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Molecular Discoveries and Treatment Strategies by Direct Reprogramming in Cardiac Regeneration

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Abstract

Cardiac tissue has minimal endogenous regenerative capacity in response to injury. Treatment options are limited following tissue damage after events such as myocardial infarction. Current strategies are aimed primarily at injury prevention, but attention has been increasingly targeted toward the development of regenerative therapies. This review focuses on recent developments in the field of cardiac fibroblast reprogramming into induced cardiomyocytes. Early efforts to produce cardiac regeneration centered around induced pluripotent stem cells, but clinical translation has proved elusive. Currently, techniques are being developed to directly transdifferentiate cardiac fibroblasts into induced cardiomyocytes. Viral vector-driven expression of a combination of transcription factors including Gata4, Mef2c and Tbx5 induced cardiomyocyte development in mice. Subsequent combinational modifications have extended these results to human cell lines and increased efficacy. The miRNAs including combinations of miR-1, miR-133, miR-208, and miR-499 can improve or independently drive regeneration of cardiomyocytes. Similar results could be obtained by combinations of small molecules with or without transcription factor or miRNA expression. The local tissue environment greatly impacts favorability for reprogramming. Modulation of signaling pathways, especially those mediated by VEGF and TGF- β , enhance differentiation to cardiomyocytes. Current reprogramming strategies are not ready for clinical application, but recent breakthroughs promise regenerative cardiac therapies in the near future.

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Keywords

Direct Reprogramming; Fibroblast; Cardiac Regeneration; Viral Vector; Cardiomyocyte

Introduction

Acute myocardial infarction (MI) occurs when a coronary artery is suddenly occluded. Lack of blood flow to the myocardium leads to tissue necrosis within minutes. The response to MI involves loss of cardiomyocytes followed by increased collagen deposition and scar formation.¹ Replacement of cardiomyocytes with scar tissue reduces cardiac function and increases demand on remaining muscle cells. This process worsens the risk for other cardiac complications including recurrent MI, heart failure, and arrhythmias. Current treatment options following MI are limited by the relative inability of the heart to endogenously regenerate itself in adults.^{1,2} A major emphasis is placed on developing therapies to induce cardiac regeneration. Different techniques are being actively studied to achieve this goal.

Tremendous advances have been made in cardiac regeneration in the past decade. One developing regenerative modality is the use of stem cell therapy. Induced pluripotent stem cells (iPSCs) can be derived from fibroblasts by exogenous transcription factor expression in vitro.³ iPSCs can then be delivered to damaged tissue. However, challenges remain before iPSCs can represent a viable treatment option. These include development of efficient delivery systems of iPSCs to damaged myocardium, improving survival and functional maturation of transplanted iPSCs, and potential tumorigenicity of iPSCs. Another area to be studied further is successful electromechanical integration of newly formed cardiomyocytes with endogenous beating cells and the risk of induced arrhythmias.⁴⁻⁶ While stem cell therapy holds great promise, clinical trials to-date have been disappointing, and challenges remain.

Another avenue for cardiac regeneration is direct conversion of native fibroblasts into induced cardiomyocyte-like cells (iCMs). Following MI, cardiac tissue is enriched in fibroblasts, an appealing potential source for reprogramming into iCMs.¹ Retroviral vector-mediated expression of transcription factors has been shown to directly reprogram murine cardiac fibroblasts into iCMs in vitro and in vivo.⁷⁻⁹ Additionally, this reprogramming has been reproduced in human fibroblasts in vitro, though conclusive clinical trials are lacking.¹⁰ Techniques are also being developed to produce iCMs using non-integrating adenoviral vectors which would improve vector safety in humans. Synthetic miRNAs and chemical cocktails can also be used to generate iCMs. These would alleviate concerns over potential immunogenic and genetic concerns associated with viral vector use, and would reduce costs and facilitate mass production of therapeutic agents.¹¹

Like stem cell therapy, challenges remain before direct reprogramming can be applied in a clinical setting. These include verifying the safety of viral vectors in vivo, assessing long term effects on cardiac function and mortality, improving the efficacy of reprogramming and maturation which remains too low in human fibroblasts, and optimizing specific delivery of reprogramming factors to damaged tissue while minimizing collateral effects. Compared to stem cell therapy, direct reprogramming, also called transdifferentiation, has certain

advantages. Direct reprogramming bypasses any stem cell like state, reducing the risk of teratoma formation. iCMs would also be formed from infarct area fibroblasts, bypassing the need for tissue transplantation and electromechanical integration with the native myocardium. Additionally, viral vector cardiac specific tropism can be exploited to enhance delivery to damaged myocardium and increase transcription factor expression.¹²

Regenerative cardiac medicine is a young and challenging field which is rapidly growing. The aim of this review is to provide a critical evaluation of current progress, remaining challenges, and future directions of direct reprogramming of fibroblasts to iCMs for treatment of cardiac diseases.

In Vitro Direct Reprogramming in Mice

Ieda et al.⁷ were the first group to successfully reprogram mouse cardiac and dermal fibroblasts into functional iCMs *in vitro*. They isolated three transcription factors necessary for transdifferentiation: Gata4, Mef2c and Tbx5 (GMT).⁷ This group of transcription factors is known to promote cardiac development as well as cardiomyocyte proliferation and function.^{13–15} Gene delivery was performed by inserting retroviral and lentiviral vectors resulting in direct reprogramming of fibroblasts to cardiomyocyte-like cells. This method bypassed intermediate reversion to a progenitor state, resulting in iCMs with a gene expression profile resembling neonatal cardiomyocytes. iCM production was measured by α -myosin heavy chain (α MHC) promoter driven expression of green fluorescent protein (GFP; α MHC-GFP). iCMs exhibited functional ability as well, including Ca^{2+} influx, electrical potentials and spontaneous beating. Though efficiency of iCM production was low, this study opened the possibility of direct reprogramming of endogenous fibroblasts into cardiomyocytes. iCM formation would skip the intermediate step of iPSC creation, and the risk of tumor formation. Additionally, autologous iCMs would presumably eliminate the need for immunosuppressive treatment and potentially other challenges of allogenic grafts.

