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Review article

Reprogramming for cardiac regeneration

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ABSTRACT

Treatment of cardiovascular diseases remains challenging considering the limited regeneration capacity of the heart muscle. Developments of reprogramming strategies to create *in vitro* and *in vivo* cardiomyocytes have been the focus point of a considerable amount of research in the past decades. The choice of cells to employ, the state-of-the-art methods for different reprogramming strategies, and their promises and future challenges before clinical entry, are all discussed here.

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INTRODUCTION

Each somatic cell is tightly programmed to perform a very specific function, and has the appropriate structure and intracellular components to perform its functions. During fetal life there is a certain degree of plasticity with cells being reprogrammed to a different type of cell during the process of organogenesis¹. This process of “natural” reprogramming stops after birth. Strategies to induce reprogramming of somatic cells after birth started in 1958, with the work of Gurdon and colleagues in Cambridge, UK, using nuclear transfer in frogs². Slow progress in the field continued until it was realized twenty years ago that the technique can be used for tissue regeneration as well as for producing *in vitro* models of disease for drug testing and genetic manipulations. This resulted in a massive expansion in the field with the publication of thousands of papers related to this topic. Several strategies have been developed for reprogramming which include nuclear transfer, and forced expression of one or more transcription factors or microRNA, to produce pluripotent cells followed by strategies to induce differentiation to the desired cell type (indirect reprogramming)³. More recently, strategies to reprogram cells from one somatic cell type to another, without passing through the pluripotent stage (direct reprogramming) has been developed.^{4,5} We here describe the evolution of the different types of reprogramming with particular reference to the heart, as well as work done at QCRC.

ADULT STEM CELLS AND REPROGRAMMING INTO CARDIOMYOCYTES

Stem cells are unspecialized cells with potentially unlimited proliferation attributes (self-renewal) and the capacity to differentiate into specialized cell types.⁶ These cells, though, can be further classified into subtypes of stem cells according to how many specialized cell types they can differentiate into, often called their “potency” or “differentiation potential” (Figure 1). From “totipotent” in the fertilized egg, cells specialize along embryo development and only “multipotent”, “oligopotent” and “unipotent” can be found in adults. These adult stem cells, however, all maintain the property of self-renewal and a certain differentiation capacity. The feasibility of cell therapy has been investigated in several of these adult stem cell populations.^{7–11} First reported in 1999,¹² adult stem cells such as bone marrow **mesenchymal stem cells** (BM-MSCs), for which the possibility of autologous stem cell isolation has long been known, were shown to be reprogrammable into cardiomyocytes (CMs). Since that time, colossal efforts have been made to employ MSCs (in particular BM-MSCs) in heart failure clinical application, and there was a focus on improving *in vitro* or *in vivo* differentiation of MSCs into CMs. Thus, the use of bone marrow cells (BMCs) for treating myocardial infarction and heart failure have been reported in a large number of clinical trials.¹³ However, conflicting results, limited *in vitro* and *in vivo* reprogramming of human MSCs into CMs and the limited clinical benefits obtained, have led to research on other adult stem cell types such as cardiac stem cells.^{14–18}

Within the heart, different populations of cardiac stem cells (CSCs) have been extensively described and isolated based on extracellular marker expression or isolation processes.^{19,20} We can quote five main types of CSCs: cardiac c-kit⁺ cells (defined by Lin⁻ c-kit⁺ markers), cardiac Sca-1⁺ progenitor cells (defined by Sca-1 expression), side-population cells (defined by their capacity to efflux Hoechst dye when analyzed in flow cytometry), cardiosphere-derived cells (CDCs) (defined by their capacity to form a sphere by tissue explanting technology) and genetically engineered cells such as Isl1-expressing cells. Among these five CSCs type described, only two populations of CSCs (c-kit⁺ and CDCs) have been escalated to phase I clinical trials, yet the clinical benefit following implantation of the c-kit⁺ CSCs has been challenged recently.^{21–23} The outcomes of these trials at phase I only delineate the safety and tolerability of transplantation of those cells but present limitations on understanding the benefits for patients means that further clinical trials will be approached cautiously.

Facing the limited benefits observed using these adult stem cells, pluripotent stem cells (PSCs) (embryonic stem cells (ESCs) and induced-PSCs (iPSCs)) retained therefore the most appealing potential for cardiac regenerative medicine. Since the first demonstration of feasibility in 1985 on murine ESCs, much effort has focused on establishing reliable and efficient differentiation strategies to produce CMs from PSCs. Presently, various cytokines and small molecules are capable of improving CM differentiation from PSCs with nearly 90% efficiency. As discussed later in this review, CMs can be derived from PSCs in a step-wise manner via sequential treatment with cytokines or small molecules.^{24, 25} Both types of PSCs present advantages and challenges regarding clinical applicability. Abundant data from preclinical studies have demonstrated the safety, feasibility, and efficacy of these cells, justifying the careful entry of PSCs based therapies into clinical trials.^{26,27}

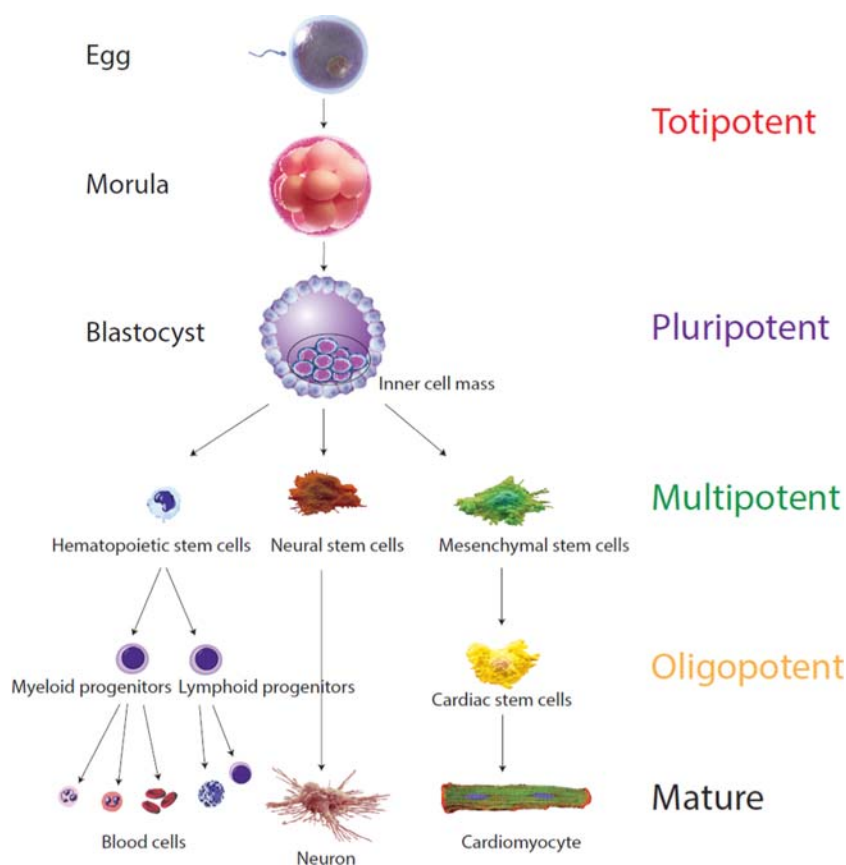


Figure 1. Different cells' "potency". The "potency" of a cell is defined by the number of cell types it has the capacity to differentiate into. The fertilized egg is "totipotent", cells having the potential to develop into an entire organism and therefore possesses the totality of potentials. This totipotent cell will divide in human for 4 days retaining this full capacity until a blastocyst develops, where these cells acquire some specialization. The cells from the inner cell mass cannot develop anymore into an entire organism, as they are unable to form the placenta but can still differentiate into all cell types within the organism. They are therefore qualified as "pluripotent". Pluripotent cells will further multiply and acquire more specialization. The resulting "multipotent" cells retain the capacity to differentiate into various cell types. They are already specialized into ectoderm, endoderm or mesoderm. Finally, cells are considered "oligopotent" when they can only differentiate into very limited cell types (adapted from Ref. ¹⁸⁰).

