Regenerating the human heart: direct reprogramming strategies and their current limitations

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Abstract

Cardiovascular diseases represent the first cause of death in the Western World as current therapies are often only palliative. As a consequence, today, heart transplantation is essentially still the only possible choice for many patients. However, several novel therapeutic approaches have been attempted in the past two decades, with quite encouraging results. Along this line, generation of induced pluripotent stem cells, through the forced expression of stem-cell specific transcription factors, inspired the most recent and attractive strategies for heart regeneration, consisting in the direct reprogramming of cardiac fibroblasts into functional cardiomyocytes. First attempts were conducted using a similar approach than the one used with transcription factors, but during years, novel strategies have been tested, e.g. miRNAs, recombinant proteins and chemical molecules. Although preliminary results on animal models are promising, the low reprogramming efficiency as well as the incomplete maturation of the cardiomyocytes still represent important obstacles before a clinical translation could be foreseen.

This review covers all the different direct transdifferentiation strategies that have been proposed and developed, illustrating the pros and cons of each approach. Indeed, as described in the manuscript, there are still many unanswered questions and drawbacks that require a better understanding of the basic signaling pathways and transcription factor networks before functional cells, suitable for cardiac regeneration and safe for the patients, can be generated and used for human therapies.

Keywords: heart dysfunction, cardiac regeneration, direct reprogramming, small molecules, cardiomyocytes, translational medicine.

Formattato: Non Evidenziato

Formattato: Non Evidenziato

1. Introduction

The latest annual World Health Statistic report of the World Health Organization (WHO, June 2016) indicates the cardiovascular diseases as the leading cause of death worldwide, resulting in 17.7 million deaths in 2015 alone and, despite new medical advances, the number is expected to grow to more than 23.6 million per year by 2030. Thus, it is quite clear that the cardiovascular diseases represent an enormous problem for the modern society, also from an economic perspective, with a cost for the world healthcare system estimated to be about 860 billions of dollars per year (http://www.who.int/en). Therefore, the development of novel, efficient, and cost-effective cardiac therapies is a fundamental goal that is evoked worldwide. Actually, it is well known that most of the heart diseases are often accompanied by a severe loss of cardiomyocytes (CMs) and by a pathological remodeling of the heart, culminating in heart failure or even sudden death [18]. As a matter of fact, one of the main problems to be addressed resides in the very limited regenerative capacity of the adult human heart. For instance, after a myocardial infarction, the injured area is replaced by a fibrotic scar, that on one side is able to protect the heart wall from rupturing, but on the other side, unfortunately, it is unable to contract and to conduct electrical signals, ultimately resulting in an elevated stress for the heart, which irredeemably affects cardiac performance [45]. As a consequence, nowadays, heart transplantation is essentially the only possible treatment for these patients. Clearly, this therapeutic approach is impracticable on a large scale, because of the shortage of heart donors, without considering the lifetime immunosuppressant therapy required after transplantation [53]. Thus, alternative regenerative therapies are extremely needed, and great attention has been focused in the past two decades on stem cell-based approaches, using different cell sources, including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), and cardiac stem cells (CSCs). Although several clinical trials with adult stem cells have now reached Phase II and III, no approach stands out, and searching for the "ideal" stem cell to be used, if it even exists, is still ongoing, as extensively reviewed in the literature [5, 14, 35, 42]. On the other hand, more recently, an alternative approach has been proposed, which is the possibility of directly reprogramming cardiac fibroblasts into

cardiomyocytes, without the need of isolating and re-injecting the stem cells. Ideally, with this approach, the scar tissue of a damaged myocardium could be reverted into functional cardiac muscle. This novel concept for heart regeneration is quite fascinating yet very challenging, and over the past few years it has been tackled in different ways. Thus, the main aim of this review is to describe all the direct reprogramming strategies available, highlighting the pros and cons of each approach, in order to have the most unbiased picture of where we are now and where we are heading to.

