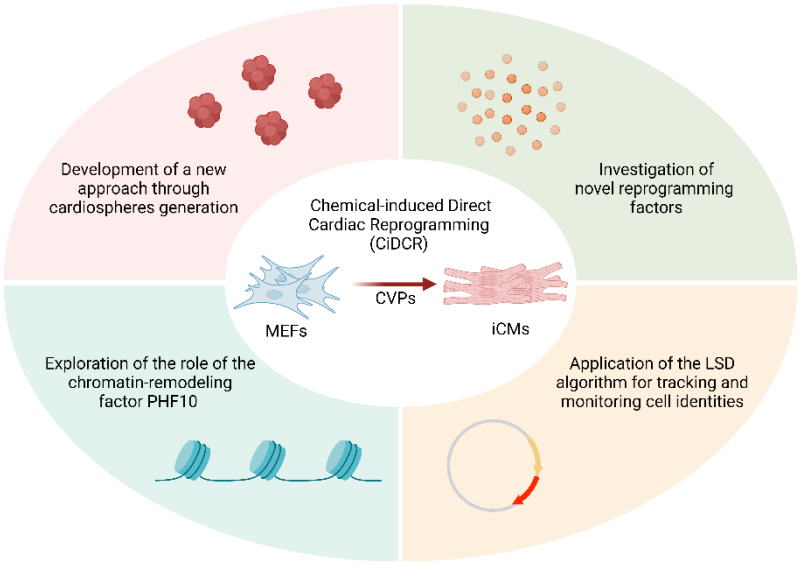
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXVI CYCLE

Giorgia Di Benedetto

Identification and characterization of new molecules that act as boosters for direct cardiac reprogramming, via the induction of cardiovascular precursors



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Identification and characterization of new molecules that act as boosters for direct cardiac reprogramming, via the induction of cardiovascular precursors

Tutor
Chiar.ma Prof. Fabiana Passaro

Candidate
Dr.ssa Giorgia Di Benedetto

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LIST OF USED ABBREVIATIONS

AMI Acute Myocardial Infarction
bHLH basic Helix–Loop–Helix
CFs Cardiac Fibroblasts
CiCMs Chemically-induced Cardiomyocytes
CMs Cardiomyocytes
CPCs Cardiac Precursor Cells
CRFVPT CHIR99021, Repsox, Forskolin, Valproic Acid, Parnate, TTNPB
cTnI Cardiac Troponin I
cTnT Cardiac Troponin T
CVPs Cardiovascular precursors
EGFP Enhanced Green Fluorescent Protein
ESCs Embryonic Stem Cells
GHMT Gata4, Hand2, Mef2c and Tbx5
GMT Gata4, Mef2c and Tbx5
HNGMT Hand2, Nkx2.5, Gata4, Mef2c and Tbx5
iCMs induced Cardiomyocytes
iCPCs induced Cardiac Progenitor Cells
iPSCs Induced Pluripotent Stem Cells
MEFs Mouse Embryonic Fibroblasts
MHC Myosin Heavy Chain
miRNAs microRNAs
MLC-2v ventricular Myosin Light Chain-2
OSKM Oct4, Sox2, Klf4 and c-Myc
PRC1 Polycomb Repressive Complex 1
PSCs Pluripotent Stem Cells
shRNA Short hairpin RNA
TFs Transcription Factors
TGFβ Transforming Growth Factor B
TTFs Tail-Tip Fibroblasts