Initial attempts to reprogram mouse fibroblasts with GMT suffered from low efficiency and there have been several attempts to make improvements (Table 1). Song et al.⁹ used retroviral delivery of the GMT and Hand2 transcription factors (GHMT) to reprogram mouse fibroblasts *in vitro*. They reported GHMT reprogramming efficiency of 9.2% (5.2%–19.7%) compared to the GMT rate of 2.9% (1.5%–5.6%) in adult mouse tail-tip fibroblasts (TTFs) as measured by α MHCGFP and cardiac troponin T expression (cTnT). The investigators also reported adult cardiac fibroblast (CF) reprogramming of 6.8% via GHMT and 1.4% with GMT. These results suggest Hand2 may interact with GMT to enhance iCM generation.⁹ While GMT is sufficient to reprogram mouse fibroblasts, addition of other transcription factors and the development of new treatment combinations hold great potential to improve efficiency.

Protze et al.¹⁶ screened different transcription factors associated with cardiac development in search of other, more effective combinations of potential transcription factors. Q-PCR analysis of multiple cardiac genes was used to assess iCM formation. They reported that using lentiviral delivery, Tbx5, Mef2c, and Myocd (3F-Myocd) reprogrammed mouse embryonic fibroblasts (MEFs) into iCMs. Furthermore, 3F-Myocd-derived iCMs expressed

more cardiac genes recorded by Q-PCR than GMT. Similarly, neonatal CFs treated with 3F-Myocd produced iCMs with greater cardiac expression than GMT. This study supported a role for Myocd in cardiac reprogramming.¹⁶ Furthermore, the 3F-Myocd protocol lacked the Gata4 component of GMT. This suggested that individual elements of GMT may not be necessary for reprogramming. The intensive transcription factor screening used here demonstrates a useful tool to identify new reprogramming factors.

Using a novel cTnT-driven GCaMP marker of transdifferentiation, Addis et al.¹⁷ compared transcription factor combinations. They found that GHMT in addition to Nkx2.5 (HNGMT) was greater than 50-fold more efficient than GMT in MEFs. While both GHMT and HNGMT were superior to GMT, HNGMT was most efficient in creating iCMs. HNGMT iCMs expressed an extensive cardiomyocyte gene profile, displayed calcium potentials and sustained spontaneous contractions. HNGMT was also more effective in reprogramming mouse adult CFs than any previously published method.¹⁷ This experiment identified Nkx2.5 as a factor that could be used to achieve improved reprogramming efficacy. Adding more transcription factors to the treatment combination would appear to potentially improve iCM yields.

Hirai et al.¹⁸ created a fusion gene between the MyoD transactivation domain (TAD) and the individual GHMT factors. Retroviral delivery of the MyoD-Mef2c fusion gene along with Gata4, Hand2 and Tbx5 produced iCMs most effectively. The authors noted that MyoD TAD fusion increased Mef2c activity greater than that of Gata4 and Tbx5. MyoD-Mef2c treated mouse TTFs were reprogrammed at a 15-fold rate, and formed larger collections of beating iCMs in less time.¹⁸ The TAD fusion gene technique was previously developed by Hirai et al.¹⁹ to generate iPSCs by increasing activity of the Oct4 transcription factor. The application of this technique to direct reprogramming is another option for enhancing iCM formation. Additionally, these findings would suggest that modulating the levels of expression of individual factors influences reprogramming.

Wang et al.²⁰ demonstrated that the relative ratios of individual transcription factor expression within a combination is another influence on the efficiency of direct reprogramming. They engineered several GMT polycistrons designed to create differential expression of Gata4, Mef2c and Tbx5. The optimal balance included higher expression of Mef2c, and lower expression of both Gata4 and Tbx5 (MGT). This combination resulted in the greatest reprogramming efficiency in vitro. MGT iCMs displayed 10-fold greater spontaneous beating and heightened cardiomyocyte gene expression.²⁰ Suboptimal balance of factor expression could help explain low reprogramming efficiency reported in numerous other studies, and optimization could improve therapeutic potential.

Zhou et al.²¹ screened protein kinases to identify molecular signaling pathways that could be targeted to improve reprogramming. They reported that addition of Akt to GHMT (AGHMT) in mouse adult CFs, MEFs, and TTFs increased the magnitude and rate of iCM formation. AGHMT iCMs of each cell line demonstrated markedly increased spontaneous contractions after two (CFs, MEFs) or three (TTFs) weeks in culture. Physiologic responses to β -adrenergic stimulation were observed. AGHMT iCMs also demonstrated a more mature cardiomyocyte phenotype evidenced by gene expression, metabolic status, cellular

hypertrophy and increased polynucleation. This was the first report of multinucleate iCM production.²¹ Akt expression modulates diverse cellular processes in the heart including growth, development and metabolic regulation. Akt is known to play a vital role in physiological cardiac hypertrophy while attenuating pathological hypertrophy. This process is dependent upon the IGF/PI3K/AKT/mTOR signaling pathway.^{22–25} Zhou et al.²¹ confirmed that enhanced reprogramming effects of Akt are mediated by IGF/PI3K/AKT/mTOR signaling, in addition to Akt inhibition of FoxO3a, but without any apparent effects on another known Akt target, Gsk3. Targeting other steps in the signaling pathway did not equal the enhancement of reprogramming from Akt.²¹ These findings suggest a central role for Akt and provide insight into the molecular mechanisms of reprogramming. Modulation of signaling pathways such as those containing Akt as done in this experiment could produce better fibroblast reprogramming. The search for optimal conditions for reprogramming is likely to identify several pathways for such treatment.

Recently, Abad et al.²⁶ reported that inhibition of the Notch pathway using DAPT, which inhibits γ -secretase Notch activation, enhanced retroviral mediated GHMT reprogramming of MEFs into iCMs.²⁶ During development, Notch signaling contributes to cardiomyocyte proliferation and development, and disruption is associated with congenital heart defects.^{27,28} DAPT increased production of iCMs and spontaneous beating, which was accompanied by increased sarcomere complex formation and calcium influx. Concurrent activation of AKT1 also improved reprogramming. The combination of DAPT, AKT1 and GHMT reprogrammed MEFs up to 70%, with 45% of reprogrammed cells demonstrating spontaneous beating. DAPT increased Mef2c binding to promoters of cardiac-related genes *Myh6*, *Tnnt2*, and *Actc2*. These results suggest complementary functions of Notch and Akt signaling in reprogramming.²⁶ Interestingly, Collesi et al.²⁹ reported that Notch1 expression stimulates proliferation in neonatal rat cardiomyocytes, attenuates fibrosis, and prevents apoptosis. Notch1 was found to maintain an immature cardiomyocyte phenotype with replicative potential in neonatal hearts. These cells were committed to a cardiomyocyte lineage, but could enter mitosis, and Notch1 expression declined with age accompanying the loss of replicative potential of the heart.²⁹ Notch inhibition also enhances iPSC production from human keratinocytes, and separately to mediate differentiation of iPSCs into neural stem cells (NSCs).^{30,31} Liu et al.³² reported that Notch signaling functions in a time- and tissue-specific manner in murine iPSCs. Notch promotes early cardiomyocyte differentiation but suppresses late stage differentiation.³² The heightened reprogramming efficiency achieved by Abad et al.²⁶ makes Notch signaling an appealing target of future therapy. However, more studies are needed to fully understand the role of Notch signaling, and the mechanistic difference between its effects on stem cell differentiation, as well as iCM induction.