2. Direct Cardiac Reprogramming

The idea of *directly reprogramming* (or *trans-differentiating*) an adult and differentiated cell into another fully mature type is truly not so novel, as the first attempt of a direct conversion was performed back in 1987. In fact, it was demonstrated that the forced expression of the muscle-specific transcription factor *MyoD* induced myogenic features in mouse fibroblasts by activating the target cell program, and concomitantly repressing the starting cell transcriptional profile [12]. However, it was only the generation of iPSCs in 2006 by Yamanaka and colleagues that really built the scientific basis for alternative reprogramming strategies [54, 55]. In fact, soon after Yamanaka's discovery, the idea of a direct conversion of terminally differentiated cell types into different ones, without passing through the pluripotent stage, started to emerge. In particular, the overexpression of specific transcription factors and/or microRNAs allowed the direct conversion of fibroblasts into several different cell lineages, including pancreatic β cells [67], neurons [59], and hepatocyte-like cells [20]. However, it soon became clear that obtaining functional cardiomyocytes directly from fibroblasts was going to be more cumbersome than expected, mainly because of the heterogeneity and complexity of the heart tissue. Indeed, in the human heart, 30% of cells are cardiomyocytes, whereas 70% are represented by several "non-myocyte" cells, the majority of which being different subtypes of cardiac fibroblasts [27]. These types of fibroblasts, which are known to express several cardiac-specific genes like *GATA4*, play a crucial role after myocardial injury, as they become active myofibroblasts, migrate to injured zones, and create a non-contractile scar that eventually

impairs cardiac functionality [39]. On the other hand, cardiac fibroblasts seem to originate from the same common progenitor as the CMs [58], thus they were soon selected as the "optimal" cell type to be reprogrammed into CMs.

In the past ten years, three main different classes of reprogramming strategies have been developed, which are divided according to the method used to obtain the fibroblast conversion: (a) purely genetical, (b) a mix of a genetical and a chemical approach, and (c) only chemical. In the next sections, each approach will be described and discussed in detail.

2.1. Genetical Direct Cardiac Reprogramming

Soon after the seminal discovery that the forced expression of four genes (SOX2, KLF4, c-MYC, OCT4) could reprogram fibroblasts into iPSCs [55], researchers have hypothesized that, with a similar approach, adult cells could be reprogrammed into other types of differentiated cells [67]. Thus, in the field of heart regeneration, efforts have been made toward the unveiling of the "*master regulators*" of cardiac differentiation by analyzing the transcriptional regulation machinery of the developing heart [44, 52]. In particular, it was soon demonstrated that two cardiac transcription factors, *GATA4* and *TBX5*, in combination with *BAF60c*, a cardiac-specific subunit of the BAF chromatin remodeling complex, could induce the formation of ectopic beating cardiomyocytes in mesodermal cells of early mouse embryos, including the normally not cardiogenic posterior mesoderm, and the extraembryonic mesoderm of the amnion, but not in adult cells [56]. In 2010, Ieda and colleagues reported the first successful reprogramming experiments of mouse fibroblasts into functional CMs, hereafter defined induced cardiomyocytes (iCMs), and identified a pool of 14 transcription factors that exhibited severe developmental cardiac defects when mutated [21]. Upon transduction with a mixture of these factors, they obtained cell expressing cardiac αMHC. Then, using a single-factor-elimination strategy, they defined the combination of three critical genes, *Gata4*, *Mef2c,* and *Tbx5* (the GMT cocktail), that were sufficient to reprogram mouse fibroblasts into iCMs after one week in culture. After retroviral/lentiviral transduction, the "GMT cocktail" was able to induce the direct conversion of cardiac and dermal post-natal murine fibroblasts in

functional CMs, expressing cardiac troponin T and with a general gene expression profile similar to neonatal CMs. Moreover, 30% of iCMs derived from cardiac fibroblasts showed spontaneous Ca^{2+} oscillations with a variable frequency and, above all, they had spontaneous contractile activity. Remarkably, the reprogramming process occurred through the generation of a cardiac precursor-like stage without an intermediate pluripotent state, as suggested by the low expression levels of the pluripotency genes such as *Oct4*, *Sox2*, *Nanog* and *Klf4*. However, the efficiency was generally low and beating cells could be observed only in iCMs derived from cardiac fibroblasts [21]. However, along this line, other groups obtained quite unsuccessful results with the GMT combination, supporting the idea that GMT factors alone were inefficient to completely convert fibroblasts into CMs, but rather produced partially reprogrammed cell types [8, 47]. Thus, several studies were performed to improve the efficiency of the reprogramming process, modifying the original GMT set, adding or substituting some transcription factors. For example, Proetze and colleagues directly screened all possible triplet combinations starting from ten candidate genes, eventually unveiling that, by combining *Myocd*, instead of *Gata4,* with *Tbx5* and *Mef2c,* significantly increased neonatal cardiac fibroblasts reprogramming efficiency, generating iCMs characterized by the expression of cardiac contractile proteins and by potassium/sodium currents typical of cardiac cells. Unfortunately, no beating cells were observed, possibly because the trans-differentiation into functional CMs was incomplete [47]. Concurrently, Olson's group developed another reprogramming strategy, adding *Hand2* to GMT, that became GHMT, inducing a 20% increase in the fibroblast conversion efficiency [51]. Moreover, a methodical study on cell markers expression of different CMs subtypes, defined a multiplex immunostaining strategy to distinguish individual CMs subtypes. Using this screening protocol, it was observed that GHMT treatment generated different CMs phenotypes in similar proportions, resembling immature forms of atrial, ventricular and pacemaker subtypes. This evidence was further confirmed by patch clamping experiments on spontaneously contracting iCMs. Moreover, even if the action potential of every subtype showed peculiar features, many differences were highlighted between iCMs and adult CMs, indicating that the reprogrammed fibroblasts were not fully electrophysiologically competent and mature [40].