1. BACKGROUND

1.1 Regenerative Medicine to Treat Cardiac Diseases

In 2016, approximately 17.9 million people died due to cardiovascular diseases, accounting for 31% of all global deaths. According to projections, 23.6 million people will die annually from cardiovascular disorders by 2030. 85% of these deaths will be related to heart attacks and strokes, which are mostly caused by a blockage of blood flow to the heart or brain (source: World Health Organization https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)). Acute myocardial infarction (AMI) occurs when there is a reduction in coronary blood flow, leading to a sudden decrease in the blood supply (ischemia) to a portion of the myocardium. After an AMI, the damage becomes irreversible, triggering tissue necrosis and resulting in the loss of up to 1 billion cardiac cells. Subsequently, AMI induces a remodeling process wherein the damaged tissue is progressively replaced by a fibrotic scar and the compensation occurs through the hypertrophy of the remaining and surrounding cardiomyocytes (Steinhauser et al. 2011). Ventricular remodeling involves the thinning of the heart wall and dilation of the ventricular cavity, which can lead to impaired cardiac function and eventually heart failure (Leancă et al. 2022). During mammalian embryogenesis, cardiomyocytes (CMs) exhibit the capacity for proliferation. However, shortly after birth, they largely lose their proliferative ability, undergoing terminal differentiation. Although there is conflicting evidence indicating slow self-renewal capabilities (Bergmann et al. 2009), strong evidence suggests that, in contrast to the regeneration capabilities shown in organisms like zebrafish and newts, the mammalian heart is incapable of efficiently and properly replacing a significant loss of cardiomyocytes following cardiac damage (Jaźwińska et. al 2016 , Laflamme et al. 2011). The treatment of AMI primarily focuses on preventing the progression of ischemic heart disease toward heart failure. Cardioprotective therapies, such as revascularization through thrombolysis, cardiac interventions and bypass surgery, have proven their efficacy in enhancing blood supply and reversing adverse remodeling processes. Pharmacological treatments have also shown promise in reducing mortality associated with heart failure. When the remodeling process has advanced and heart failure becomes chronic, mechanical support therapies like left ventricular assist devices and cardiac resynchronization therapy yield positive outcomes. However, it's important to note that current therapeutic approaches for heart failure are primarily palliative rather than curative. Heart transplantation is the only definitive cure available. Nevertheless, this option is limited to severe cases due to donor scarcity, surgical complexity and immunocompatibility challenges.

As cardiovascular diseases continue to claim millions of lives each year and the limitations of current treatments for acute myocardial infarction become increasingly evident, the pursuit of innovative therapies becomes all the more

urgent. While existing interventions offer valuable support, the inability of the mammalian heart to fully regenerate underscores the critical need for revolutionary approaches in regenerative medicine. While heart transplantation remains a definitive solution, its constraints emphasize the imperative for ongoing research into regenerative strategies to handle the escalating global burden of cardiovascular diseases.

1.2 Cell reprogramming for heart regeneration

Cell reprogramming has emerged as an attractive strategy for generating new cardiac lineages, including cardiomyocytes (CMs) and cardiac progenitor cells (CPCs), with broad applications in stem cell therapy, disease modeling, drug screening and developmental biology (Robinton et al. 2012, Carvajal-Vergara et al. 2010, Parrotta et al. 2020). CMs and CPCs produced through cell reprogramming can be expanded or scaled up, exhibit immunocompatibility, effortlessly integrate and synchronize with the host myocardium. The generation of these cardiac lineages from adult somatic cells can be achieved through various cell reprogramming methods:

- Induced Pluripotent Stem Cells (iPSCs): iPSCs are established by overexpressing Oct4, Sox2, Klf4 and cMyc (OSKM), followed by subsequent differentiation into cardiac lineages (Takahashi and Yamanaka 2006).
- Direct Cardiac Reprogramming: This approach entails the direct conversion of cells into specific cardiac lineages, such as induced cardiomyocytes (iCMs) or induced CPCs (iCPCs) (Figure 1). Bypassing the pluripotent stage, this method avoids the tumorigenic risks associated with pluripotent stem cells (PSCs).

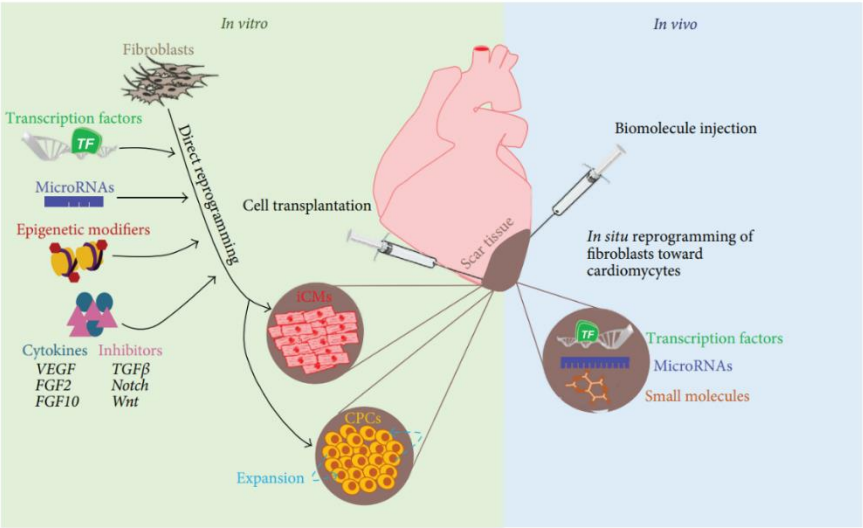

Figure 1. Direct Cardiac Reprogramming strategies.

Schematic representation showing the current and future strategies of direct cardiac reprogramming. [Image extracted from Engel, J. L., and Ardehali, R. (2018). Direct Cardiac Reprogramming: Progress and Promise. Stem cells international, 2018, 1435746.]