Epigenetic alterations can also enhance direct reprogramming. Zhou et al.³³ screened for epigenetic regulators of iCM formation. Bmi1 was identified as a critical obstacle early in the cellular reprogramming process. Bmi1 inhibition via shRNA markedly improved efficiency of reprogramming by GMT. In the absence of shRNA, Bmi1 repressed cardiac loci, preventing reprogramming.³³ Interestingly, Bmi1 has been shown to promote formation of cardiomyocytes from cardiac stem cells (CSCs) following MI,³⁴ and to generate iPSCs when combined with transcription factor expression.³⁵ Bmi1 may have a novel function in

direct reprogramming separate from its effects related to iPSC production and CSC differentiation warranting further study. Additionally, the use of epigenetic modulation with shRNA to enhance fibroblast reprogramming is an important advancement in cardiac reprogramming. Other molecules may inhibit or promote reprogramming via epigenetic mechanisms. Elucidation and manipulation of these interactions may unleash improved iCM yields.

In Vivo Direct Reprogramming in mice

Following successful direct fibroblast reprogramming in vitro, several groups attempted to reproduce the feat in vivo (Table 1). Qian et al.⁸ first reported that retroviral delivery of GMT induced reprogramming of murine CFs into iCMs in vivo. Lineage tracing experiments were performed to track the origin of iCMs that demonstrated sarcomere formation, action potentials and contractions in response to electric stimulation. iCMs expressed N-cadherin and Cx43, prominent gap junction proteins. Additionally, Ca²⁺ potentials were propagated between iCMs and native cardiomyocytes suggesting syncytia formation and electrical coupling with native cardiac muscle. GMT was delivered to injured hearts following coronary artery ligation in a MI model. Cardiac function was assessed by stroke volume (SV), cardiac output (CO), and ejection fraction (EF) using MRI and echocardiography 12 weeks post-MI. GMT treatment was associated with improved cardiac function in all measures, as well as decreased area of infarction and scar formation. Addition of thymosin β 4 to GMT further enhanced cardiac function and limited the area of infarction.⁸ Thymosin β 4 is known to activate Akt and provide a protective effect to cardiomyocytes as well as promote repair following MI in mice.³⁶ These findings strengthen the in vitro data of Zhou et al.,²¹ suggesting a role for thymosin β 4 and Akt regulation in clinical applications. Most importantly, these findings demonstrate that GMT treatment induces fibroblast to cardiomyocyte reprogramming. Critically, this study establishes that fibroblast reprogramming can be used in a mouse MI model with improvement of in vivo mouse cardiac function.

Song et al.⁹ used retroviral delivery of GHMT to produce mouse iCMs in vitro and in vivo. Hand2 acts downstream in the Notch signaling pathway discussed previously and is essential during cardiogenesis. Hand2 defects disrupt cardiogenesis and alter trabeculae formation leading to impaired cardiac function.^{37,38} In a mouse LAD ligation MI model, GHMT reduced post-infarct fibrosis and enhanced cardiac function. GHMT treated mice had significantly attenuated loss of cardiac function as measured by MRI and echocardiography at 6 weeks. Control mice reached a stable functional status, while GHMT demonstrated continued increase of function at 12 weeks with a roughly 2-fold ejection fraction as well as half the area of fibrosis compared to control mice. GHMT enhanced cardiac function more efficiently than GMT alone. These results confirm the findings of Qian et al.⁸ and reinforce the role of Notch signaling, in this case mediated by Hand2, as a potential enhancer of reprogramming efficacy in vivo.⁹

Interestingly, both of the studies by Qian et al.⁸ and Song et al.⁹ found that reprogramming efficiency, and maturity of iCMs was greater in vivo than in vitro. The composition of the natural cardiac tissue environment may be more permissive for cardiomyocyte

differentiation than tissue culture. Several possible explanations for this observation could include the effects of growth factors, extracellular matrix, and local autonomously beating cardiomyocytes. Despite these advantages over in vitro conditions, in vivo efficiency of reprogramming remained low. Additionally, while a correlation between the concurrent reprogramming of fibroblasts to iCMs and improvement in cardiac function is suggested, a causal relationship cannot be definitively proven. Song et al.⁹ reported that GHMT treatment only converted between $2.4 \pm 1.5\%$ to $6.5 \pm 1.2\%$ of fibroblasts to iCMs in vivo. However, measures of cardiac function following treatment improved to almost double those of the untreated group.⁹ The striking improvement in cardiac function despite modest iCM yields indicate that fibroblast reprogramming may not be the sole contributing factor. Other influences may be responsible, at least in part. Alteration of fibroblast activity and attenuation of scar formation are likely to be involved in this process.

Inagawa et al.³⁹ created a retroviral polycistronic GMT vector and reported murine iCM generation in vitro and in vivo. iCMs displayed a more mature phenotype than those created with three separate monocistrons for each transcription factor. Similarly, Mathison et al.⁴⁰ reported that a lentiviral polycistronic GMT vector enhanced reprogramming in vitro and in vivo in rats. Additionally, they reported that a single polycistronic GMT vector improved cardiac function measured by EF significantly more in a rat MI model ($37\% \pm 10\%$) compared to 3 separate monocistronic Gata4, Mef2c and Tbx5 vectors ($13\% \pm 7\%$) and to control vector ($9\% \pm 5\%$).⁴⁰ Creation of polycistronic vectors may enhance reprogramming and cardiac function by inducing iCM formation following fibroblast infection by a single viral vector as opposed to requiring three infections from each of the monocistronic vectors. Decreased need for infection may also reduce the immunogenicity of treatment, lowering side effects and resistance to transdifferentiation.