Despite these numerous challenges and uncertain results, direct reprogramming approaches were tested *in vivo* on mice models using GMT [48] or GHMT [51] vectors. Both groups reported evidences that fibroblasts conversion into CMs was more efficient *in vivo* than *in vitro*. Moreover, since the retroviruses used to deliver GMT directly into the heart can only integrate in dividing cells, the reprogramming factors may affect only cardiac fibroblasts. These cells, once transformed into iCMs, were able to ameliorate cardiac function after myocardial injury, decreasing infarct size and improving cardiac parameters, such as the fractional shortening and the stroke volume. In particular, the analysis of the cells phenotype revealed that iCMs generated *in vivo* were more mature than the ones obtained *in vitro*, perhaps due to the cardiac microenvironment that could facilitate their differentiation [48, 51]. Moreover, it was reported that the efficacy of *in vivo* cardiac fibroblasts reprogramming could be enhanced by infarct pretreatment with pro-angiogenic and fibroblast activating peptides, such as Thymosin β4 [51] or vascular endothelial growth factor (VEGF) [33]. Both treatments resulted in fibrosis reduction and in cardiac function improvement. More recently, a novel strategy for cardiac reprogramming of fibroblasts into iCMs has been introduced, and it is based on the use of a polycistronic vector to transduce target cells, instead of all single constructs. In particular, supported by the positive results achieved in iPSCs generation [65], Inagawa and colleagues applied the same approach for cardiac reprogramming, using a polycistronic retrovirus expressing GMT. Unfortunately, they obtained only marginal positive effects in fibroblasts conversion into iCMs, as compared to the single vector strategy [23]. Surprisingly, the optimal balance among the transcriptional factors emerged as a crucial point for the reprogramming efficiency [62]. In particular, polycistronic vectors with all the possible combinations of the G-M-T factors were generated and tested. The combination that gave the highest MEF2C protein levels, the M-G-T vector, improved by ten folds the reprogramming efficiency as compared to the original GMT set. CMs obtained with this approach showed a more mature phenotype, characterized by the expression of Connexin43, the protein of the gap junctions, and by the sarcomeric structures typical of fetal CMs and by Ca^{2+} periodic oscillations.

Unfortunately, switching from mouse to human cells was all but straightforward, as several attempts with the GMT combination failed [16, 41, 60]. Thus, researchers had to go back from scratch to search for a new combination of factors. After many attempts, they eventually fairly succeeded using a new complex "cocktail" that was identified from fourteen transcriptional factors known to be crucial during heart development, and three muscle-specific miRNAs [41]. Thus, the transduction of a combination composed by *GATA4, TBX5, HAND2, MEF2C, MYOCARDIN*, miR-1, and miR-133 was sufficient to obtain 20% of troponin T positive cells from neonatal human foreskin fibroblasts, but only rare beating foci after a long period of culture [41]. Moreover, the conversion efficiency markedly decreased when using adult cardiac or dermal fibroblasts, possibly because adult cells own a more stable epigenetic state which counteract the reprogramming process [41]. Many other attempts have been conducted to improve this conversion by modifying the transcription factors combination. However, the reprogramming process was still characterized by a general low efficiency [16, 38, 60].

An alternative strategy to obtain iCMs without the use of transcription factors was reported by Jayawardena and colleagues, who employed miRNAs [25]. They identified six miRNAs important for cardiac muscle development and differentiation and, by a combinatorial strategy, they tested 41 different combinations of two or three miRNAs. The combination of miR-1, miR-133, miR-208 and miR-499 was able to induce the direct transdifferentiation of transiently transfected murine fibroblasts into iCMs expressing specific cardiac proteins and showing sarcomeric structures and calcium fluxes [25]. More recently, the same group tested the potential of their miRNAs cocktail to induce fibroblast reprogramming directly *in vivo* [26]. The intramyocardial delivery of the miRNAs generated iCMs resembling mature ventricular CMs for many characteristics, especially regarding the action potential. Moreover, miRNAs iCMs ameliorated the fractional shortening, decreased the left ventricular mass and reduced the grade of fibrosis, finally promoting the functional recovery of the damaged myocardium [26].

