation and contributes to cardiac fibrosis [77, 78]. TGFβ also behaves as a repressor for embryonic cells differentiation toward cardiomyocytes [79]. Generally, TGFβ superfamily members bind to TGFβ type II receptor, which subsequently recruits and triggers phosphorylation of TGFβ type I receptor. Phosphorylated type I receptor activates SMAD molecules and leads to formation of SMAD complex. Activated SMAD complex translocates into nucleus and interacts with other DNA binding factors, transcription factors, thus regulates the transcription of target gene [80]. One of the most commonly used TGFβ inhibitors is SB-431542 that selectively blocks the TGF-β

type I receptor including ALK4 and ALK5, as well as ALK7 [81]. Both Srivastava group [53] and Gearhart [57] group screened out this inhibitor for its application in iCM reprogramming, while Song group [55] identified another TGF-β inhibitor termed as A8301 that inactivates similar receptors for its use in enhancing iCM induction. Through disturbing TGFβ signaling with chemical inhibitors, all three researches achieved much greater reprogramming quality. Most iCMs generated with the help of TGFβ inhibitors were relatively more reprogrammed beating cells with transcriptome more similar to adult cardiomyocytes [53, 55, 57].

4.5 Direct Cardiac Reprograming in Human Cells

Compared to the rapid advances of murine iCM programming, generation of human iCMs in vitro is more complicated thus much delayed. Neither GMT nor GHMT was sufficient to induce human iCMs [82–84]. Screening additional transcription factors finally led to successful induction of cardiomyocyte-like cells from human fibroblasts. Fu et al. discovered that the combination of GMT with ESRRG, MESP1 was sufficient to turn on cardiac specific markers in transduced human fibroblasts. Addition of myocardin and ZFPM2 further enhanced the reprogramming and resulted in iCMs exhibiting calcium flux and action potential [82]. More recently, with the help of two chemical inhibitors (TGFβ inhibitor and WMT inhibitor), the seven transcription factors (7c) induced reprogramming was further accelerated [53]. In addition, the 7c cocktail could be cut down to a four-factor recipe (GMT plus myocardin) with the two inhibitors, indicating the critical role of TGFβ and WNT signaling for human iCM reprogramming. Wada et al. showed that addition of Mesp1 and Myocd to GMT (referred to as GMTMM) cocktail transformed HCF (human cardiac fibroblasts) and HDF (human dermal fibroblasts) to iCMs that expressed a broad panel of cardiac markers, exhibited calcium oscillation and contracted synchronously when co-cultured murine primary CMs [84]. Later they demonstrated that miR-133 mediated snail1 inhibition in human fibroblasts is as important as that for mouse iCM reprogramming. Inclusion of miR-133 or snail1 depletion promoted GMTMM induced human iCM reprogramming [54]. Nam et al. showed that combination of GHMT with myocardin generates few beating cells after 11 weeks in culture [83]. Addition of miR590 to GHMT with myocardin upregulated cardiac gene expression and further suppressed fibroblast marker genes by directly inhibition of Sp1 (specificity protein 1) expression [85].

Generation of expandable cardiac progenitor cells (CPC) from fibroblasts shed lights on the production of CMs through de-differentiation. CPCs can differentiate into three major cell types of heart- endothelial cells, CMs and smooth muscle cells [86, 87]. Transcription factors ETS2 and MESP1 have been reported to transdifferentiate HDF into cardiac progenitors [88]. Lalit et al. reprogrammed adult mouse fibroblasts into induced CPCs (iCPCs) with a cocktail of five transcription factors (Mesp1, Tbx5, Gata4, Nkx2.5, and Baf60c) with two compounds (BIO and LIF) [89]. iCPCs were capable of proliferation and differentiation into all three cell types

and generated new myocardium post MI [89]. In comparison, Zhang et al. transiently expressed four Yamanaka factors (Oct4, Sox2, Klf4 and c-myc) in fibroblasts, cultured these primed cells in conditioned medium and ended with acquisition of iCPCs [90]. However, the precise outcome of the final differentiation is difficult to be controlled, with the likelihood of contamination of cells from non-cardiac lineages. Independent of transcription factors, Cao et al. demonstrated that 9 chemical compounds, called 9c, successfully transformed human foreskin fibroblasts to cardiomyocyte like cells [91]. The chemically induced iCMs (ciCM) sequentially expressed mesoderm, CPC and CM genes and eventually became spontaneously beating cells. It appears that ciCMs acquire similar transcriptional and epigenetic signatures, as well as functional properties that are similar to human CMs.

In summary, current studies (summarized in Table 4.2) paved a great foundation for future translational applications that require generation of mature human cardiomyocytes from different resources. Induction of human iCMs takes longer time and requires more factors; in addition human iCMs are far less mature, all of which suggest the need of further refinement from laboratory work. Research using cells from large animals in particular non-human primates could serve as an alternative to study the combination of factors with small molecules on the outcomes of iCM reprogramming, the outcome of which may facilitate the ultimate goal of effectively generating human iCMs.

4.6 Conclusions and Perspectives

Direct cardiac reprogramming holds great potential for regenerative medicine by offering an alternative strategy for treatment of heart disease and disease modeling. Recent studies indicated that the reprogramming efficiency is steadily increased by utilizing multiple strategies and through understanding the molecular mechanisms. Although it has been progressed rapidly, there are still challenges for this field.