Direct Reprogramming of Human Fibroblasts

Following the success of reprogramming of mouse fibroblasts in vitro and in vivo, attempts were made to reprogram human fibroblasts (Table 1). Wada et al.¹⁰ reported that unlike in mice, panretroviral GMT expression alone is insufficient for direct conversion of human fibroblasts into iCMs. However, addition of the Mesp1 and Myocd transcription factors to GMT (GMTMM) produces iCMs from human CFs and dermal fibroblasts in vitro.¹⁰ MESP1 drives differentiation into hematopoietic, cardiac, and skeletal muscle tissues in a temporal and environmentally dependent manner. MESP1 loss of function is associated with congenital cardiac defects.^{41,42} Myocd contributes to cardiac development by influencing proliferation and apoptosis of embryologic cardiomyocytes.⁴³ GMTMM overexpression causes formation of sarcomeric structures and contractile activity. The GMTMM iCMs appear similar to immature cardiomyocytes as demonstrated by morphological and physiological measurements. Interestingly, by culturing treated human CFs and dermal fibroblasts with mouse cardiomyocytes, iCMs demonstrated some increased maturity, with action potentials and spontaneous contractions.¹⁰ These findings suggest potential paracrine interactions with the cardiac environment that favor differentiation and maturation. Conversion of human fibroblasts represents a key step in the advancement of the field. However, as evidenced by the inability of GMT to reprogram human fibroblasts alone, and the immature phenotype of iCMs, human fibroblasts appear more resistant to

reprogramming than mouse fibroblasts. This difference is likely to pose a significant challenge to the translation of techniques found to be effective in mice towards their use in clinical trials.

Islas et al.⁴⁴ had previously reported that lentiviral induction of ETS2 and MESP1 transdifferentiates human dermal fibroblasts into cardiac progenitor cells in vitro.⁴⁴ Neither ETS2 or MESP2 is sufficient to reprogram human fibroblasts, but the combination activates a specific cardiac gene expression profile. They also reported that upregulation of ETS2 induces expression of the Gata4, Mef2c, Tbx20, Mesp1/2 and Nkx2.5 transcription factors. The iCMs produced by this combination display an immature phenotype that likely could be enhanced by culture in their native environment.⁴⁴ Additionally, Fu et al.⁴⁵ demonstrated that retroviral GMT in addition to estrogen-related receptor- γ (ESRRG), an essential transcription factor in cardiac metabolism and function,⁴⁶ and MESP1 (5F) reprogram human fibroblasts to iCMs. Addition of 2 other factors, Myocd and ZFPM2, (7F) significantly improves reprogramming. ZFPM2 is a transcription factor that interacts with Gata4 during cardiogenesis.⁴⁷ Compared to 5F, 7F enhances formation of sarcomeres, Ca²⁺ currents, and action potentials. However, reprogramming efficiency is still low, and most cells undergo only partial reprogramming.⁴⁵ While these findings expand our understanding of human fibroblast reprogramming, the low efficacy of 5F and 7F reinforce the challenges to achieving results sufficient for clinical translation.

Nam et al.⁴⁸ found that retroviral induced expression of miRNAs, miR-1 and miR-133, which will be discussed in greater detail below, along with GMT, improved human fibroblast to cardiomyocyte reprogramming. Additionally, miR-1 and miR-133, which are regulated by Mef2c, removed the requirement for Mef2c expression. However, reprogramming still took longer than in mouse cell lines, and spontaneous beating was rare.⁴⁸ Muraoka et al.⁴⁹ similarly found that miR-133 improved reprogramming efficiency of GMT, and GMTMM in adult mouse and human fibroblasts. miR-133 in addition to either GMT or GMTMM decreased expression of fibroblast transcription factors, specifically Snai1 (also referred to as Snail), a regulator of epithelial to mesenchymal transition (EMT). miR-133 mediated Snai1 inhibition decreased fibroblast gene expression and increased cardiac gene transcription. This effect was attenuated by overexpression of Snai1 in both mouse and human fibroblast cell lines.⁴⁹ Snai1 activation has also been shown to suppress iPSC formation, suggesting a conserved mechanism in iPSC and iCM formation.⁵⁰ Isolated Snai1 inhibition did not achieve as efficient of reprogramming as miR-133, suggesting other functions of miR-133 in reprogramming.⁴⁹ Utilization of miRNA to induce greater cardiac gene expression is another promising tool to overcome the significant barriers to human fibroblast reprogramming.

Christoforou et al.⁵¹ demonstrated the most efficient transcription factor mediated iCM generation from human fibroblasts yet reported. A combination of GMT, Myocd, and Nkx2.5 (GTMMN) transcription factors in addition to a single treatment with miR-1 and miR-133 promoted iCM formation. Supplemental inhibition of either JAK1 or Gsk3 significantly improved the reprogramming efficiency, as did addition of NRG1, a critical effector in cardiac development.^{51,52} Reprogramming of human fibroblasts remains a significant challenge. The optimal reprogramming technique will likely combine several

different modalities, including transcription factors, miRNA, small molecules and epigenetic regulation. Despite advances, further progress must be made to significantly improve reprogramming efficacy, and the maturation of iCMs in humans before clinical application.

Adenoviral Vector-Mediated Direct Reprogramming

A recent study by Mathison et al.⁵³ demonstrated the viability of using adenoviral vectors to reprogram rat fibroblasts into iCMs using a rat MI model in vivo. Though transient transcription factor expression has been shown to induce gene product synthesis, it had never been used for stable reprogramming into iCMs. Using an adenoviral, non-integrating vector (AdV), transient expression of GMT was sufficient to induce reprogramming in rats. Previously, reprogramming of fibroblasts into iCMs had been performed with lentiviral and retroviral vectors, as well as adeno-associated virus, all of which integrate into the host genome and induce chronic gene expression. Using these vectors in a clinical setting would risk mutagenesis and adverse events from random gene insertion.¹² Additionally, chronic gene expression following insertion may have unanticipated deleterious effects on generation of the desired phenotype as seen in iPSCs.⁵⁴ Direct reprogramming with only transient expression of transcription factors could avoid these complications improving safety and efficacy of treatment. Adenoviral and lentiviral vectors were used to deliver GMT (AdGMT, LentiGMT). MRI, echocardiography and histological analyses showed similar efficacy between the vectors. Both methods induced cTnT expression in 6% of fibroblasts, while AdGMT produced greater MHC expression. Echocardiography revealed an increase in EF of $21\% \pm 3\%$ in AdGMT treated rats, and $14\% \pm 5\%$ in the LentiGMT treatment group compared to controls 4 weeks following treatment.⁵³ AdV vectors have shown adequate long term safety,⁵⁵⁻⁵⁸ and promising ability to promote gene expression and product secretion with beneficial outcomes in clinical trials.⁵⁹ This study demonstrated that acute expression of the GMT transcription factors using an AdV vector is a viable alternative to chronic gene expression via an integrating viral vector. This advancement represents a decisive step in cardiac reprogramming toward clinical application.