REPROGRAMMING

Indirect reprogramming

iPSCs

The derivation and clinical use of human ESCs are limited by ethical problems and immunological barriers respectively. To circumvent these issues, a sizable number of researchers have dedicated considerable effort to deriving PSCs from adult, non-pluripotent somatic cells, which display the same characteristics as ESCs. This was first achieved in mice in 2006, and in humans in 2007, by Yamanaka *et al.* The first iPSCs were generated following retroviral transfection of adult fibroblasts with 4 transcription factors previously known to be involved in the maintenance of pluripotency (Oct3/4, Sox2, Klf4, and c-Myc, the OSKM factors).²⁸ Yamanaka discovered that the iPSCs he generated are similar to ESCs by their common expression of stem cell genes and proteins, open epigenetic configuration, growth capacities, embryoid body and the ability to form teratomas, and most importantly having similar potency and differentiation capacities. Hence, Yamanaka's work has transformed our understanding of genetic reprogramming of somatic cells to a pluripotent state, and set the ground for considerable work aimed at bringing this technology to clinical applications. Since this breakthrough, multiple alternative approaches and technologies have been developed to generate hiPSCs. The OSKM

factors can be replaced by an alternative set of genes (Oct3/4, Sox2, Nanog, and Lin^{28,29,30}), avoiding the use of the oncogene c-Myc. The concerns of genomic instability due to viral delivery and integration of those factors have also been circumvented by the development of non-viral, non-integrative approaches.^{31–44} Common hiPSCs culture methods utilize a feeder layer of inactivated MEFs to support self-renewal of hiPSCs and to maintain their undifferentiated state. However, the use of MEFs exposes the cultured hiPSCs to animal contaminants. As reported for hESCs, autologous skin fibroblasts isolated from the same donor/patient can efficiently replace the MEFs.^{45,46} Additionally, Matrigel-coated surfaces, which is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, is also commonly used.⁴⁷ However, the use of Matrigel may entail a risk of contamination with animal pathogens and can be substituted by vitronectin and synthemax®.⁴⁸ hiPSCs have also been generated from various tissues including skin fibroblasts,^{28,49–51} keratinocytes,⁵² neural stem cells,⁵³ melanocytes,⁵⁴ cord blood cells,^{55,56} peripheral blood cells⁵⁷ and adipocytes.⁵⁸ This progress made possible the development of clinical grade strategies to generate autologous iPSCs with potential for clinical use.

Very recently, a highly controversial publication by Obokata *et al.* sent shock waves throughout the scientific community. The publication, rapidly withdrawn, stated that “IPS-like” cells could also be derived without introduction or delivery of any transcription factors. This controversial approach, dubbed “stimulus-triggered acquisition of pluripotency” (STAP), stated that when cells are subjected to a strong external stimulus such as transient low-pH, somatic cells (from neonatal mice) could be reprogrammed into a pluripotent state. Even if unclear, the proposed mechanism of the phenomenon was that the stimulation resulted in a decrease of DNA methylation in the regulatory regions of pluripotent transcription factors and markers. This mechanism was recently successfully achieved by Lee *et al.*,⁵⁹ demonstrating that external stimulation (*via* TLR3 ligand) activating the cells’ inflammatory pathway, led to global changes in the expression of epigenetic modifiers, which augmented chromatin remodeling and nuclear reprogramming. Although the “STAP” study could not be confirmed to date and raised numerous ethical and scientific suspicions by other groups, it has nevertheless elevated the possibility of iPSCs derivation without genetic modification and strengthens more than ever iPSCs into the race for clinical application.

Alternative approaches: Nuclear Transfer Embryonic Stem Cells

Somatic cell nuclear transfer embryonic stem cells (SCNT-ESCs) are cells that are obtained by substituting the oocyte genome with a somatic cell donor genome.⁶⁰ Once artificially activated, embryonic development begins. As the embryo reaches the blastocyst stage, and as is typical in embryonic stem cell derivation, the inner cell mass gives rise to patient-specific pluripotent stem cells (Figure 2). This method involves various chemicals such as caffeine, fetal bovine serum, puromycin, kinase inhibitor 6-DMAP, HDAC inhibitor scriptaid, and calcium depletion, which all intersect at different stages during development of SCNT-ESCs.

Given the recent successes in the derivation of patient-specific pluripotent stem cells via somatic cell nuclear transfer, groups have begun to decipher the epigenetic differences at the mechanistic level between SCNT-ES and iPSCs. Understanding the molecular determinants for these two cell types should further elucidate methodologies that will improve current limitations of pluripotent cell derivation for both *in vitro* disease modeling and cellular replacement strategies. Ma *et al.* performed this much-needed comprehensive experiment that profiles the molecular characteristics of iPSCs and SCNT-ESCs. These experiments compared and contrasted iPSCs, SCNT-ESCs and *in vitro* fertilization (IVF) produced ESCs. They demonstrated that iPSCs derived with transcription factor-based methodologies displayed inadequate epigenetic reprogramming. While the same somatic cells derived via SCNT were epigenetically and transcriptionally similar to control IVF-ESCs, there is residual DNA methylation in cells derived by transcription factor-based reprogramming. It is interesting to note that genome-wide microarray-based DNA methylation techniques demonstrated that iPSCs, at a frequency eightfold higher than SCNT-ESCs, contain somatic patterns of CpG methylation, which is consistent with previous studies demonstrating that iPSCs retain an “epigenetic memory” of the somatic cells they are derived from.^{61,62} Hence, SCNT-ESCs are better at retuning genomics of reprogramming more faithfully than iPSCs. Taken together, these subsequent data highlight the importance of investing more in studies that will lend insight into improving current reprogramming paradigms. Methodologies of reprogramming will have to take into account reprogramming factors upstream of pluripotency. One place to look in order to identify possible strategies will be the ooplasm which may possibly contain processes that demethylate the somatic genome more faithfully compared with the more subdued or

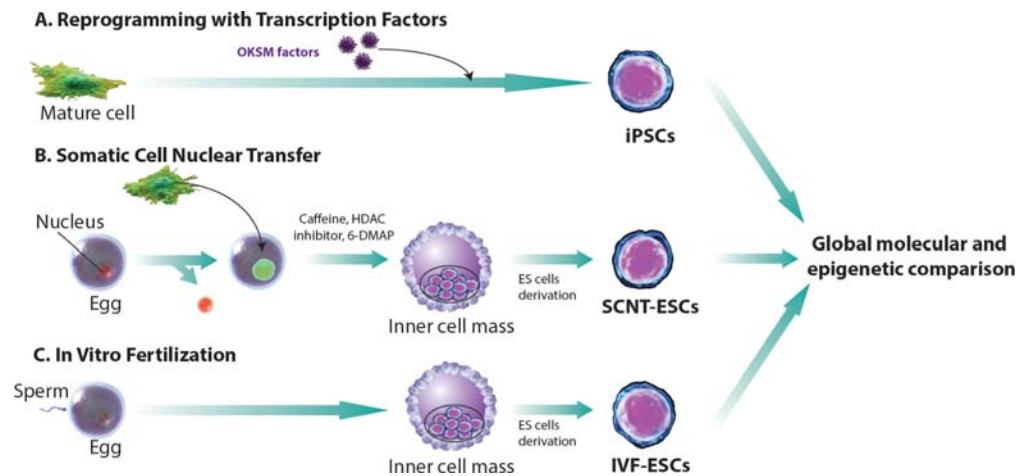


Figure 2. Comparing techniques for generating stem cells. There are three distinct ways to derive pluripotent stem cells *in vitro*. (A) iPSC cell derivation is achieved via conversion of somatic cells with the addition of a cocktail of transcription factors, originally described as “Yamanaka factors”. (B) Traditional embryonic stem cell derivation can be augmented by replacing the nucleus of an egg with a nucleus from a somatic cell at an early stage, as the cells mature and form the blastocyst. The inner cell mass (ICM) is isolated to form Nuclear Transfer embryonic stem (NT ES) cells. (C) *In vitro* fertilization is performed and ES cells are derived when reaching the blastocyst level to obtain IVF-ESCs (adapted from Ref. ⁶¹).