First, the reprogramming efficiency is still low and varies between labs. The majority of reprogramed cells are not functionally fully matured cardiomyocytes. It's undoubtedly required to further optimize the platform and remove the molecular barriers so as to obtain sufficient highly matured iCMs for drug screening and disease modeling. One of the intriguing areas is to identify the contributing factors by evolving "omics" technologies, such as genomics, transcriptomics, proteomics and metabolics, which would reveal the genetic and epigenetic regulation, protein interaction network and metabolite profiles. Interrogating data from these techniques can help to understand more thoroughly about the events and mechanisms underlying the iCM reprogramming. Recent single cell RNA sequencing analysis (scRNA-seq) offers another opportunity to dissect the reprogramming trajectory, profile dynamic gene expression and identify cell fate determinants. Researches from Treutlein et al. set an example as how to use scRNA-seq to gain mechanistic understanding for induced neuron reprogramming [93].

Table 4.2 Direct programming of human fibroblasts into iCMs

Transcription factors	microRNAs	Supplements	Cell source	Phenotypes	Reference
GMT, ESRRG, MESP1, Myocardin, ZFPM2			Human fibroblasts derived from ESCs, HDFs and HCFs	Around 10% cTnT+ for ESC derived fibroblasts; around 4% with HDF, calcium transients in 4 weeks and action potentials in 10 weeks	[82]
GHMT, myocardin			Neonatal HFF	Around 20% cTnT+, beating in 11 weeks	[83]
GHMT, myocardin	miR-1 and miR-133		HFF, adult HCFs and HDFs	Around 10% cTnT+ for HCFs and around 4.4% cTnT+ for HDFs(2 week), calcium transients in 8 weeks	[83]
GHT, myocardin	miR-1, miR-133		HFF, adult HCFs and HDF	Around 13% cTnT+ for HCFs and around 9.5% cTnT+ for HDFs(2 week); around 35% cTnT (4w) for HFF	[83]
GMT, Mesp1, Myocd			HCF and HDFs	Around 5% cTnT+; calcium transients in 4 weeks; contact with co-cultured CMs	[84]
GMT, Mesp1, Myocd	miR-133		HCFs	23–27% cTnT+	[54]
GHMT, Myocardin	miR-590		HCFs	Around 6% cTnT+	[85]
GMT, Myocd, NKX2-5	miR-1, miR-133	JAK1i, GSK3i; IGF1 and NRG	HDFs	Calcium transients in 1 week	[92]
GMT, ESRRG, MESP1, Myocardin, ZFPM2		TGFbi (SB431542) and WNTi (AXV939)	Immortalized HCFs	Around 12% cTnT+, calcium transients in 10 days	[53]

(continued)

Table 4.2 (continued)

Transcription factors	microRNAs	Supplements	Cell source	Phenotypes	Reference
GMT, Myocardin		TGFbi (SB431542) and WNTi (AXV939),	Immortalized HCFs	Around 12% cTnT+, calcium transients in 10 days	[53]
		CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632, OAC2, SU16F and JNJ10198409	HFFs	Around 6.6% cTnT+ at day30, form beating clusters	[91]

Second, most studies used virus-based strategies to deliver reprogramming factors, which inevitably raises up the safety issues such as genomic integration and subsequent tumorigenesis [6]. To address this concern, safer delivery vectors, such as bio-safe Adeno-associated virus (AAV) based vectors [94], could be developed and optimized of reprogramming. Moreover, the use of small molecules would be another promising way for the clinical application of iCM reprogramming.

Last but not least, direct in vivo reprogramming yields iCMs at higher efficiency, better quantity and quality than iCMs generated from in vitro [8], clearly suggesting the fundamental effective amelioration of environmental niches. Mechanical forces, inflammatory responses, angiogenesis and extracellular matrix could be potential contributors for iCM maturation. Studies using growth factors and small peptide to mimic the environmental changes showed the enhancement of iCM reprogramming, however, the detailed mechanism remains unclear [8, 49, 51]. Identification and understanding the role of these environmental factors could further benefit iCM production and harnessing this approach for regenerative purpose. An alternative approach to identify microenvironmental clues is the application of recently advanced bioengineered materials. Biomaterials have been shown to influence cell fate and behavior through mutual interaction between cells and their environment [95]. Manipulation of the biophysical and biochemical properties of certain biomaterials leads to an improvement of iCM reprogramming [27, 96–98], indicating that it can be used to engineer the niches and realize the controllable release of reprogramming factors and small molecules in situ for in vivo reprogramming. One more consideration for experimental biologists is the utilization of large animal species for in vivo iCM reprogramming, such as the porcine model that raises exciting prospect for future iCM based therapies. Compared to murine models, pigs are anatomically and physiologically more similar to humans in cardiovascular, skeletal muscle, immune, and metabolic systems. It can not only solve the aforementioned biosafety issues and explore environmental niches, but also allows researchers to overcome the possible adverse effects of arrhythmia caused by newly formed iCMs in the scar region of myocardium. In particular, efficient and improved genetic engineering

approaches for pigs are now available, facilitating the establishment of tailored animal models for mimicking human diseases.

In summary, direct cardiac reprogramming converts injury-activated fibroblast into terminally differentiated cardiomyocytes in situ, holding tremendous potential for healing the injured heart. After better understanding the molecular mechanisms and overcoming the obstacles discussed, we anticipate that we can ultimately harness the iCM reprogramming and translate it to mend the broken heart.

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