In addition to the results of Mathison et al.,⁵³ Merentie and colleagues reported that AdV mediated gene transfer was more effective in inducing transdifferentiation than lentivirus or adeno-associated virus (AAV) gene therapy in a mouse MI model. Vectors were delivered using echocardiogram guided intramyocardial injection. AdV vectors had the greatest levels of transduction, with $19 \pm 7\%$ of the LV area transduced six days following injection, compared to $10 \pm 8\%$ with AAV and $7 \pm 3\%$ with Lentivirus. However, AdV was also associated with increased cardiac fibrosis and inflammation. These were accompanied by thinning of the myocardium with scar formation, as well as systolic and diastolic dysfunction and EKG changes proportional to the degree of transduction. Lentivirus demonstrated the least efficient transduction but was also associated with less fibrosis and no significant impact on LV function. The authors also compared systemic and local administration via intramuscular injection of AAV vectors. Local administration was associated with greater efficiency, as well as decreased fibrotic response.⁵⁷ These results support AdV as a possible therapeutic option for cardiac regeneration. However, further advances will be required to minimize inflammation and fibrosis following treatment, and to

optimize cardiac function. Further development of the third-generation AdV vectors may address these challenges in the coming years.^{60,61}

Hartikainen et al.⁶² recently reported the results of a phase I/IIa clinical trial assessing use of intramyocardial injection of AdV vector VEGF-D^{N C} to treat refractory angina. At a 1-year follow up time point, treatment was well tolerated and safe, with no significant increase in major complications. Treatment was also associated with improved perfusion and reduced angina symptoms. However, 54% of patients developed an increase in anti-AdV antibodies. While this did not cause any significant adverse clinical events to be reported, antibody formation could limit efficacy in subsequent treatments for refractory disease. Any conclusions from this trial are limited by small sample size (control n = 6, AdV VEGF-D^{N C} n = 24) and lack of long term data.⁶² These results support the potential safety and efficacy of intramyocardial AdV injection for cardiac reprogramming. Reprogramming of human fibroblasts remains inefficient, and despite marked improvement in cardiac function in rats, AdV reprogramming of in vivo mouse models has not improved cardiac function substantially.^{53,57} Further optimization of treatment protocols is required before AdV direct reprogramming will be a clinical treatment option. Despite these challenges, the development of AdV vectors safe for human therapy, and direct reprogramming via transient transcription factor expression using AdVs represent a breakthrough in regenerative cardiac medicine.

miRNA-Mediated Direct Reprogramming

Direct fibroblast to cardiomyocyte reprogramming can be achieved using miRNA delivery in place of transcription factor activation. Jayawardena et al.⁶³ used a lentivirus vector to deliver different miRNA combinations to reprogram mouse CFs in vitro and in vivo. The miR-1 was sufficient to cause reprogramming alone, however efficacy was greatest with a combination of miR-1, miR-133, miR-208, miR-499. Addition of JAK-1 inhibitor enhanced cardiomyocyte formation 8–10 fold.⁶³ The four miRNAs used are expressed in adult myocardium, mediate cardiac development and regulate cardiomyocyte function. Additionally, injury such as MI is known to disrupt their expression.⁶⁴ These experiments established direct reprogramming of murine fibroblasts to iCMs in vitro and in vivo by upregulation of miRNA.⁶³ Jayawardena et al.⁶⁵ subsequently used the same miRNA combination to assess the morphological and functional efficacy of reprogramming in vivo. miRNA combination treatment yielded iCMs resembling mature cardiac myocytes as measured by sarcomere creation, excitation to contraction coupling and action potential generation. The miRNA combination decreased fibrosis and steadily improved ventricular pump function as assessed by repeat echocardiography with effects peaking at about 3 months, suggesting sustained functional improvement. miRNAs produced iCMs within a time period similar to those reported with transcription factor mediated reprogramming.⁶⁵ These results established the viability of miRNA treatment to induce direct reprogramming in the absence of transcription factor delivery.

The development of miRNAs represents an intriguing alternative to the use of transcription factor expression for direct cardiac reprogramming. miRNAs can be delivered with a viral vector. Viral vectors are size limited, making it difficult to program large combinations of

transcription factors that may be needed to achieve sufficient efficacy into a single vector. The small size of miRNAs could allow multiple transcripts to be encoded in a single vector, delivering a large combination of miRNAs. Additionally, their ability to regulate several transcription factors gives miRNA's tremendous potential to enhance reprogramming efficiency.⁶⁵ Synthesis of chemical miRNAs would obviate the need for viral vectors and associated side effects. Synthetic miRNAs have been used to reprogram human fibroblasts into other cell types, and have demonstrated adequate safety in animal studies.^{66–68} Additionally, synthetic miRNAs have shown promise in treating human cancers based on in vitro studies.⁶⁹ In a phase 1 clinical trial a miRNA mimic, MRX34, was administered to patients with solid tumors. The trial was halted after five serious immune related adverse events, however, MRX34 administration with prior dexamethasone treatment demonstrated acceptable safety and efficacy.⁷⁰ Hurdles remain before miRNA represent a viable therapeutic option for cardiac regeneration, but strides have been made and clinical application in the future is an intriguing possibility.

Small Molecule-Mediated Direct Reprogramming

Small molecules are capable of augmenting or speeding up transcription factor-mediated iCM production. Wang et al.⁷¹ tested varying small molecule cocktails in the presence of selected transcription factors to induce iCMs. The group identified a combination of SB431542, an inhibitor of ALK4/5/7, of which ALK5 is a TGF- β receptor, CHIR99021, a GSK3 inhibitor which functions similar to Wnt3a, parnate, an LSD1/KDM1 inhibitor, and forskolin, an adenylyl cyclase inhibitor (SCPF). SCPF enabled mouse fibroblast to cardiomyocyte reprogramming, only requiring the presence of the transcription factor Oct4 which has also been used to form iPSCs.⁷¹ Oct4 regulates pluripotency and functions to promote lineage determination and cardiac formation.⁷² These results indicated that small molecules have potential to augment reprogramming, and can eliminate the requirement of certain transcription factors.