The genetical direct cardiac reprogramming described approaches are summarized in Table 1.

2.2. Genetical/Chemical Direct Cardiac Reprogramming

A different approach for fibroblasts transdifferentiation into CMs was proposed by Efe and colleagues, the so-called *Cell-Activation and Signaling-Directed* (CASD) Lineage Conversion Method [13]. The new approach consists in exposing the cells to be reprogrammed to an initial *epigenetic activation phase*, which is meant to facilitate the successive differentiation toward the cardiac phenotype, using a chemically defined cardiogenic medium. In particular, cell activation was obtained overexpressing *Oct4*, *Sox2* and *Klf4*, three of the reprogramming genes established in the standard iPSCs generation protocol [55], and maintaining the cells in feeder-free culturing conditions without leukemia inhibitory factor (reprogramming medium) to avoid the generation of pluripotent cells [13]. During the first nine days of treatment, mouse embryonic fibroblasts, transduced with the three factors, were continuously maintained in the reprogramming medium in the presence of the JAK inhibitor JI1, and then shifted for five days to the cardio-inductive medium supplemented with BMP4. Following this approach, it was possible to observe an up-regulation of mid-stage cardiac markers, such as *Flk-1*, *Gata4* and *Nkx2.5*, starting from day ten, whereas, from day eleven onwards, the expression of late-stage markers including Troponin T, α-MHC and α-actinin resulted increased. Moreover, many converted fibroblasts started to express the gap junctions' protein Connexin43, and simultaneously several colonies showed spontaneous waves of contractions. Interestingly, the obtained iCMs expressed only the atrial isoform of the myosin light chain (MLC-2a), suggesting that the reprogrammed fibroblasts principally acquired the atrial phenotype [13].

The relevance of the CASD approach was confirmed by other independent studies, where the method was used to generate several cell subtypes, including neural [68], endothelial [28] and pancreatic cells [29], in all cases without going through the induction of a pluripotent state. However, the CASD approach requires a genetic manipulation step, that rises questions about safety and efficiency, in the perspective of a possible clinical translation of this strategy. Indeed, many research groups are persuaded that eliminating all genetic factors in the reprogramming process represents a fundamental step for the development of a safe therapeutic approach. Along this line,

the first attempt to reduce, at least partially, the genetic manipulation in cardiac reprogramming was performed by Wang and colleagues [61]. Starting from the established CASD approach [13], they screened small molecules that could replace the pluripotency transcription factors to activate mouse fibroblasts and convert them into CMs. Combining iPSCs inducing/enhancing small molecules together with cardiogenic small molecules to activate the cells and then to direct the differentiation process toward the cardiac phenotype, they defined a cocktail of compounds consisting of SB431542 (ALK4/5/7 inhibitor), CHIR99021 (GSK3 inhibitor), parnate (LSD1/KDM1 inhibitor), and forskolin (adenylyl cyclase activator) (SCPF), which was sufficient to convert mouse embryonic fibroblasts (MEFs) or mouse tail-tip fibroblasts (TTFs) into iCMs in combination with only one pluripotency transcription factor (*Oct4*) [61, 68]. In particular, converted cells expressed cardiac-specific genes, such as *Myh6*, *TnnT2*, *Ryr2*, *Gata4*, *Nkx2.5*, and the first beating cluster appeared around day twenty after treatment. Interestingly, immunostaining analysis revealed the presence of the myosin light chain-2v (MLC2v), which is a ventricular specific marker, indicating that most of the iCMs were of the ventricular subtype, as confirmed also by the action potential measurements [61]. Since ventricular cardiomyocytes are the cell population that is typically lost during myocardial infarction, the results obtained by this modified CASD approach could represent the basis for a considerable step forward in the bench to bed translational process of direct cardiac reprogramming strategies.

The combination of the genetic and chemistry characteristics of the CASD approach was applied also with the more "classical" cardiac transcription factors cocktail (GMT). In fact, several studies demonstrated that reprogramming efficiency of genetical reprogramming could be increased with the use of small molecules to induce or inhibit specific molecular pathways. In particular, it was demonstrated that, during GHMT reprogramming, suppression of pro-fibrotic signaling by TGF-β inhibitors SB431542 or A-8301, or by ROCK inhibitor Y-27632 increased the conversion efficiency 5-fold in both embryonic and adult mouse fibroblasts [22], and enhanced the kinetics of the reprogramming process, with spontaneously contracting CMs emerging in less than two, instead of four, weeks [66]. The group of Yamakawa [63] screened eight cardiogenic compounds used to