1.3 Direct cardiac reprogramming of murine fibroblasts

In 2006, Takahashi and Yamanaka discovered that conversion of somatic cells into pluripotent cells could be accomplished by simultaneously expressing four transcription factors (TFs). They effectively converted mouse fibroblasts into iPSCs by utilizing the transcription factors Oct4, Sox2, Klf4 and c-Myc (OSKM). As reprogramming efforts evolved, the focus shifted towards identifying multiple factors that, when combined, could activate the cardiac program for cardiomyocyte transdifferentiation.

In 2010, Ieda et al. identified a trio of transcription factors, Gata4, Mef2c and Tbx5 (GMT), which were sufficient to convert mouse cardiac and dermal fibroblasts into functional cardiomyocyte-like cells in vitro. To identify these GMT reprogramming factors, the authors developed an in vitro assay to determine the minimum number of factors required to induce a nonmyocyte cell to adopt a cardiomyocyte fate. Neonatal transgenic mice expressing enhanced green fluorescent protein (EGFP) under the α-myosin heavy chain (αMHC) promoter, active in mature cardiomyocytes, were used to isolate cardiac fibroblasts. The EGFP-negative/Thy1+ fibroblasts were selected using fluorescence-activated cell sorting (FACS). Fourteen transcription factors, known for their significance in cardiac development, were then retrovirally overexpressed in the isolated cardiac fibroblasts. A small population of EGFP-positive cells indicated successful cell fate conversion. By progressively eliminating individual transcription factors from the initial set of 14, the authors identified the crucial role of the three transcription factors, GMT. Together, GMT was capable of inducing EGFP expression in 15-20% of cells, resulting in induced cardiomyocytes (iCMs). Although some iCMs expressed cardiac markers such as cardiac Troponin T (cTnT), sarcomeric α-actinin, assembled sarcomere structures and exhibited a global transcriptome profile resembling that of cardiomyocytes, the majority of iCMs were likely only partially reprogrammed. Nevertheless, many iCMs displayed Ca^{2+} transients, and 4 weeks post-reprogramming, approximately 0.5% of αMHC-EGFP+/cTnT+ iCMs exhibited spontaneous beating. These beating iCMs also exhibited action potentials similar to those of adult ventricular cardiomyocytes (Ieda et al. 2010). Furthermore, iCMs displayed epigenetic changes toward a neonatal cardiomyocyte-like state at cardiac-specific genes Actn2, Ryr2 and TnnT2, indicating that reprogramming with the GMT factors induced alterations in the epigenetic status of the reprogramming cells. Additionally, the GMT factors effectively reprogrammed 15% of neonatal tail-tip fibroblasts (TTF) into αMHC-EGFP+ iCMs, which expressed cTnT and generated Ca^{2+} transients. However, these TTF-derived iCMs did not exhibit spontaneous beating and their generation efficiency was reduced. Despite these challenges, these findings

represented significant progress in direct cardiac reprogramming, albeit with a low in vitro efficiency (<1%), which was comparable to the efficiency of iPSC generation (Ieda et al. 2010 , Takahashi and Yamanaka 2006).

Shortly thereafter, in 2012 Song et al. discovered that the addition of Hand2, a bHLH transcription factor, to the GMT combination (collectively termed GHMT) could induce adult TTFs into beating iCMs. In their study, cardiac fibroblasts were isolated from αMHC-GFP transgenic mice and retrovirally expressed six core transcription factors involved in cardiac development: Hand2, Mesp1, Nkx2.5 and the GMT factors. Serial elimination of transcription factors revealed that the GHMT cocktail was identified as producing a 4-fold increased conversion of adult TTFs to αMHC-EGFP+/cTnT+ iCMs compared to GMT reprogramming of neonatal TTFs. These TTF-derived iCMs formed sarcomeres, generated Ca^{2+} transients and exhibited a transcriptome profile reminiscent of cardiomyocytes. Furthermore, 5 weeks after GHMT reprogramming of TTFs, a small population of these iCMs exhibited spontaneous beating, demonstrating that GHMT could directly reprogram TTFs into functional iCMs.