passive demethylation process during transcription factor-based reprogramming. In any case, the development of hiPSCs has emerged as a promising source of PSCs for tissue engineering, cell-based therapies, novel drug screening, and disease modeling. The drastic improvement of hiPSCs reprogramming^{63,64} has reduced the time and cost of iPSCs generation and has fostered even more the enthusiasm toward PSCs use in cellular therapy. Beside the improvement of hiPSCs generation, major improvements have been made in the differentiation, purification, and maturation of iPSCs derived CMs, precluding iPSCs cardiac clinical use.

Cardiomyocyte generation from PSCs

Lessons from developmental biology

In order to achieve cardiac lineage reprogramming and to derive efficient differentiation protocols, investigators took lessons from developmental biology and the mechanisms of cardiac commitment in early embryos. Gastrulation begins with mesoderm induction through Nodal signaling in the primitive ectoderm. Nodal, a cytokine belonging to the TGF- β superfamily, plays a crucial role in the formation of the primitive streak and germ layers. As gastrulation proceeds, cardiac progenitor cells are among the first cell lineages to be established from mesoderm cells emerging from the primitive streak. These cells express mesoderm gene inducers and transcription factors such as BMP4, Wnt3, Brachyury T, and MESP1.^{65–67} The latter acts as a key regulator of cardiovascular lineage commitment and drives expression of various transcription factors including NKx2.5, Gata4, Mef2c, and Tbx5 for cardiac differentiation and maturation.^{68,69} MESP-1 also represses early mesoderm induction through direct inhibition of Wnt and Nodal signaling pathways by DKK1 and CER1^{70,71} (Figure 3A). Accordingly, induction of cardiac differentiation of human PSCs can be initiated by sequential stimulation with specific recombinant growth factors such as basic fibroblast growth factor (bFGF), BMP4, Wnt3, and Activin A, followed by addition of DKK1 or other Wnt inhibitors.^{72–74} Further cytokines have been reported to increase the differentiation efficiency such as the vascular endothelial growth factor (VEGF),⁷⁴ Noggin⁷⁵ (BMP antagonist), and IWR-1/IWP-2⁷⁶ (inhibitors of Wnt) among others. Taken together, these findings have led to the establishment of efficient protocols allowing the production of CMs from PSCs with yields close to 90%.^{16,25} Nevertheless, much effort is still needed to improve purification and maturation of the derived CMs.^{77,78}

Improvement of CMs differentiation

Many strategies have been proposed to improve the differentiation and maturation of iPSCs derived CMs. In particular, hypoxic culture conditions,^{79,80} knockdown of selected genes,⁸¹ and forced aggregation of iPSCs-derived EBs in a chemically defined medium⁸² have shown promising results.

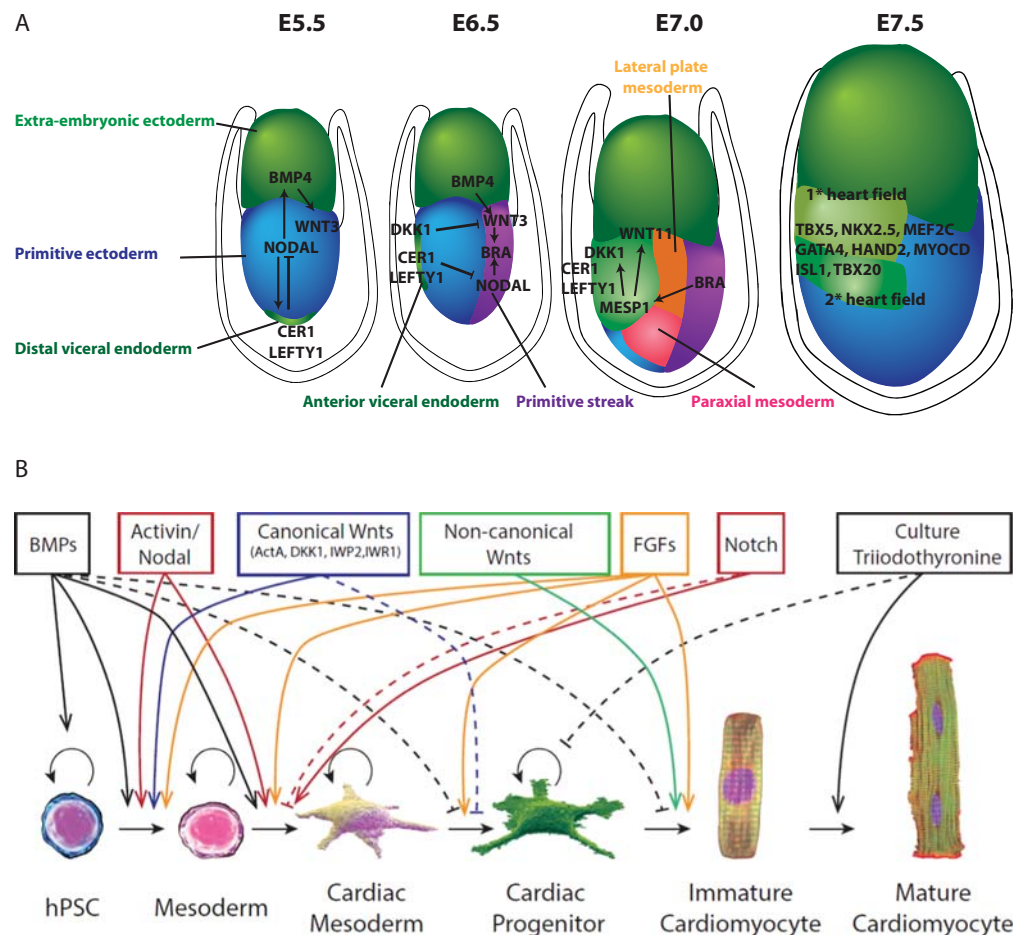


Figure 3. Lesson from embryology and factors involved in hPSC cardiac differentiation. **(A)** During mouse embryonic development at E5.5 gastrulation occurs by the formation of the primitive streak. An epithelial to mesenchymal transition (EMT) of anterior primitive ectoderm allows cells to move laterally between primitive ectoderm and visceral endoderm. At E6.5 cells located proximally to the primitive ectoderm go on to form the extraembryonic mesoderm. Cells adjacent to this zone form heart, blood and mesoderm derivatives. The most distal portion of the primitive streak gives rise to endoderm cells. At E7.0 the lateral plate mesoderm is formed which delaminates to form two layers. Cardiac mesoderm goes on to form the first heart field (FHF) laterally and more ventrally the second heart field (SHF) is formed both by coordinated expression of Dkk1, MESP1, Nodal and WNT signaling. Other cardiogenic signals, such as BMP and FGF, activate cardiac-specific transcription factors such as Nkx2.5, GATA4, HAND2, which coordinate to move both heart fields to the midline. Whereby at E7.5, the FHF progenitors form the heart tube which later contributes to the left ventricle. SHF progenitors join with the CMs of the FHF which leads to the rightward looping of the cardiac tube which eventually progresses towards formation of cardiac chambers. **(B)** Schematic representation of a family of factors reported to trigger progression from pluripotent state to CM (adapted from Ref.²⁴).