differentiate pluripotent stem cells in combination with GMT cocktail. They found that the combination of fibroblast growth factor 2 (FGF2), FGF10, and VEGF, greatly improved the quality of cardiac reprogramming of mouse fibroblasts, activating p38 mitogen-activated protein kinase and phosphoinositol 3-kinase/AKT pathways and also removing Gata4. A screening of 5,500 compounds in GMT-overexpressing murine fibroblasts, identified two molecules (the TGF-β inhibitors SB431542 and the WNT inhibitor XAV939) that increased the reprogramming efficiency by eight folds, reducing also the time of transdifferentiation. *In vivo*, the heart treated with GMT and molecules, showed a significant improvement after infarction, as compared to a heart exposed to GMT alone. It was also demonstrated that this reprogramming protocol ameliorated the transdifferentiation of human fibroblasts [36]. Modulation of other pathways showed an increase in the efficiency of fibroblast reprogramming [1]. In particular, the inhibition of the Notch pathway with the N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), doubled the conversion of mouse fibroblasts with the GMHT cocktail, increasing the binding of MEF2C to the promoter regions of cardiac structural genes [1]. These studies demonstrated the importance and the potential of small compounds in controlling and promoting the differentiation process.

The described genetical/chemical direct cardiac reprogramming approaches are summarized in Table 2.

2.3. Chemical Direct Cardiac Reprogramming

As mentioned before, a purely chemical approach for direct reprogramming could intrinsically be safer than the genetic one. Along this line, a series of very recent studies reported the direct conversion of mouse and human fibroblasts into CMs without the use of viruses. In particular, several groups tried to directly convert fibroblasts to CMs by simply delivering specific transcription factors inside the cell, avoiding the use of viral vectors and exogenous DNA integration. Because proteins with high molecular weight cannot easily pass through the cell membrane, different delivery methods have been used to introduce these transcription factors inside the cell to induce the reprogramming process. The first attempt to transdifferentiate fibroblasts to

cardiomyocytes using recombinant proteins was made by Islas and colleagues in 2012 [24]. They showed that the combination of Mesoderm Posterior BHLH Transcription Factor 1 (MESP1), which is a key transcription factor in the development of cardiac mesoderm, together with the ETS Proto-Oncogene 2 (ETS2), a transcription factor required for the mesoderm initiation from the epiblast, was able to convert normal human dermal fibroblasts into cardiac progenitors, expressing α-striated actin, troponin T and troponin I. They fused MESP1 and ETS2 to a short fragment of the transactivator of transcription protein (TAT) to increase the cell permeability but the conversion efficiency still remained very low [24]. More recently, Li *et al* showed that individually modifying the four cardiac transcription factors GATA4, HAND2, MEF2C, and TBX5 (mGHMT), through a recently developed protein transduction reagent with high delivery efficiency and low toxicity (QQ-reagent; US patent 2009/0298111 A1), they were able to transduce more than 96% of human dermal fibroblasts. In particular, combining mGHMT with a cardio-inductive medium supplemented with BMP4, activin A, and basic fibroblasts growth factor (bFGF), they generated functional human cardiac progenitor cells (piCPC) after 28 days in culture that were similar to cardiac progenitors in morphology, colony formation capability, expression of cardiac endogenous markers, and cardiac lineage differentiation potential. Moreover, mGHMT treatment was able to induce epigenetic modifications in the enhancer region of the cardiac progenitor-specific gene *Nkx2.5*. In fact, the enrichment of trimethylated histone H3 lysine 4 and monoacetylated histone H3 lysine 9 proved the presence of transcriptionally active chromatin. The piCPCs were also injected into a rat heart tissue after myocardial infarction, and they played an important role in moderating the left ventricular remodeling. Thus, both ejection fraction and fractional shortening were improved in rats transplanted with piCPCs, together with a significant decrease in fibrosis, as compared to control animals at four weeks after MI [30].

Finally, the most recent approach for directly reprogram fibroblasts is based on the use of chemical cocktails of small molecules. One of the earliest evidence, even before iPSCs breakthrough, showing how chemistry could play a crucial role in the reprogramming strategies, was obtained with the synthetic purine *reversine* [10]. The molecule induced the de-differentiation of lineage-

committed murine myoblasts, and reversine-reprogrammed mouse and human fibroblasts could be induced to differentiate into skeletal myocytes both *in vitro* and *in vivo* [4]. More recently, promising results were obtained by Hou and collaborators, who demonstrated the possibility to convert mouse somatic cells into pluripotent cells using a combination of seven small-molecules alone [19]. These chemically induced pluripotent stem cells (CiPSCs) [3] were very similar to ESCs in terms of gene expression profiles, epigenetic status and differentiation potential. Thus, treatment with small molecules could make exogenous transcription factors overexpression completely dispensable for cell fate reprogramming [19].