In a different study based on GHMT factors, Hirai et al. (2013) fused the GHMT factors to the MyoD activation domain, resulting in an accelerated reprogramming and an increased number of beating iCMs. Nam et al. (2014) demonstrated that GHMT reprogramming of fibroblasts into cardiomyocytes yielded a range of phenotypes, including immature forms of atrial, ventricular and pacemaker cells. Using multiplex immunostaining and patch clamping, the authors identified distinct constellations of cardiac markers and electrical activity that reliably defined atrial, ventricular and pacemaker cardiomyocyte subtypes in Hcn4-GFP reporter mice, where GFP expression is driven by the promoter of Hcn4, a pacemaker-specific potassium channel gene. At the single-cell level, GHMT reprogramming of MEFs derived from Hcn4-GFP reporter mice resulted in iCMs exhibiting immature phenotypes of all three major cardiac cell types, demonstrating a remarkable degree of plasticity inherent to GHMT reprogramming and offering a starting point for investigating cardiac cell subtype-specific reprogramming.

The methods described by Ieda et al. (2010) and Song et al. (2012), for identifying reprogramming factors, relied on the activation of the αMHC-EGFP reporter. This approach could potentially introduce bias by favoring factors that efficiently activated the αMHC promoter while excluding those promoting full reprogramming. To address this concern, Protze et al. in 2012 screened 120 different triplet combinations of 10 candidate reprogramming factors to assess their ability to activate the expression of five cardiac genes (Myh5, Myl2, Actc1, Nkx2.5 and Scn5a) in mouse fibroblasts. Among these, the expression of Tbx5, Mef2c and Myocd collectively upregulated the majority of cardiac genes examined. Although fibroblasts transduced with these three factors expressed cardiac cell fate indicators, such as contractile proteins, cardiac-like sodium and potassium currents and inducible action potentials, these iCMs did not exhibit spontaneous beating.

Other research groups adopted different criteria for screening reprogramming factor combinations. In 2013, Christoforou et al. evaluated different combinations of 10 transcription factors for their ability to induce cardiomyocyte conversion in MEFs. Their criteria for identifying factor combinations included the activation of cardiac-specific promoters and specific cardiac genes, morphological cardiac features and functional characteristics, including resting membrane potential and spontaneous beating. Through this approach, they determined that Myocd and SRF could enhance GMT reprogramming, although no spontaneous contractile activity was observed. In another study, Addis et al. (2013) aimed to optimize direct cardiac conversion by employing a quantifiable calcium reporter, GCaMP driven by the cTnT promoter, to screen candidate factor combinations for their ability to induce functional transdifferentiation in MEFs. Using this method, Hand2, Nkx2.5, Gata4, Mef2c and Tbx5 (HNGMT) were identified as the optimal combination for inducing robust calcium oscillation and increasing the number of spontaneously beating iCMs compared to GMT reprogramming alone. In a subsequent study using the same calcium reporter, Ifkovits et al. (2014) demonstrated that the small molecule TGF- β Inhibitor SB432542 enhanced the efficiency of HNGMT reprogramming by 5-fold.

While most of these studies primarily focused on transcription factor-mediated reprogramming, Jayawardena et al. (2012) reported the successful conversion of neonatal mouse fibroblasts into cardiomyocyte-like cells in vitro through the use of four miRNAs: miR-1, -133, -208 and -499. The addition of a JAK inhibitor (JI1) led to increased reprogramming efficiency and the generation of spontaneously beating iCMs. Extending the capabilities of miRNA-mediated reprogramming, Muraoka et al. (2014) demonstrated that adding miR-133a to the combination of GMT in MEFs resulted in a 7-fold increase in beating iCMs compared to GMT reprogramming alone. MiR-133a was found to repress Snai1, a master regulator of epithelial-to-mesenchymal transitions, suggesting that suppressing fibroblast signatures may play a crucial role in reprogramming fibroblasts into iCMs.

In 2006, Takahashi and Yamanaka revolutionized cell biology by discovering that somatic cells could be reprogrammed into pluripotent cells by expressing four transcription factors simultaneously. This groundbreaking work paved the way for direct cardiac reprogramming efforts, focusing on identifying factors capable of activating the cardiac program. In 2010, Ieda et al. identified Gata4, Mef2c, and Tbx5 (GMT) as sufficient to convert fibroblasts into cardiomyocyte-like cells. Shortly after, Song et al. (2012) enhanced reprogramming efficiency by adding Hand2 to the GMT combination (GHMT). Other studies explored refinements, such as using small molecules like TGF-β inhibitor SB432542, and miRNA-mediated reprogramming. These collective efforts have advanced our understanding of direct cardiac reprogramming, offering valuable insights into the intricate mechanisms underlying cell fate conversion.

1.4 Chemically induced Direct Cardiac Reprogramming

Despite the relatively high transfection efficiency associated with viral protocols, persistent concerns regarding insertional mutagenesis, residual expression and genetic/epigenetic abnormalities limit their clinical applicability (Thomas et al. 2003, Wang et al. 2014, Efe et al 2011). Consequently, there has been a growing interest in small molecule–driven, non-viral and transgene-free strategies to enhance the safety profiles of reprogramming protocols (Wang et al. 2021, Zhou et al. 2022).