Further, a high-throughput screening system has been established to identify small molecules that promote the differentiation of CMs from iPSCs. Among these, vitamin C,⁸³ cyclosporine A⁸⁴ (immunosuppressant), and triiodothyronine⁸⁵ (T₃, thyroid hormone) showed a marked induction of cardiac differentiation. Of note, the exogenous expression of human Apolipoprotein-A1⁸⁶ or induction of the Wnt/ β -catenin signaling pathway⁸⁷ have been shown to enhance cardiac differentiation and maturation of hESCs and hiPSCs. These cardiogenic effects are thought to be mediated by the BMP4/SMAD signaling pathway. A recent publication of highly reproducible and efficient differentiation protocols, *via* temporal modulation of canonical Wnt signaling (90% of differentiation),^{16,25} sets the path for clinically scalable applications (Figure 3B).

Aside from all the different iPSCs to CMs differentiation protocols that are being considered, a crucial parameter influencing the differentiation potential is the cellular origin of the iPSCs.⁸⁸ This is likely to be due to the “epigenetic memory” of iPSCs, which manifests as differential gene expression and variable

differentiation potential.^{88–90} In brief, the two major factors determining iPSCs differentiation to CMs are the starting cell population and the cardio-inductive growth factors used.

Purification and enrichment of iPSCs derived CMs

Following differentiation, even if great yields are obtained, only pure CMs could potentially be used clinically. The iPSCs derived CMs therefore need to be purified and enriched before potential implantation to avoid risks of teratoma formation or inappropriate tissue engraftment. Frequently used methods include manual dissection of spontaneously beating CMs using a pulled-glass micropipette,⁹¹ cell separation based on density gradient,⁹² and fluorescence-activated cell sorting (FACS).^{93,94} Physical enrichment by manual dissection or density gradient separation has limited success due to their low yield. The FACS technique relies on a positive selection of CMs cells that are phenotypically different from other cells. This can be realized by tagging cardiac-specific proteins with fluorescent antibodies followed by a subsequent step of detection and sorting using a FACS machine. Hence, a panel of surface markers is being used for the enrichment of CMs. This panel includes the following surface markers: CD166,⁹⁵ vascular endothelial growth factor 2 (VEGFR2) and platelet-derived growth factor- α (PDGFR- α),¹⁷ elastin microfibril interface 2 (EMILIN2),⁹⁶ signal regulatory protein- α (SIRPA- α),⁹⁷ and vascular cell adhesion protein1 (VCAM1).^{97,98} A major limitation for this approach is the lack of specific CMs surface markers that could specifically identify and select only cardiac cells from a pool of differentiating/undifferentiating cells.⁹⁹ Therefore, genetically modified hESCs lines have been developed to select the terminally differentiated CMs based on the expression of a luminescent reporter gene (e.g the green fluorescent protein, GFP) coupled to the regulatory sequence of a cardiac-specific gene like MYH6,¹⁰⁰ Nkx2.5,⁹⁸ myosin light chain 2V (MLC2V),¹⁰¹ and insulin gene enhancer protein 1 (ISL1).¹⁰² The expression level of the reporter fluorescent proteins reflects the transcriptional activity of the attached cardiac gene. Although the latter method showed a marked CMs purification and enrichment, they obviously cannot be used on a larger scale or for clinical applications. Interestingly, Hattori *et al.* reported that a fluorescent dye that labels mitochondria may serve as a selective marker of hESCs/iPSCs derived CMs. The authors claimed that a significant enrichment of CMs with a high degree of purity (>99%) could be obtained by FACS with this dye.⁹³

Purification and enrichment of PSCs derived CMs is an important limiting step for cell-based therapy for cardiac regeneration. Though different protocols for purification and enrichment of CMs have been proposed, their efficiency is controversial. An efficient method of purification should be fast, specific, and scalable with no genetic modifications. Such a method would greatly enhance the potential of iPSCs derived CMs in the cardiology clinic.

Maturation of iPSCs-derived CMs

Beyond the differentiation protocol improvements, much work focused on the maturation of PSCs derived CMs. Indeed, the maturity of generated CMs remains a critical bottleneck. For instance, despite the fact that iPSCs derived CMs present most of the characteristics of adult CMs (self-induced action potential, proteins/genes expression), the generated cells lack maturity for the most part (non-expression of mlc2v contractile protein, lack of organization, absence of M-band etc) and more closely resemble embryonic CMs than adult CMs.¹⁰³ A remarkable publication by Murry and colleagues¹⁰⁴ showed that implantation of a large number of human embryonic stem cell-derived CMs in non-human primates following heart failure could lead to re-muscularization of infarcted heart and even electromechanical junctions with surrounding muscle, but even over a three month period, cells failed to completely mature, and the non-human primate ultimately experienced arrhythmic complications. Long term culture of PSC derived CMs has been shown to improve the maturity of differentiating cells, but remains of restricted interest for potential clinical applications.¹⁰⁵ Our group and others have previously demonstrated that a forced alignment of the cells improves the maturity of the CMs generated^{78,106} (Figure 4). The mechanical and electrical stimulation of derived CMs has also been proven to favor maturation of cells toward an adult phenotype.^{107,108} Besides, the use of scaffolds for maturation of CMs may lead to their potential interest for clinical use.

iPSCs and targeted gene therapy

While iPSCs propose to circumvent the autologous hurdle of cell therapy, this technique cannot be used straight away for patients presenting disorders linked to genetic abnormalities. The ability to generate gene targeted mutations or manipulations in iPSCs has opened new areas of research in