Hou et al.⁷³ demonstrated that chemically induced pluripotent stem cells (CiPSCs) could be generated from mouse fibroblasts using a cocktail of small molecules consisting of CHIR99021, RepSox, Forskolin, VPA, Parnate, TTNPB, and DZnep (CRFVPTZ) without the need for transcription factors.⁷³ Subsequently, Fu et al.⁷⁴ modified the CRFVPTZ cocktail to include just CHIR99021, RepSox, Forskolin, and VPA (CRFV) in mice. CRFV was sufficient to produce chemically induced cardiomyocytes (CiCMs) in vitro. CiCMs resembled mature cardiomyocytes morphologically and physiologically. Of the individual components of CRFV, RepSox was found to be most important.⁷⁴ RepSox is known to inhibit the TGF- β R1 and can replace the transcription factor Sox2 in murine iPSC production.⁷⁵ Additionally, Rolipram and Cilomilast, PDE4 inhibitors were found to enhance CiCM formation.⁷⁴ These results established CiCM production as an alternative means of cardiac regeneration.

Cao et al.⁷⁶ reported that small molecules can produce CiCMs from human foreskin fibroblasts (HFFs) and human fetal lung fibroblasts (HLFs). Starting with the SCPF cocktail developed previously, the group screened small molecules known to enhance reprogramming and discovered a new combination sufficient to generate human CiCMs consisting of seven

molecules (7C): CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632 and OAC2. Addition of two other molecules, SU16F and JNJ10198409, (9C) which are PDGF signaling inhibitors, dramatically improved reprogramming with $6.6 \pm 0.4\%$ of HHFs forming CiCMs as measured by cTnT expression. 9C treatment in an optimized reprogramming media induced expression of the mesoderm marker KDR in $27.9 \pm 4\%$ of cells. CiCMs showed homogenous expression profiles as opposed to the more heterogeneous ones seen following transcription factor mediated reprogramming. Impressively, 97% of CiCMs spontaneously contracted with action potentials resembling a ventricular phenotype. CiCM production from human fibroblasts was markedly more efficient than transcription factor promotion of iCM formation. Additionally, CiCMs grew when transplanted from culture into mouse hearts post-MI, suggesting the ability to develop in vivo. CiCMs still demonstrated a relatively immature phenotype, necessitating further refinement of CiCM techniques.⁷⁶ However, these results suggest that the protocols for reprogramming fibroblasts to CiCMs hold tremendous promise when compared with other methods.

Small molecule derived CiCMs have potential advantages over other methods of direct reprogramming. Chemical cocktail treatment obviates the need for viral vectors, avoiding possible side effects from viral mediated immunogenicity. Delivery of transcription factor or miRNA transcripts by inserting viral vectors could also result in random genomic insertion events and mutagenesis, in addition to possible deleterious effects of primary viral infection.¹² Chemical cocktails are also cheaper to produce on a large scale, and easier to control in terms of temporal delivery and optimization of component concentration ratios. Challenges facing clinical application of chemical-mediated direct reprogramming include spatial homing and delivery of small molecules to target tissues, and correspondingly limiting off target effects.^{71,74,76} Chemically- induced reprogramming must be performed in vivo to assess the efficacy of CiCM formation in vivo, safety, and benefits to cardiac output before the technique can be considered for clinical trials. Development of optimal delivery mechanisms, especially devices for local administration, will also be crucial. However, CiCMs represent an intriguing alternative in cardiac regeneration.

Tissue Culture Environment

Culture environment can dramatically influence direct reprogramming. Wang et al.⁷⁷ reported that culture media could be optimized to improve miRNA combination reprogramming of mouse CFs and TTFs. A reprogramming media (RM) was developed which improved reprogramming as measured by cardiac marker expression by 5 to 15-fold. Selenium was identified as the critical element in RM, and removal of selenium attenuated the effects of RM.⁷⁷ Li et al.⁷⁸ developed a 3D hydrogel tissue culture environment that significantly increased iCM production from mouse CFs and TTFs in vitro. 3D gel culture was associated with increased expression of MMPs, while inhibition of MMPs prevented the effects of 3D culture on iCM production.⁷⁸ Sia et al.⁷⁹ demonstrated that culturing GMT treated cells on a microgrooved substrate enhanced reprogramming of dermal fibroblasts and CFs in vitro. iCMs displayed greater structural maturity, and cardiac gene expression. Mechanical interaction with the environment activated Mkl1, which regulates cardiac hypertrophy and response to stress through GATA-4 modulation.⁸⁰ Similarly, Morez et al.⁸¹ reported that a microgrooved culture enhanced reprogramming with 3F-Myocd and

promoted cellular organization along parallel microgrooves reminiscent of ventricular myocardial tissue (Figure 1).⁸¹ These results demonstrate the importance of the culture environment in direct reprogramming and suggest that chemical and physical factors contribute to enhanced efficiency. This may further explain increased reprogramming efficiencies observed in vivo compared to in vitro. Further studies are warranted to identify environmental factors that are conducive or hinder reprogramming. Optimization of tissue environment may boost reprogramming and iCM maturation.

VEGF and FGF Signaling

Mathison et al.⁸² reported that pretreatment with AdV-VEGF following MI improved subsequent GMT mediated direct reprogramming and improved cardiac function in vivo using a rat model. AdV-VEGF was given after coronary ligation, with lentiviral GMT given 3 weeks later. EF increased by 12.3% overall following GMT treatment and decreased 7.3% in controls. Pretreatment with AdV-VEGF enhanced the effects of GMT 4-fold. VEGF-GMT increased EF 17.2% compared to 4.1% in the GMT only group. VEGF treatment also increased vascularization in the infarct area.⁸² VEGF has been shown to induce differentiation of CSCs and embryonic stem cells (ESCs) into cardiomyocytes through PI3K and ERK-Flk-1 activation respectively.^{83,84} Improvements in cardiac function with VEGF angiogenic prevascularization were larger than predicted based on the relatively modest increase in iCM production. VEGF pretreatment also markedly attenuated fibrosis. Possible explanations for this discrepancy include paracrine effects of iCMs and direct effects of VEGF on fibrotic pathways and resident cardiomyocytes.⁸² These findings demonstrate that VEGF enhances in vivo efficacy of reprogramming in a rat model, though the underlying mechanism was not established.