A purely chemical cocktail, named CRFVPT, has been demonstrated to functionally substitute the ectopic expression of transcription factors (TFs) in inducing beating clusters of cardiomyocytes CMs. The generation of chemically-induced cardiomyocytes (CiCMs) was pioneered by Fu et al. in 2015. CRFVPT consists of CHIR99021 (C), Repsox (R), Forskolin (F), Valproic Acid (V), Parnate (P) and TTNPB (T) (Figure 2). During small molecule iPSCs reprogramming, cell clusters resembling cardiomyocytes emerged, with beating cells unintentionally observed between 6 and 8 days post-treatment with CRFVPT. To enhance stable and effective induction of CiCMs, Fu et al. devised a two-step strategy, formulating a cardiac-reprogramming medium primarily based on CRFVPT (Figure 3). They discovered that bFGF was not necessary for CiCMs generation and optimizing the combination of fetal bovine serum (FBS) and knockout serum replacement (KSR) led to more efficient beating cell generation. Additional improvements, including the addition of N2 and B27, were made to enhance induction efficiency. Reprogramming on matrigel-coated dishes and the subsequent transition to a maintenance medium resulted in a significant increase in the number of beating cells. As maintaining the cardiac-reprogramming medium for more than 16 days did not enhance efficiency, CRFVPT was removed and known maintenance factors for cardiomyocytes were added (PD0325901, a MEK1/2 inhibitor and insulin). Consequently, a significant increase in the number of beating cells was observed. Fu et. al attempted CiCMs reprogramming of neonatal mouse tail-tip fibroblasts but found that the reprogramming efficiency was lower than it had been for the MEFs. These CiCMs expressed cardiomyocyte markers such as αactinin, cardiac troponin-T (cTnT), cardiac troponin-I (cTnI), α-Major Histocompatibility Complex (α-MHC) and accurately exhibited cardiac electrophysiological characteristics.

In the pursuit of safer and more efficient reprogramming strategies, researchers have turned their attention to small molecule-driven approaches, aiming to overcome the limitations associated with viral protocols. Among these innovative methods, the CRFVPT cocktail has emerged as a promising contender, offering a transgene-free solution for generating chemically-induced cardiomyocytes (CiCMs).