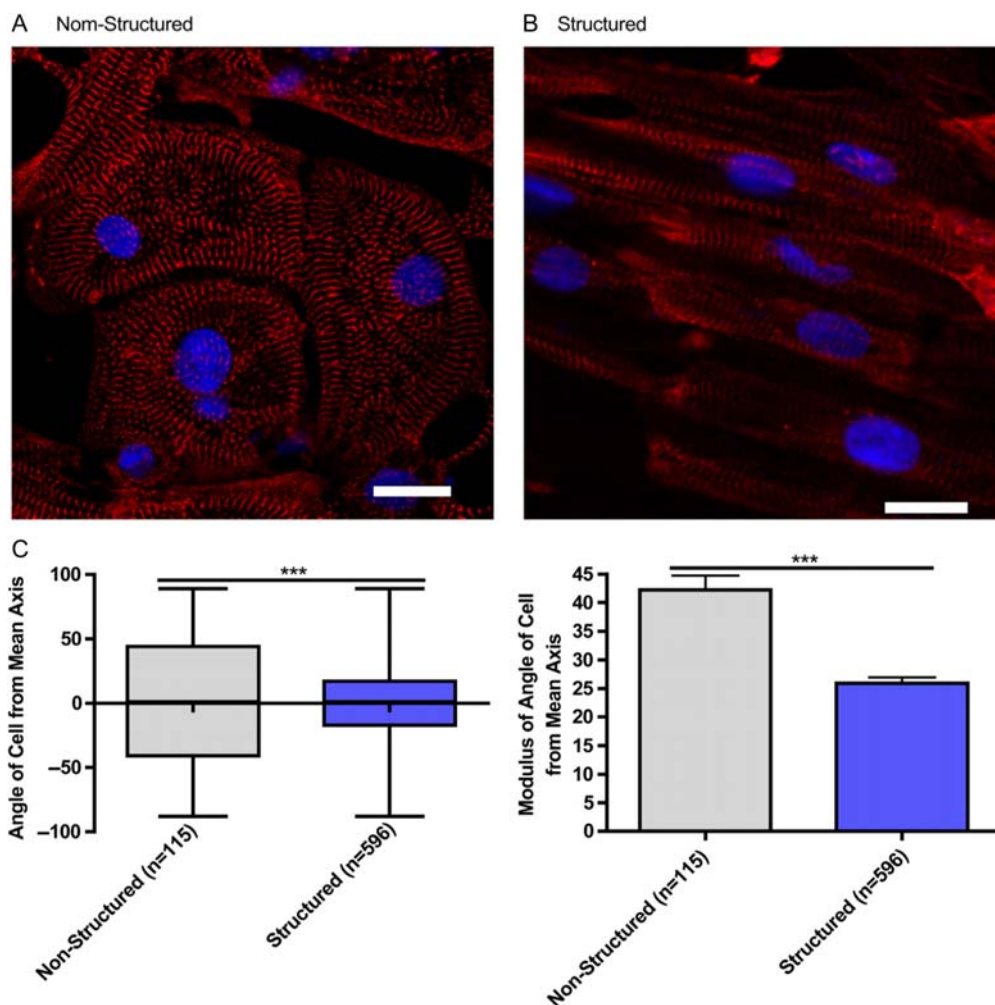


Figure 4. Microgrooved culture substrates' effect on calcium cycling of cardiac myocytes derived from human-induced pluripotent stem cells. Representative immunofluorescence of iPSC-CM cultured on unstructured polydimethylsiloxane (A) and microgrooved polydimethylsiloxane (B), Red - sarcomeric α -actin, Blue - DAPI, scale bar 20 μ m. Quantification of cell alignment iPSC-CM on structured and unstructured constructs (C). (Adapted from Ref.⁷⁸)

dissecting multifaceted genetic interactions. It has become increasingly easy to utilize genome engineering techniques which involve site-specific nucleases acting as DNA scissors that introduce double strand breaks, allowing expression of nucleotide alterations, knockin reporters, and small insertions or deletions to study loss of function mutations.

The possibility of using iPSCs for targeted gene therapy reflects further their promising therapeutic potential. Hockemeyer *et al.* demonstrated in 2011 the possibility of genetic engineering of hESCs and hiPSCs using TALE nuclease.¹⁰⁹ Reversion of disease mutation was further demonstrated in hPSCs by various groups utilizing CRISPR-Cas and TALENs.^{110–114} For example, Jessup *et al.* showed that overexpression of SERCA2a via gene therapy can improve the contractility of iPSCs derived CMs of dilated cardiomyopathy patients.¹¹⁵ Jiang *et al.* demonstrated that the allele specific silencing of a dominant mutation could suppress hypertrophic cardiomyopathy condition in mice.¹¹⁶ Subsequently, other groups have introduced conversion of normal genes to disease models by genome editing, targeting specific mutations to mimic known diseases and syndromes in hPSCs.^{117,118} Gonzalez *et al.*, have even combined two gene editing tools, TALEN and CRISPR/Cas systems, developing a more efficient platform which can rapidly and simultaneously introduce multiple gene alterations along with stage specific inducible genetic alterations which they named the iCRISPR platform.¹¹⁹ Thus, the possibility to use the high proliferative capacity of iPSCs would enable the expansion of clonal populations of genetically modified iPSCs *ex vivo* followed by their subsequent differentiation and

selection of the “corrected” cells for transplantation.¹²⁰ This approach would therefore resolve the issue of non-specific targeting, which is one of the drawbacks of conventional gene therapy.

This genetic editing could also be of great interest for clinical applications as previous studies have shown that even autologous mouse iPSCs are rejected by an immune response.¹²¹ Perhaps the combination of this new wave of technology platforms for engineering genetic alterations and better understanding reprogramming may circumvent strategies that will have to be employed to produce iPSCs which need to evade immune rejection in the therapeutic context.

Immunogenicity of iPSCs

Recently, human ESCs have been used in phase-I clinical trials to treat acute spinal cord injury¹²² and Stargardt’s macular dystrophy.¹²³ In preclinical studies, they have also been directed to differentiate into CMs for *in vivo* studies.^{104,124,125} hESCs derived CMs were demonstrated to reduce LV remodeling and promote LV systolic function in rat hearts following myocardial infarction.^{124,125} More recently, hESCs derived CMs were also shown to generate extensive vascularized cardiac muscle in the infarcted hearts of non-human primates.¹⁰⁴ Despite the advantage of using hESCs to generate large numbers of human CMs for clinical transplantation, these cells are non-self and therefore contribute to immune rejection unless with courses of immunosuppression. Similar to human ESCs, human iPSCs would have a better potential in cell replacement therapy by virtue of their “autologous” nature; however, in any transplantation setting, the histocompatibility of iPSCs must be considered before transplantation.

The immunogenicity of human iPSCs has not been carefully examined and whether they are truly “autologous” remains controversial. In one study, teratomas derived from syngeneic murine ESCs were accepted while those derived from syngeneic murine iPSCs were rejected by activated T cells, suggesting that syngeneic iPSCs are more immunogenic upon transplantation compared with their syngeneic ESC counterparts.¹²⁶ Later, Abe *et al.* showed that teratomas derived from integration-free, syngeneic murine iPSCs did not trigger immune rejection and had the same rate of success following transplantation compared with their syngeneic ESC counterparts.¹²⁷ In addition, Boyd *et al.* also demonstrated that tissues of the three embryonic germ layers (endothelial cells, hepatocytes and neuronal cells) derived from syngeneic murine iPSCs did not trigger immune rejection following transplantation.¹²⁸ More recently, Wu *et al.* have shown that syngeneic murine iPSCs were rejected but their differentiated progeny such as endothelial cells were accepted following intramuscular injection.¹²⁹

We are still unsure why syngeneic iPSCs were accepted in some transplantation settings but not in others. It could be due to expression of non-self-proteins during the reprogramming process. Xu *et al.* discovered that syngeneic murine iPSCs express minor antigens such as Zg16, Hormad1, and Retn, which are not normally found in murine ESCs¹²⁶ (Figure 5). Indeed, forced expression of Zg16 and Hormad1 could induce teratoma regression. Overexpression of these minor antigens in some iPSC lines is believed to be a result of mutations in the coding sequences of iPSCs¹³⁰ or the epigenetic difference between iPSCs and ESCs.¹³¹ Moreover, the viral vectors carrying pluripotency-inducing transgenes for reprogramming could also trigger antiviral immunity or anti-DNA antibody production. Therefore, the use of nonviral and integration-free methods for reprogramming could be a safer option to prevent immune surveillance. For instance, we could use modified mRNAs which have been shown to induce iPSC reprogramming with very high efficiency and minimal immune recognition. Nevertheless, more work is still needed to examine whether human iPSCs are truly “autologous” and whether the degree of immunogenicity would be regained following cardiomyocyte differentiation for transplantation.¹³²

Direct reprogramming

Both types of PSCs (ESCs and iPSCs), cultured or induced, prior to CMs differentiation, trigger the risk of teratoma formation, inappropriate tissue engraftment and immune rejection when later implanted into the patient. This issue remains one of the major drawbacks of these approaches in clinical trials. Even if new strategies and drugs are tested specifically to eliminate undifferentiated cells and therefore their tumorigenic risk, the path remains long before a potential clinical application.¹³³ Therefore, development of strategies avoiding the pluripotent stage raised great enthusiasm. The publication of direct genetic reprogramming approaches (known as “trans-differentiation” or “direct conversion”) brought exciting perspectives for cardiac cellular regeneration. This genetic modification allows the direct conversion of a terminally differentiated cell type into so-called *induced cardiomyocytes* (iCMs) (Figure 6).