Regarding CMs generation, two independent groups obtained cardiomyocytes-like cells starting from mouse fibroblasts with two different cocktails of small molecules. The You's group achieved chemical-induced cardiomyocyte-like cells (CiCMs) selecting twelve small molecules involved in the induction of the reprogramming process or in the maintenance of the pluripotency of ESCs. To identify which combination of molecules was responsible for the generation of the spontaneously contracting cell population, each of the twelve small molecules was removed from the culture medium, and they eventually defined a combination of five small molecules (forskolin, A-8301, SC1, Chir99021 and BayK 8644 (FASCB)) that are sufficient to convert mouse fibroblasts into cardiomyocyte-like cells. In particular, these five small molecules were able to modulate different cellular targets such as the glycogen synthase kinase 3 (GSK-3), the activin receptor-like kinase 5 (ALK5), the cyclic AMP [57], the ras GTPase activating protein (Ras-GAP)/extracellular signal-regulated kinase (ERK), and calcium channels, suggesting that the reprogramming process is complex and mediated by several cellular signaling pathways [46]. At the same moment, Xie's group identified another small molecule combination (Chir99021, RepSox, forskolin, VPA, Parnate, TTNPB, DZnep (CRFVPTZ)) that was able to induce murine fibroblasts conversion into chemically induced cardiomyocytes (CiCMs). Moreover, they showed that the combination of a four-core chemicals (CRFV) was sufficient to promote fibroblasts conversion. This cocktail of small molecules was used as the basal induction system for the screening of several compounds, including

modulators of cardiac development or somatic cell reprogramming, which could enhance the efficiency of the transdifferentiation process [17].

Taken together, all these chemical approaches demonstrated the possibility to convert murine fibroblasts into automatically beating cardiomyocyte-like cells. In both cases, the resulting CiCMs expressed cardiac specific markers, possessed the epigenetic status typical of cardiac genes, and showed the subcellular structures similar to CMs. Moreover, these cells were characterized by a typical cardiac calcium flux and electrophysiological features as spontaneous contractility [17, 46].

Very recently, S. Ding's group, a pioneer and leader in the field, for the first time successfully performed the chemical conversion of human fibroblasts into functional beating cardiomyocytes [7]. Their strategy was based on a cocktail of small molecules able to induce or enhance cellular reprogramming (cell activation), in combination with several cardiogenic molecules, such as activin A, BMP4 and VEGF, to induce their differentiation towards the cardiac cell phenotype. The pivotal principle of their approach, as for the other attempts discussed above, is to initially promote an epigenetic state in the cell to be reprogrammed, which is characterized by an open chromatin. Thus, cells become more responsive to stimuli with extrinsic cardiogenic factors that could bind the promoter/enhancer regions of key genes, modulating fundamental pathways such as TGF-β, Wnt, and GSK3β [31, 32]. In particular, Cao and colleagues screened several sets of compounds, in addition to the SCBF combination composed of SB431542, CHIR99021, parnate and forskolin. Finally, they optimized a cocktail composed of nine small molecules (9C) that was able to convert human foreskin fibroblasts into CiCMs by a sequential induction of mesoderm, cardiac progenitor cells, and finally cardiomyocytes, reproducing the physiological cardiogenesis [6]. CiCMs showed a well-organized sarcomere structure and expressed specific morphological and functional cardiac markers, such as cardiac troponin T and I, connexin43, atrial natriuretic factor and MLC2v. Moreover, 97% of these CiCMs spontaneously beat *in vitro*, thus demonstrating that they were functional and possessed electrophysiological characteristics similar to CMs. Importantly, CiCMs were directly reprogrammed without passing through a pluripotent cell-like state and maintained the parental genomic stability. Furthermore, when transplanted into infarcted mouse hearts, 9C-treated

fibroblasts differentiated into CMs and partially re-muscularized the injured area [7]. These results opened new insights for a completely drug-based direct reprogramming of human cells into CMs, even if many efforts are still needed to optimize these procedures before their possible use in clinical trials.

The described chemical direct cardiac reprogramming strategies are summarized in Table 3.