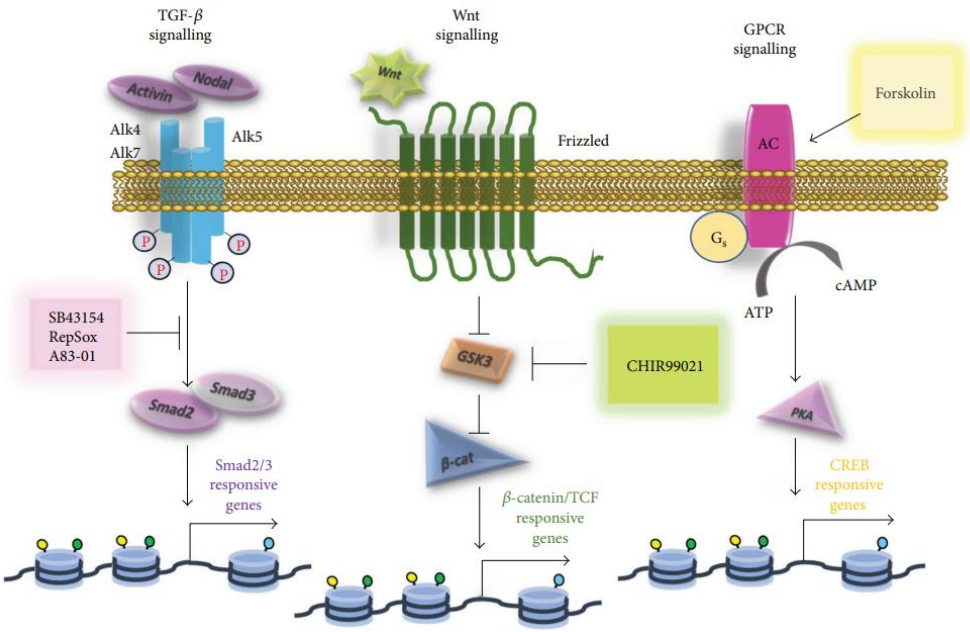

Figure 2. Key pathways modulated by CRFVPT.
Comprising six chemicals (C–CHIR99021, R–RepSox, F–Forskolin, V–Valproic Acid, P–Parnate, T–TTNPB), CRFVPT includes a GSK3 inhibitor, a TGFβR1 inhibitor, a cAMP synthesis sustainer, an histone deacetylases inhibitor, an inhibitor of lysine-specific demethylase 1 and a selective analogue of retinoic acid. CHIR99021 and RepSox, as two 'mesenchymal to epithelial transition' modulators, are thought to suppress the fibroblast phenotype. Conversely, Parnate and VPA, as two epigenetic modulators, are believed to assist in overcoming epigenetic barriers in various cell types. The final two factors seem capable of inducing features characteristic of CM-like cells. [Image extracted from Passaro, F., Testa, G., Ambrosone, L., Costagliola, C., Tocchetti, C. G., di Nezza, F., Russo, M., Pirozzi, F., Abete, P., Russo, T., & Bonaduce, D. (2017). Nanotechnology-Based Cardiac Targeting and Direct Cardiac Reprogramming: The Betrothed. Stem cells international, 2017, 4940397].

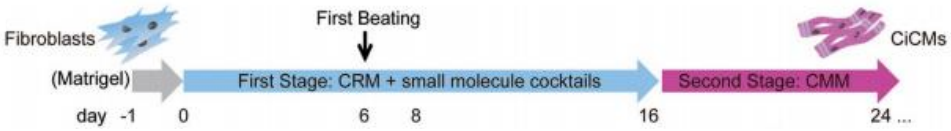

Figure 3. Schematic representation of Chemically-induced Direct Cardiac Reprogramming protocol, divided into stages.
Fibroblasts were plated on Matrigel. The next day, the medium was replaced with cardiac reprogramming medium (CRM), containing the chemical cocktail CRFVPT. The medium was changed every four days with fresh CRM. Clusters of beating cells with the characteristic morphology of cardiomyocytes can be observed on days 6-8. On the sixteenth day, CRM is

replaced with maintenance medium for cardiomyocytes (CMM). [Image extracted from Fu, Y. et al. Direct reprogramming of mouse fibroblasts into cardiomyocytes with chemical cocktails (2015)].

An enriched chemical cocktail consisting of nine molecules (CHIR99021, A83-01, SC1, OAC2, Y27632, BIX01294, AS8351, SU16F, JNJ10198409), partially overlapping with the one used for mouse fibroblast reprogramming, has also been reported to reprogram human fibroblasts into beating cardiac cell clusters in vitro (Cao et al. 2016).

More recently, our research group demonstrated that the efficacy of the CRFVPT cocktail could be enhanced by pharmacologically inhibiting the epigenetic modulator Bmi1. This resulted in the repression of the JAK/STAT3 and MAPK/ERK1/2 pathways in induced cardiomyocytes (iCMs) generated from mouse CFs, leading to increased spontaneous beating and elevated levels of mature cardiac phenotype markers, MLC-2v and cTnT (Testa et al. 2020).

1.5 Barriers to Cardiac Reprogramming

Better understanding of the molecular mechanisms behind direct cardiac reprogramming paves the way for developing novel approaches to enhance the effectiveness of reprogramming. To overcome the challenges associated with direct reprogramming in cardiac cells, potential strategies can include optimizing reprogramming cocktails, modifying signaling pathways and inducing epigenetic alterations.

A significant barrier lies in the fibroblast signatures, which must be suppressed to ensure an efficient and successful reprogramming. Notably, the TGF-β and Wnt pathways play pivotal roles in fibroblast activation. Studies have demonstrated their signaling inhibition increases cardiac reprogramming by silencing fibroblast signatures (Figure 4).

In the process of direct reprogramming involving GHMT, miR-1 and miR-133, inhibition of TGF-β promotes the interaction of Gata4 with the H3K27me3 demethylase Kdm6b/JMJD3 and the SWI/SNF subunit Brg1. Consequently, it enhances Gata4 access to its targets. Conversely, TGF-β activation has been observed to impede H3K27me3 demethylation and hinder Gata4 binding to its targets, establishing a connection between the repressive modification and resistance to reprogramming (Riching et al. 2021).

Subsequently, Mohamed et al. (2017) confirmed that inhibition of TGF-β and Wnt signaling enhanced cardiac-reprogramming efficiency and maturation. Therefore, overcoming these mechanistic hurdles allowed successful reprogramming of efficient and functionally mature iCMs.