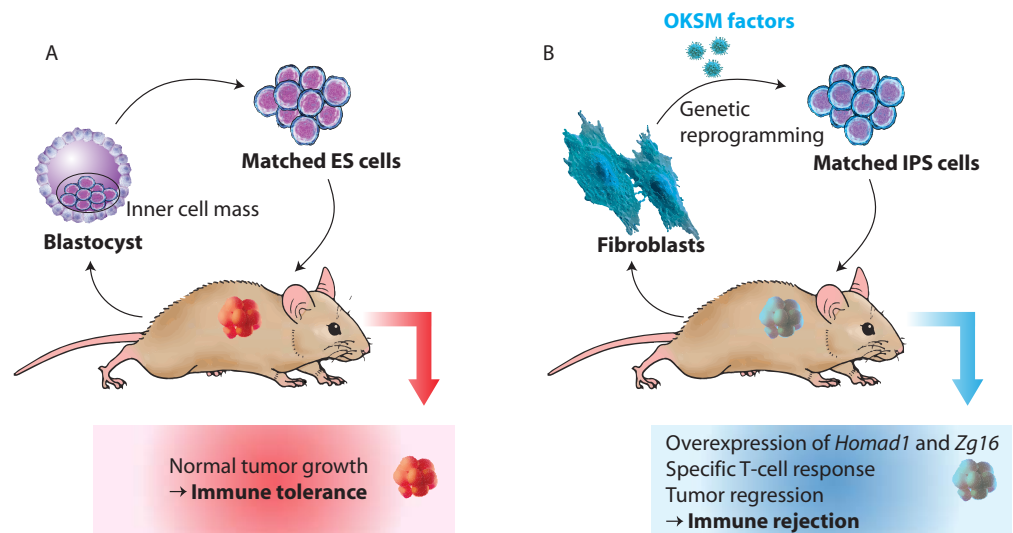


Figure 5. Immunogenicity of induced pluripotent stem cells. (A) Zhao *et al.* Performed transplantation studies assessed by injections of embryonic stem cells from the same genetic background found that ES cell-based transplants formed normal tumor growth. Hence, autologous ES cell transplantation evades immune system rejection. (B) Conversely, autologous iPS cells, derived from the same fetal fibroblasts, exhibited immunological rejection upon transplantation whilst in the same genetic background (adapted from Ref.¹⁸¹).

Reprogramming of fibroblast toward cardiac fate

The use of transcription factors (TFs) to induce the expression of specialized genes and therefore the reprogramming of adult fibroblast was first demonstrated by Weintraub *et al.* in 1987 using the transcription factor (myogenic regulatory factor) MyoD to induce skeletal muscle cells

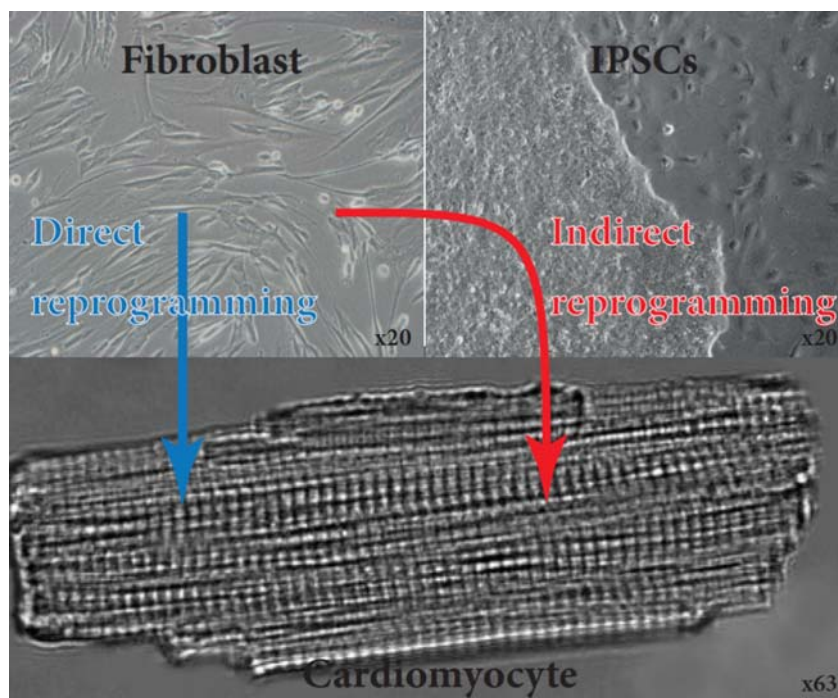


Figure 6. Generation of functional cardiomyocytes by direct and indirect reprogramming of fibroblasts. Fibroblasts derived from skin biopsy from a patient. These cells were then reprogrammed using OSKM factors towards induced pluripotent stem cells. These are then directed to differentiate towards CMs. Fibroblasts can also be directly reprogrammed towards CMs.

trans-differentiation.^{134,135} Subsequently, the identification of only a single factor driving cardiac fate has long been elusive. Yamanaka's seminal discovery that necessitates co-transfection of multiple factors simultaneously to reprogram fibroblasts into iPSCs, has led to subsequent studies to identify a cohort of TFs essential for direct cardiac reprogramming.³ The first demonstration of fibroblasts reprogramming into CMs was published in 2010 by Ieda *et al.*⁵ Working on neonatal and adult mouse fibroblasts, they originally tested 14 central TFs for cardiac differentiation and serially eliminated factors to finally establish that at least 3 TFs were necessary for CM trans-differentiation.⁵ They demonstrated that transduction of the following TFs GATA4 (member of the GATA family of zinc finger transcription factors), Mef2C (Myocyte-specific enhancer factor 2C) and Tbx5 (T-box transcription factor 5) (the three of them are now commonly referred as the GMT factors), successfully converted 15% of mouse cardiac fibroblast into α myosin heavy chain (α -MHC) expressing cells, out of which 20% also expressed cardiac troponin T (cTnT). They described these cells as "cardiomyocyte-like" cells. This first demonstration was later scrutinized when Chen *et al.* published that using the same GMT factors in adult fibroblast, no expression of α -MHC or NKX2.5 (Homeobox protein NKX2.5 often used as an early cardiac differentiation marker⁹⁸) could be detected.^{136,137} However, feasibility is now confirmed by different groups,^{138–141} but a broad range of efficiency is reported using the GMT factors. The heterogeneous nature of reported results could in part be explained by the variation in genetic background of mice used, the fibroblasts isolation protocols, the culture methods, the promoter employed for expression of the TFs as well as the type of virus used (lentivirus, retrovirus) and their respective efficiency of transduction.