3. Considerations on Direct Cardiac Reprogramming

Yamanaka's revolutionary discovery of reprogramming somatic cells into iPSCs suggested the possibility to identify novel approaches to directly convert fibroblasts into functional cardiomyocytes without going through the pluripotent state (Figure 1). Despite an initial enthusiasm about the tremendous potential of direct reprogramming, there are many questions and technical challenges to be overcome before a real clinical application can be developed. The first concern regards the efficiency of the reprogramming process. The conversion rate of iCMs ranges from 5% to 20%, which is still too low to be sufficient for a full regeneration of an injured myocardium. In fact, as directly reprogrammed cells exit the cell cycle and stop proliferating, the initial reprogramming rate, as well as the speed and the quality of the conversion, have to be high enough to reach the cell numbers required for the development of a therapeutic strategy. It is estimated that at least 50% of the starting cells should be reprogrammed into mature CMs to be considered relevant in a post myocardial infarction state [9]. Moreover, another important concern is the fact that iCMs are still immature cells, rather distant from fully differentiated CMs. The percentage of functional iCMs, that are spontaneously beating and showing action potential, dramatically lowers to 0.01 - 0.1% [50]. Moreover, it was demonstrated that iCMs beat *in vitro* in a non-rhythmic way, typical feature of early embryonic CMs [50]. This point represents an important safety issue for future applications, because partially reprogrammed cells are not electrically coupled with cardiac resident cells, and could potentially generate disturbances of the physiological cardiac rhythm [50].

Another important aspect to be considered is the subtype of cardiomyocytes that are generated by direct reprogramming. Most of the transcription factors and small molecules combinations tested

generated only atrial-type cells [13, 21, 38, 40]. These results could represent a substantial limitation for the applicability of direct fibroblasts conversion strategies because the majority of cardiac cells lost during myocardial infarction that need replacement are of the ventricular subtype. However, it has been demonstrated that the GHMT strategy induced immature atrial, ventricular and pacemaker CMs in the same proportions [40]. Thus, in the future, it will be interesting to identify key factors for the determination of each different CMs subtype in order to apply specific direct reprogramming approaches to generate the correct cell type for each cardiac disease. Furthermore, several groups also tried to understand whether the reprogrammed cells passed through a pluripotent or progenitor state before becoming iCMs. This is an important aspect to be considered for a clinical application, because it would be preferable to generate CMs without inducing any pluripotent feature, in order to avoid the risks of teratomas formation and of excessive proliferation. Interestingly, direct fibroblasts conversion with transcription factors does not seem to depend on the pluripotent/progenitor state [16, 38, 40], whereas fibroblasts transdifferentiation with microRNAs passed rapidly through a progenitor state, as indicated by the upregulation of *Mesp2*, a cardiac mesodermal marker [25]. CASD approach, which employed pluripotency factors [13, 61], showed ISL1 positive cells mitotically active, but no OCT4, NANOG, or REX1 positive cells. The authors stated that no pluripotent intermediate was generated during the process [13, 61], despite another group, using the same protocol, observed NANOG positive cells during the differentiation process, indicating a transient acquisition of pluripotency [34]. Thus, it seems clear that a more accurate understanding of this aspect will be mandatory before any possible clinical application, in order to minimize the safety risks for the patients. Strictly connected with the pluripotent/progenitor state concern, another major obstacle for a future clinical translation of the direct reprogramming approach is the use of viruses carrying genetic material as the delivery method. Indeed, the risk of random genomic integration of overexpressed transgenes, that modify the genomic stability of the parental cells, could dramatically increase the probability of adverse effects and tumor formation [9, 37]. Indeed, there is an urgent need to optimize the gene delivery systems with non-integrating vectors, or to replace the reprogramming transcription factors with small molecules cocktails. In

particular, small molecules seem to be really appealing because they offer numerous advantages as compared to virus-based approaches: they exert cellular effects in a transient and dose-dependent manner, and their action could be finely regulated. Moreover, they are non-immunogenic, cost-effective, and could be structurally modified to improve potency, selectivity, or pharmacological properties [7]. *In vitro* experiments of both murine and human fibroblasts reprogrammed into iCMs [7, 17, 46] showed promising results, although the methods still need to be optimized to increase the conversion efficiency and to obtain iCMs with fully-mature cardiomyocytes characteristics. To date, there is no report of successful direct-chemical reprogramming of cardiac fibroblasts *in vivo*. This is probably due to several critical challenges of the approach, such as the development of specific delivery strategies of the reprogramming molecules *in vivo*. In particular, the ideal delivery system should allow the temporal and spatial control of compounds-release, in order to control the reprogramming process and, even more challenging, to direct the reprogramming molecules only to cardiac fibroblasts, to avoid undesired side-effects on other cells.