Understanding the molecular intricacies of direct cardiac reprogramming offers insights into improving its efficiency. Suppressing fibroblast signatures, notably through inhibiting TGF-β and Wnt pathways, enhances reprogramming success. Studies show inhibiting TGF-β boosts Gata4 accessibility to its targets,

crucial for effective reprogramming, while its activation hinders this process. Overcoming these hurdles leads to the generation of mature induced cardiomyocytes, advancing regenerative medicine prospects.

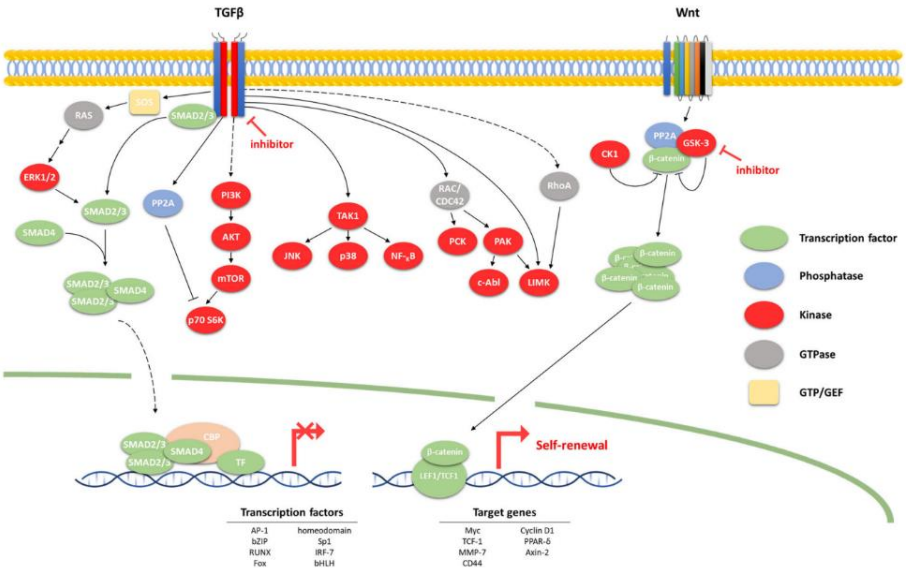

Figure 4. Signaling pathways for Wnt and TGF-β.
The TGF-β and Wnt signaling pathways are summarized in this image. All TGF-β-related signaling is regulated by tiny compounds preventing the TGF-β receptor from functioning. On the other hand, β-catenin phosphorylation by GSK-3 inhibits Wnt signaling. However, GSK-3 can be blocked by small molecules, which will allow β-catenin to enter the nucleus and start transcription. Hence, during reprogramming, the target genes of Wnt signaling are transcribed while the expression of TGF-β target genes is inhibited in all cell types. [Image extracted from Kim, Y., Jeong, J., & Choi, D. (2020). Small-molecule-mediated reprogramming: a silver lining for regenerative medicine. Experimental & molecular medicine, 52(2), 213–226.]

1.6 Epigenetic Regulation in Direct Cardiac Reprogramming

Epigenetic regulation plays a pivotal role in coordinating cellular plasticity and the conversion of cell fate (Wamstad et al. 2012). The epigenetic landscape assumes a crucial role in determining the efficiency of reprogramming, as the accessibility of transcription factors (TFs) to their DNA targets is paramount (Figure 5). Throughout the reprogramming process, epigenetic marks such as histone methylation, acetylation and ubiquitination must be added and removed from fibroblast- and cardiac-specific genes. These modifications serve to suppress the expression of fibroblast genes and activate cardiac genes by reshaping chromatin structure, thereby allowing or restricting TFs' access to their target genes.

Almost every cocktail known to enhance direct cardiac reprogramming has demonstrated mechanistic changes in the chromatin accessibility of cardiac enhancers. These modifications aid in loading or reinforcing the binding of transcription factors to cardiac genes and regulatory elements crucial for lineage definition. Many of these mechanistic studies have observed demethylation of the repressive H3K27me3 and the induction of H3K4 methylation to initiate reprogramming with cardiac transcription factors (TFs), miRNAs or chemicals. However, only recently genome-wide studies have provided insights into the enhancer landscapes of reprogrammed induced cardiomyocytes (iCMs) and the global binding sites of cardiac TFs during reprogramming (Stone et al. 2019), (Hashimoto et al. 2019).