The direct reprogramming of mouse fibroblast was also later performed using alternative combination or additional TFs. Indeed, Song *et al.* showed that the addition of a basic helix-loop-helix transcription factor Hand2 to the GMT factors could increase the percentage of cells co-expressing α -MHC and cTnT.¹³⁸ More recently, Addis *et al.* showed that the addition of Hand2 and NKX2.5 (used here as a transcription factor to induce differentiation) could increase by > 50 fold the cardiac reprogramming of fibroblasts.¹³⁹ Similarly, Christoforou *et al.* showed that the addition of the following TFs Mesp1, MYOCD (Myocardin), Smarcd3 (Baf60c) and Srf (serum response factor) could also enhance the efficiency of GMT factors.¹⁴⁰ Protze *et al.* demonstrated that the replacement of Gata4 by MyoCD gave more cardiac gene expression rate compared to GMT factors.¹⁴¹ Alternatively, Jayawardena *et al.* demonstrated that a combination of miRNA with addition of JAK (Janus protein tyrosine kinase 1) inhibitor 1 (to inhibit the JAK/STAT pathway) could also successfully convert fibroblast into iCMs.¹⁴² It is now accepted that in appropriate conditions, iCMs can be derived from mouse fibroblasts.^{143–145} The efficiency of those various combinations remains difficult to compare since various promoters, fibroblasts and viral deliveries were employed. In addition, the analysis of physiological parameters like calcium oscillation and spontaneous action potential were not performed in all of these studies. The evaluation of the efficiency should give greater consideration to these physiological parameters over cardiac genes expression. This *in vitro* demonstration in mouse still remained to be translated into human. The first report in August 2012 by Islas *et al.* demonstrated that neonatal foreskin fibroblast could be reprogrammed into cardiac progenitors using the transcription factors MESP1 and ETS2.¹⁴⁶ However, it was only early this year that Nam *et al.* revealed the possibility of reprogramming adult cardiac or dermal fibroblast into cardiac fate using the optimized combination of GMT factors with Hand2, MYOCD and two microRNAs miR-1, and miR-133.¹⁴⁷ The evidence presented confirms that the expression of proteins and microRNAs produces cells that display calcium transients, sarcomere-like structures, and spontaneous contractility. This indicates that human fibroblasts are amenable to reprogramming by forced expression of cardiac-specific transcription factors with muscle-specific microRNAs in order to produce cardiac-like myocytes. Further work in elucidating the exact mechanisms and role of these factors and microRNAs will represent a possible step toward the clinic and its therapeutic applicability. Confirmation of these results was done by Wada *et al.* a few months later, where they document that the addition of Mesp1 and MyoCD induced reprogramming of cardiac fibroblast into iCMs presenting cardiac gene expression, Ca²⁺ oscillation and action potential amenable to synchronous beating when co-cultured with murine CMs.¹⁴⁸ A few months later, Fu *et al.* reported that fibroblast from human neonatal skin or fetal heart could be reprogrammed using GMT factors with the addition of ESRRG and MESP1. This resulted in global cardiac gene-expression and phenotypic shifts.¹⁴⁹ The demonstration of feasibility on human fibroblasts encourages further investigation towards this approach.

What kind of fibroblasts should be targeted?

The *in vitro* demonstration of direct fibroblast reprogramming previously discussed was performed on embryonic, neonatal, adult tail-tip, skin and cardiac fibroblasts.^{5,141,142} Fibroblasts have long been considered as of uniform cell type across different tissues.^{150,151} Morphologically they are flat, spindle-shaped cells with multiple processes emanating from the main cell body. They can be defined as mesenchymal cells producing extracellular matrix (ECM) components like collagens and fibronectin.¹⁵² This simple morphological definition highlighted a mistaken identity with mesenchymal stromal/stem cells (MSCs).¹⁵³ This view has now been challenged by the demonstration of phenotypic heterogeneity of fibroblast from different tissues in different conditions.¹⁵¹ This heterogeneity poses an active area of investigation for direct reprogramming methods to focus on the most appropriate fibroblasts. But which fibroblast populations are the most appropriate to focus on? To answer this question, we might first need to address the question of the different strategies that can be foreseen using this direct reprogramming technique. Indeed, two types of therapy (summarized in Figure 7) can be envisioned using direct reprogramming.

The first strategy would be a cell-replacement based therapy, where functional iCMs are generated *in vitro* and then injected to the site of injury. This approach relies on the same concepts developed with a strategy using PSCs. Advantages of this alternative approach in lieu of PSCs are the avoidance of potential teratoma formation and an increase in kinetics that would be gained from avoiding a PSCs intermediate state. In this case, the most promising autologous cells would most probably be, as for iPSCs, the use of adult skin fibroblasts. Similarly the choice for derivation of iPSCs from skin fibroblasts is driven by the easy access to skin fibroblast cells for personalized medicine with autologous cell-based treatment. The possibility of an *in situ* reprogramming approach is the most appealing aspect in direct reprogramming. In this case, using viral delivery of TFs and specific targeting, the fibroblasts would be reprogrammed directly in place. This would present the considerable advantage of replacing the lost contractile tissues without any *in vitro* manipulations and hazardous cell injection. In this case, cardiac fibroblasts would represent the target of choice. A first obvious reason to justify this choice comes from the composition of human hearts. Indeed, cardiac fibroblasts represent the largest cell population of the heart (~50–60% of cells), while CMs represent only 40% of cells.¹⁵⁴ A second characteristic that makes cardiac fibroblasts a target of choice for direct reprogramming is their ideal location. Indeed, after infarction, lost CMs are slowly replaced by fibrotic tissues caused by fibroblast invasion. Therefore, these fibroblasts are ideally placed, with ideal homing abilities to be converted in

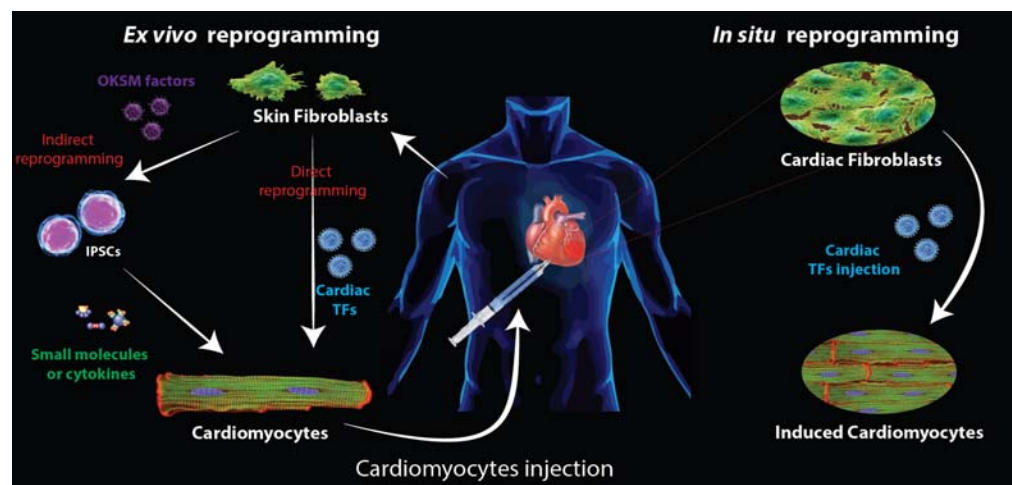


Figure 7. Schematic representation of heart repair strategies using reprogramming technologies. Two strategies to repair post myocardial infarction are envisioned using *in vitro* reprogramming techniques starting from skin fibroblast. First fibroblasts are reprogrammed into iPSCs before being differentiated into CMs using small molecules or cytokines. The second approach consists of a direct reprogramming of the skin fibroblast into iCMs. In both cases, the CMs produced *in vitro* have to be injected into the patient. When considering *in situ* reprogramming, the transcription factors (carried by viruses) are directly delivered to the patient and TFs will be expressed in cardiac fibroblasts, reprogramming them directly into iCMs. OKSM factors: Oct4, Sox2, Klf4, c-Myc.

iCMs to reconstitute the lost contractility. However, it is still to be proven that these fibroblasts invading the post-myocardial infarction area are, as normal heart fibroblasts, prone to cardiac reprogramming.

Long believed to be simple “gap filler” and matrix producer, the perception of cardiac fibroblasts is now considerably evolved. Fibroblasts are now known to play an important active role in heart function, facilitating the intercellular communication between myocytes and endothelial cells and even between fibroblasts themselves.¹⁵⁵ Moreover, they have been shown to affect the electrophysiological properties. A direct interaction between myocytes and fibroblasts occurs via gap junction connexins (Cx40, Cx43 and Cx45) to facilitate the electrical conduction in the heart.^{150,151,155,156} They also express cadherin (notably N and T-cadherin) which play a critical role in heart development and function.¹⁵⁷ This *in situ* strategy is the spearhead of direct reprogramming strategy, particularly since proof of concept has been achieved.