Another interesting, yet puzzling result from these studies is that the reprogramming process seems to be more efficient *in vivo* than *in vitro* [26, 48, 51]. Indeed, cardiac microenvironment that provides soluble co-factors and extracellular matrix (ECM) connections in an organized 3D structure could play a crucial role, thereby facilitating a more rapid and efficient conversion of resident cells into more mature iCMs. Moreover, cardiac fibroblasts are exposed to mechanical forces inside the heart that might positively influence the reprogramming process [37]. Another aspect that may contribute to the increased efficiency *in vivo* could be associated to a self-concentration of the virus in relatively small extracellular areas inside the heart, with consequent increased biological effects [9]. However, many critical points still remain pending, including the number of newly generated iCMs *in vivo,* which is difficult to be predicted and determined. The effect of reprogramming on the cardiac functionality principally depends on the efficiency of the conversion. Indeed, even if the efficiency is higher *in vivo*, it is commonly accepted that the number of iCMs is still too low to be responsible for the improvement detected in the cardiac function. A possible explanation could be that viral infection modifies the amount and the paracrine behavior of

the “scar-producing” fibroblasts, ultimately reducing the size of the scarring zone [38, 64]. In addition, the integration with the resident CMs is another critical point of the reprogramming strategies: newly formed iCMs have to integrate in the heart general structure and become electrically coupled with the native CMs. On the contrary, if iCMs remain isolated, they could have the propensity of triggering arrhythmic events, worsening an already compromised heart [49]. Therefore, the optimization of the *in vivo* reprogramming approach, in order to generate higher numbers of more mature iCMs is still an important goal to be achieved before a functional integrated myocardial tissue could be obtained. Moreover, from a clinical perspective, there is another very important aspect to be considered, which is the timing of intervention. The general principle of direct reprogramming is the notion that inducing the process, while the scar in the infarcted area is still forming, should give better results. However, it is still unclear how this would affect the healing process of the heart. The answer is difficult to predict, although it has been demonstrated that the absence of cardiac fibroblasts, due to loss of *Tcf21* transcription factor, resulted in a complete perinatal lethality [2]. Moreover, the generation of ECM is fundamental for the maintenance of the 3D cardiac structure and its synthesis and degradation are highly dynamic processes regulated by fibroblasts [15]. Reduction of ECM production has been shown to be harmful for heart morphology, as it can cause CMs slippage, and a consequent chamber dilation and systolic dysfunction [43]. On the other hand, a reduction of fibroblasts in damaged areas will reduce local fibrosis, with positive effects for cardiac function [48]. However, it seems quite difficult to perfectly balance all different cell types present in a normal heart, using the current direct reprogramming strategies.

Another important and critical point about the time of intervention is when the reprogramming should be performed. In fact, during the *in vivo* experiments, reprogramming factors are injected during ligation of the left anterior descending coronary [25, 48, 51]. However, this scenario is quite different from real life, as patients typically arrive to the hospital at least a few hours after an acute myocardial infarction. Furthermore, the earliest stages of healing, characterized by ECM synthesis, are fundamental in preventing cardiac rupture [11]. On the contrary, if the intervention occurs too

late, the ECM-rich environment may impair fibroblasts conversion and iCMs survival. Thus, it is clear that further studies are mandatory to better characterize these aspects for determining the best timing for intervention.

4. Conclusions

Despite decades of progress in modern medicine, heart disease is still one of the leading causes of death worldwide and there is essentially any cure for a failing heart. As heart failure is mainly characterized by loss and dysfunction of cardiomyocytes, efforts have been directed to identify strategies for replacing dead or damaged CMs with newly synthesized cardiac cells. As described in this review, direct cell reprogramming represents an attractive and valuable alternative to cell therapy, which may have several advantages over cell-transplantation strategies. Ideally, the process is simpler, does not require a surgical approach, and intrinsically should not have problems of immunogenicity or tumorigenesis. Nonetheless, although preliminary results are very promising, the reprogramming protocols still show many critical points that need to be addressed.

However, the astonishing ongoing efforts worldwide are unprecedented, and it can be easily predicted that many answers will be provided in the nearest future, and that we are not very far from novel regenerative therapies.

5. Conflicts of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Direct Reprogramming Approaches for Cardiac Regeneration

Direct reprogramming of fibroblasts into iCMs was achieved with different strategies:

Ex vivo approaches achieved by retro-/lentiviral infection of combinations of cardiac TFs or microRNAs; chemical reprogramming with proteins or small molecules cocktails; cell activation followed by chemical differentiation (CASD). iCMs were then intramyocardially injected in the failing heart.

In situ lineage conversion of scar fibroblasts was induced directly by TFs or microRNAs intramyocardial delivery with retro-/lentiviral vectors.

The small molecules approach has not been yet tested *in vivo* and could represent a future perspective to treat cardiac dysfunction.