Yamakawa et al.⁸⁵ corroborated the results of Mathison et al.⁸² that VEGF improved GMT efficacy. They used a combination of FGF2, FGF10 and VEGF (FFV) to enhance reprogramming. FGF2 and FGF10 regulate embryonic cardiogenesis and cardiomyocyte proliferation but are absent from embryonic fibroblasts.⁸⁶ FFV increased production of spontaneously contracting cells in vitro by 100-fold. Mechanistically, FFV increased expression of p38MAPK and PI3K/AKT which activated several transcription factors associated with iCM formation including Gata4, Gata6, Hand2, Nkx2.5, Hey2, Ppargc1a, Smyd1, Sirt3, and Srf. Additionally, FFV enabled transcription with only Mef2c and Tbx5, eliminating the requirement of exogenous Gata4. FFV induced MAPK and PI3K signaling also converted partially reprogrammed cells similar to those seen in other studies into mature iCMs.⁸⁵ VEGF and FGF enhance iCM formation through MAPK and PI3K activation, and may play a useful role in optimizing reprogramming strategies (Figure 1).

Fibrosis and TGF- β Signaling

Fibrotic signaling pathways are upregulated in the heart following MI, and play a role in direct cellular reprogramming to iCMs (Figure 1).⁸⁷ Fu et al.⁴⁵ reported that addition of TGF- β to 5F increased reprogramming of human dermal as well as cardiac fibroblasts comparably to 7F. However, TGF- β did not improve 7F reprogramming. Additionally, SIS3, an inhibitor of SMAD3, a transcription factor downstream of TGF- β ,⁸⁸ significantly

attenuated 7F reprogramming, and the effects of adding TGF- β to 5F.⁴⁵ These results suggest a role for TGF- β in reprogramming mediated by SMAD3 signaling, potentially in place of the activity of the transcription factors, Myocd and ZFPM2, as discussed in the section of direct reprogramming in mice and human fibroblasts.

Ifkovits et al.⁸⁹ examined the effect of adding small molecules to HNGMT expression. Interestingly, adding the TGF- β inhibitor, SB431542, enhanced reprogramming in both embryonic and adult mouse fibroblasts by 5-fold. This would seem to contradict the findings of Fu et al.⁴⁵ that TGF- β increased 5F efficiency in human fibroblasts. Possible explanations include differences in TGF- β signaling between species, and differential TGF- β pathway effects in 5F and HNGMT treated cell lines. Mohamed et al.⁹⁰ screened thousands of signaling pathways to identify potential targets to improve reprogramming efficacy. Similar to the findings of Ifkovits et al.,⁸⁹ TGF- β inhibition dramatically improved mouse iCM production in vitro and in vivo. The optimal combination included GMT, SB431542, and the Wnt inhibitor XAV939. Wnt/ β -catenin signaling regulates fibrosis, ECM deposition by fibroblasts and hypertrophy following cardiac insult.⁹¹ In vitro, spontaneously beating cells developed by the end of 1 week as opposed to 6–8 weeks with GMT alone and In vivo cardiac function was markedly improved with TGF- β and Wnt inhibition following MI in an LAD ligation model. Additionally, TGF- β and Wnt inhibition enhanced reprogramming of human fibroblasts, and required only GMT and Myocd.⁹⁰ Despite previous findings of Fu et al.,⁴⁵ These results suggest the critical role of antifibrotic signaling in iCM formation in both mouse and humans. TGF- β may have different functions following cardiac insult in reprogramming based on environmental and temporal changes. Further characterization of the in vivo fibrotic response to cardiac insults, and how this may impact efforts at reprogramming are needed.

Additionally, Zhao et al.⁹² reported that fibrotic signaling impairs direct reprogramming. Viral encoded GHMT in vitro was used to develop iCMs. Addition of mir-1 and mir-133 (m2), known cardiogenic and antifibrotic mediators, to GHMT (GHMT2m) increased cardiac gene expression while decreasing fibroblast gene expression. GHMT2m dramatically increased iCM production, reprogramming 60% of MEFs. TGF- β and rho-associated kinase (ROCK) signaling interact and mediate pathological fibrosis in response to MI and responses to injury.⁹³ Upregulation of TGF- β and ROCK diminished reprogramming via GHMT, and attenuated GHMT2m iCM creation. Conversely, A83–01 and Y-632, inhibitors of TGF- β and ROCK signaling, markedly increased iCM formation and spontaneously contracting cells at 4 weeks, though no further benefit was achieved by combining the two. GHMT2m and A83–01 induced 7,000 beating cells per cm² compared to 1,000 per cm² following GHMT2m treatment alone in one trial.⁹² Fibrotic signaling appears to be a key obstacle in iCM formation.

Mathison et al.⁹⁴ recently reported that Gata4 has antifibrotic function through downregulation of the profibrotic transcription factor Snail. They demonstrated that Gata4 expression alone is sufficient to decrease post-infarct cardiac fibrosis in a rat ligation model. In vivo administration of Gata4 or the GMT combination improved post-infarct ventricular function equally. Given that Gata4 alone is insufficient to produce iCMs, the improved post-infarct cardiac function induced by GMT, GMTMM or other combinations including Gata4

may not entirely be due to iCM production, but rather to the antifibrotic effects of Gata4. They suggest incorporation of Gata4 into reprogramming cocktails in vivo.⁹⁴ Further characterization of the function of Gata4 is necessary to elucidate mechanisms of improved cardiac function separate from its reprogramming effects.