***In situ* reprogramming promises in animal models**

The successful *in vitro* trans-differentiation led rapidly to attempts at direct *in situ* reprogramming. To date, five studies demonstrated the feasibility of such a technique in murine models.^{138,142,158–160} In three of the mouse models, the specific expression of the TFs in fibroblasts was ascertained by the use of cre-recombination under the control of a fibroblasts-specific promoter like fibroblast specific protein 1 (FSP1), periostin or transcription factor 21 (Tcf21) promoters. This approach ensured the targeted conversion of non-myocyte fibroblast into iCMs and avoided analysis of ectopic activation of TFs in neighboring CMs. In their rat model, Mathison *et al.* chose an over expression system using CMV promoter which nevertheless reported overall ejection fraction improvement.¹⁵⁹

The demonstration of effective reprogramming into functional cardiac-like myocytes with evidence of electrical coupling and modest but significant improvement of cardiac function was ultimately reported. These trials are a great demonstration of proof of principle that cardiac fibroblasts can be reprogrammed into CMs-like cells in their native environment which is shaping the way towards a blueprint for future clinical translation. Regardless of the modest improvement observed, the data suggest that optimization of the TFs cocktails or co-injection with various pro-angiogenic molecules and fibroblast-activating peptide should further be pursued.^{158,159}

Sinoatrial cells direct reprogramming

In mice and rats, cellular transplantation experiments using PSCs derived CMs can improve function after myocardial injury. However, two critical bottlenecks related to its maturation and electrophysiological behavior remain unresolved. First, current understanding of the relationship between cardiac-specific genes and functionally distinct cardiac myocytes (nodal-like, atrial-like, and ventricle-like) is poorly understood. Secondly, the electromechanical integration and arrhythmogenic potential of these PSCs derived CMs need to be further defined. Previous demonstration of direct and indirect reprogramming into CMs almost systematically produced mixed atrial, ventricular and nodal-like CMs. A little more than a year ago, Kapoor *et al.* paved the way for a different type of cardiac direct reprogramming. They elegantly demonstrated *in vitro* that the transfer into ventricular CMs of Tbx18 (a critical gene for early sinoatrial node (SAN) cells specification) could convert ventricular CMs in 2 to 4 days into cells displaying “spontaneous electrical firing, physiologically indistinguishable from that of SAN cells”. They also demonstrated in their publication that this approach could be used *in situ* as they established in guinea-pig that focal Tbx18 gene transfer could correct bradycardia phenotype.¹⁶¹ In a more recent publication this year, Josowitz *et al.*, using bacterial artificial chromosome (BAC) reporter constructs, were able to identify and purify hPSC-derived atrial-like CMs based on sarcolipin expression. This allowed for delineating cells via flow cytometry, whereby cells with high fluorescence specifically expressing atrial-specific gene sarcolipin (SLN) were isolated. These cells exhibited atrial specific calcium handling and electrophysiological characteristics.¹⁶² These sets of data should glean methodologies that will pave the way for more concrete specific CM subtype generation as well as for developing ways to study chamber-specific pathologies and lineage development.

Importance of the niche

We previously discussed that reprogramming and differentiation is based on transcription factors and cytokines/small molecules treatments attempting to mimic naturally occurring development. However, such simple culture conditions often fail to give cells the full environment necessary for appropriate differentiation, leading to partial reprogramming and immature differentiation. Much research focused

on recreating this more complex and multifactorial environment, or “niche”, in which the cells are naturally placed. We previously discussed that maturation of PSCs derived CMs is more efficient when cells are subjected to an electro-mechanical stimulating environment as encountered *in vivo*. Besides, complex molecular stimulus which can be provided by the niche is hardly reproducible artificially. For instance, direct differentiation of MSCs into CMs is often unsuccessful or at least inefficient in human.^{163–165} However, an acquisition of CM features is observed when MSCs are simply co-cultured with neonatal CMs, revealing a complex autocrin/paracrine system of CMs.^{166–168} Similarly, clinical trial using MSCs injection following ischemic heart failure demonstrated modest benefit.¹³ It is now accepted that, rather than direct differentiation of these MSCs into CMs, it is the paracrine effect of the MSCs and their influence on the microenvironment that drive those beneficial effects by stimulating endogenous cardiac progenitors and fibroblasts.^{13,169–172} Fioret *et al.* recently demonstrated that endothelial cells are also a key component of the cardiac ventricular myocyte niche.¹⁷³ The extracellular matrix, and more particularly its content in metalloprotein MMP9, has also recently been demonstrated as an essential component that can influence the differentiation of hESCs into CMs.¹⁷⁴ Sandler *et al.* demonstrated recently that direct reprogramming of endothelial cells into hematopoietic stem cells was made possible when using vascular cells as a feeder to recreate the vascular niche.¹⁷⁵ Transferring this approach into direct cardiac reprogramming or the possibility of using feeders to recreate a more “natural” niche for PSCs cardiac differentiation is certainly a promising area of research.¹⁷⁶

FUTURE CHALLENGES

After the first retinal cells derived from iPSCs were transplanted into a woman with eye disease earlier this year,¹⁷⁷ reprogramming into iPSCs before differentiation into CMs seems to be the most advanced area of research for cardiac cellular therapy. However, many problems remain to be addressed before that stage can be reached with cardiomyocytes. Firstly, even if differentiation efficiency has been achieved recently, the purification of genetically unmodified cardiomyocytes needs to be worked out. Even if teratoma formation encountered with PSCs is avoided and time gain is anticipated, the path remains long before application of PSCs becomes a clinical reality for cardiac injuries. The cell maturity prior to implantation definitely needs to be improved. The potential immune-rejection of cells, even autologous, needs to be further studied and anticipated. Beyond these capital points, an optimization of cell delivery with potential tissue engineering and scaffolds has to be addressed, as this could affect the maturity, the engraftment capacity, the electro-mechanical coupling and the survival of the cells. Nevertheless, the subsequent milestones achieved over the past decades make PSCs clinical use for cardiac regeneration more promising than ever, and efforts should be pursued in that direction.

As exciting as direct reprogramming is, this approach could be even more challenging. When considered for primary *in vitro* differentiation before cell-based therapy, one might consider that differentiation of PSCs into CMs is a more advanced field. The same hurdles encountered with PSCs derived CMs will have to be ruled out before clinical translation of directly reprogrammed cells can be envisioned. Besides, even at the *in vitro* level, it remains difficult to compare the various TFs cocktails reported and future work should attempt to compare those cocktails side by side. Also, despite successful expression of cardiac markers, not all studies could demonstrate functionality of the iCMs¹⁴¹ and the focus should now be on functionality (analyzing spontaneous contractility or induced action potential measurements) rather than on single gene expression. Similarly, characterization of iCMs subtype (atrial, ventricular or nodal) and their maturity needs to be addressed and fine-tuned. The efficiency of differentiation also remains to be improved in order to reach clinical levels and close the gap with PSCs differentiation efficiency. The possibility of an *in situ* direct reprogramming represents an exciting blueprint for this technique. Even if some of the drawbacks previously discussed were avoided, a large number remain. Besides, in contrast to problems encountered in gene therapies, other efforts need to be made to optimize direct reprogramming before any treatment can be envisioned. Indeed, the first lesson from mice models could be that the cocktail of TFs used in the human context might need to be validated and consolidated for humans. In lieu of mouse studies, human reprogramming towards CMs has been less straightforward. Successful direct reprogramming of human cells had initially been discovered in making functionally immature neurons^{178,179} with lower efficiencies than in mouse. Therefore, it seems that human cells are typically more resistant and require activation of innate immunity for efficient reprogramming.⁵⁹ It is prudent to speculate that human reprogramming requires more acute control of regulatory elements to convert cells towards a different cell fate lineage. Early larger mammalian animal models would deliver more certainty regarding the validity of this

approach in humans. Finally, specific delivery of TFs needs to be optimized and validated for clinical use by drug dosing and toxicology testing. Optimization of the promoter used (allowing for the gene expression to be turned off after differentiation) also needs to be performed, and similarly to PSCs derived CMs cell-based therapy, the maturity of the cells achieved *in situ* needs to be validated.

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