During the body's response to MI, inflammatory and fibrotic signaling is markedly upregulated in the heart.⁸⁷ These findings by Mathison et al.⁹⁴ suggest that targeting fibrotic response signaling pathways following MI and other cardiac insults with Gata4 may represent a promising therapeutic option. A strategy upregulating Gata4 expression in the absence of reprogramming may even yield efficacy approaching that of reprogramming, with fewer technical hurdles to overcome. More research is necessary to separate the primary effects of reprogramming and antifibrotic signaling on post-infarct cardiac function with Gata4 treatment. Undiscovered synergistic interactions between iCMs and antifibrotic signaling may play a role as well. Despite the promise of antifibrotic treatment to improve outcomes in acute MI, clinical trials have largely disappointed.⁹⁵ Additionally, the cardiac fibrotic response is evolutionarily conserved and not completely understood. Antifibrotic treatment may disrupt the normal healing process following MI, and thus worsen clinical outcomes.⁹⁶

Conclusion

Direct reprogramming of cardiac fibroblasts to iCMs is a new and promising strategy for cardiac regenerative therapy. Several protocols have been developed for reprogramming of fibroblasts in mouse and human cell lineages. Additionally, in vivo reprogramming has been achieved in mouse MI models with improvements in cardiac function. Several advancements in the field have been made in recent years. Ongoing discovery of more efficacious combinations of delivered transcription factors, the use of miRNA, shRNA, and small molecules has improved our understanding. Developments in viral vector technology may make clinical application more effective and safe. Reprogramming using miRNA and chemical cocktails open the door for reprogramming without the need for delivery of transcription factors. Further discoveries have been made about the impact of the extracellular environment and signaling pathways that promote growth or fibrotic processes. Direct reprogramming can be mediated by transcription factor activation, miRNA expression and small molecules. Combining these modalities improves efficacy, suggesting the presence of complementary effects. Combinations can also eliminate requirements for other factors indicating possible shared or redundant mechanisms.

Challenges remain before direct reprogramming will be ready for adoption as a viable treatment modality. Primarily, reprogramming efficiencies need improvement, which is especially true of human cell lines which are more resistant to reprogramming, and more importantly the development of optimal techniques for transdifferentiation. The basis for in vivo improvement of cardiac function should also be better characterized. Currently, the impact of different factors such as genuine iCM muscular function and reduction of fibrotic response are not well known. Safety concerns, especially regarding treatment vectors and delivery methods also need to be addressed. Finally, the successful generation of iCMs that

are phenotypically similar to endogenous cardiomyocytes must be parlayed into improved outcomes, and results must be translated from animal models to human clinical trials.

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Figure 1: The schematic diagram

depicts factors and pathways that contribute to direct reprogramming of fibroblasts to iCMs. Pretreatment vascularization enhances reprogramming of fibroblasts in vivo. The tissue environment also plays a significant role in reprogramming. Selenium has been identified as a key component of in vitro reprogramming media. The presence of a microgrooved 3D substrate can enhance reprogramming and organization. Activation of transcription factor signaling, including combinations of Gata4, Mef2c, Tbx5, MESP1, Myocd, Nkx2.5, ETS2, ESRRG, and ZFPM2 mediate direct reprogramming. Treatment with miRNA, including miR-1, miR-133, miR-208, and miR-499 can enhance or drive reprogramming alone. VEGF, FGF2, and FGF10 promote reprogramming through MAPK- and PI3K-mediated pathways separately from angiogenesis-promoting effects. Abrogation of fibrotic signaling through several pathways has been demonstrated to improve reprogramming. These include SB431542 and A83-01 to inhibit TGF- β , Y-632-induced inhibition of ROCK, XAV939-induced inhibition of Wnt, and Gata4-induced inhibition of Snail.

Table 1.

Protocols for direct reprogramming of fibroblasts to iCMs

Mouse in vitro direct fibroblast reprogramming				
Cell Origin	Culture Environment	Treatment Components	Name	Reference
Mouse	in vitro	Gata4, Mef2c, Tbx5	GMT	7
		Gata4, Hand2 Mef2c, Tbx5	GHMT	9
		Tbx5, Mef2c, Myocd	3F-Myocd	16
		Hand2, Nkx2.5, Gata4, Mef2c, Tbx5	HNGMT	17
		MyoD-Mef2c, Gata4, Hand2, Tbx5		18
		Mef2c, Gata4, Tbx5	MGT	20
		Akt, Gata4, Hand2 Mef2c, Tbx5	AGHMT	21
		DAPT, Akt, Gata4, Hand2 Mef2c, Tbx5		26
		Bmi1 shRNA, Gata4, Mef2c, Tbx5		33
Mouse and rat in vivo direct fibroblast reprogramming				
Mouse	in vivo	Gata4, Mef2c, Tbx5, Thymosin β 4	GMT	8
		Gata4, Hand2 Mef2c, Tbx5	GHMT	9
		Gata4, Mef2c, Tbx5	GMT Polycistron	39
Rat	in vivo	Gata4, Mef2c, Tbx5	GMT Polycistron	40
Human in vitro direct fibroblast reprogramming				
Human	in vitro	Gata4, Mef2c, Tbx5, MESP1, Myocd	GMTMM	10
		ETS2, MESP1		44
		ESRRG, MESP1, Gata4, Mef2c, Tbx5	5F	45
		Myocd, ZFPM2, ESRRG, MESP1, Gata4, Mef2c, Tbx5	7F	45
		miR-1, miR-133, Gata4, Mef2c, Tbx5		48
		miR-133, Gata4, Mef2c, Tbx5		49
		miR-133, Gata4, Mef2c, Tbx5, MESP1, Myocd		49
		Gata4, Mef2c, Tbx5, Myocd, Nkx2.5, miR-1, miR-133	GTMMN	51
Rat adenoviral vector reprogramming in vivo				
Rat	in vivo	Gata4, Mef2c, Tbx5	AdGMT	53, 57
Mouse miRNA reprogramming in vitro and in vivo				
Mouse	in vitro / in vivo	miR-1, miR-133, miR-208, miR-499		63
Mouse and human small molecule reprogramming in vitro				
Mouse	in vitro	SB431542, CHIR99021, Parnate, Forskolin, Oct4	SCPF	71
Mouse	in vitro	CHIR99021, RepSox, Forskolin, VPA, Parnate, TTNPB, Dznep	CRFV	74
Human	in vitro	CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632, OAC2	7C	76
Human	in vitro	CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632, OAC2, SU16F, JNJ10198409	9C	76

Mouse in vitro direct fibroblast reprogramming				
Cell Origin	Culture Environment	Treatment Components	Name	Reference
Reprogramming utilizing inhibition of fibrotic signaling				
Mouse	in vitro	FGF2, FGF10, VEGF, Mef2c, Tbx2		85
Human	in vitro / in vivo	Gata4, Mef2c, Tbx5, Myocd, SB431542, XAV939		90
Mouse	in vitro	Gata4, Hand2 Mef2c, Tbx5, miR-1, miR-133	GHMT2m	92