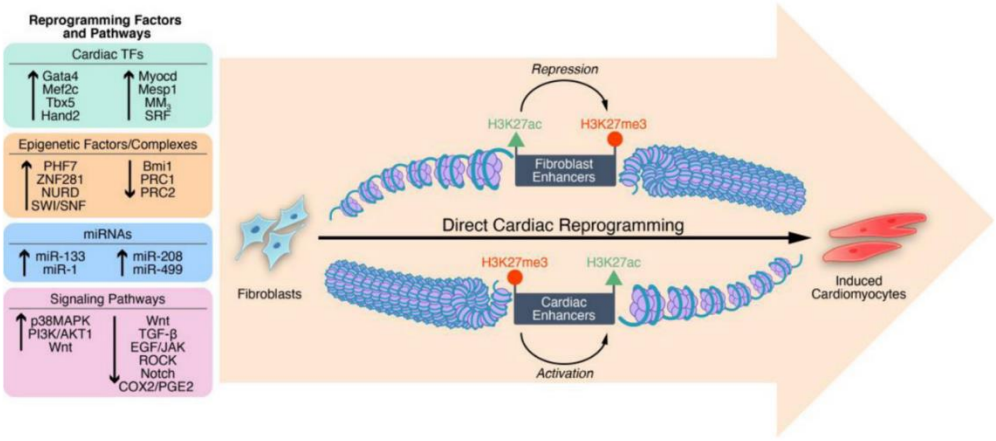

Figure 5. Chromatin accessibility as the crucial regulator for effective cellular reprogramming.
Reprogramming factors and signaling pathways involved in cardiac reprogramming (depicted on the left) have been demonstrated to intricately alter chromatin accessibility at specific enhancers. This involves the elimination of inhibitory epigenetic modifications, such as H3K27me3 and the addition of activating modifications, such as H3K27ac, at regulatory regions associated with cardiac functions. [image extracted from Garry, G. A., Bassel-Duby, R., & Olson, E. N. (2022). Direct reprogramming as a route to cardiac repair. Seminars in cell & developmental biology, 122, 3–13.]

Zhou and colleagues conducted an shRNA-based loss-of-function screen to identify chromatin remodeling factors influencing reprogramming barriers. Knockdown of 11 out of 35 tested factors significantly reduced reprogramming efficiency, underscoring their essential role in cardiac TF action at cardiac enhancers (Figure 6). Notably, knockdown of the PRC1 subunit Bmi1 resulted in the most significant increase in reprogramming efficiency. Mechanistically, Bmi1 binds to cardiac loci marked by monoubiquitination of histone H2A at lysine 119 (H2AK119ub), thereby suppressing the expression of target genes, including Gata4 and Tbx20. Knocking down Bmi1 activity increases the active histone mark H3K4me3 and reduces the repressive H2AK119ub mark, resulting in enhanced cardiac gene expression at critical loci (Zhou et al. 2016).

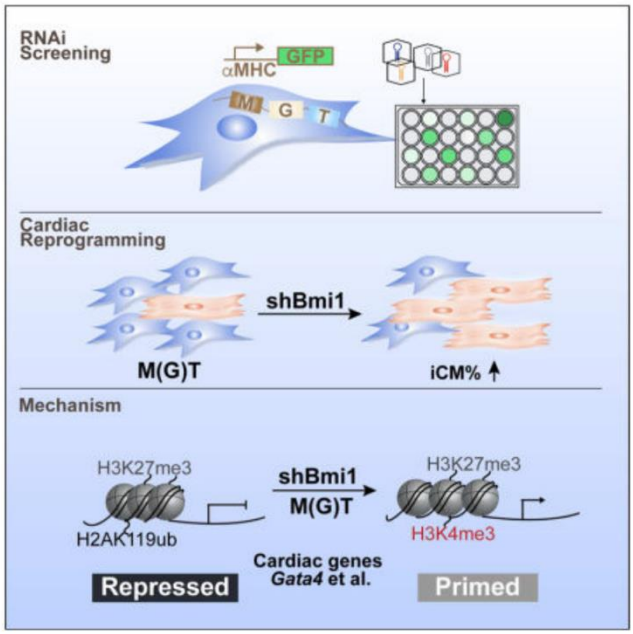

Figure 6. Epigenetic regulators loss-of-function screening.
Zhou et al. conducted a loss-of-function screening through shRNA targeting various epigenetic regulators to identify those capable of influencing reprogramming using the GMT protocol, converting fibroblasts into cardiomyocytes with spontaneous contractile ability. Experimental evidence suggests that the inhibition of Bmi-1 leads to an improvement in reprogramming efficiency, associated with the derepression of key cardiogenic loci. [Image extracted from: Zhou, Y., Wang, L., Vaseghi, H. R., Liu, Z., Lu, R., Alimohamadi, S., Yin, C., Fu, J. D., Wang, G. G., Liu, J., & Qian, L. (2016). Bmi1 Is a Key Epigenetic Barrier to Direct Cardiac Reprogramming. Cell stem cell, 18(3), 382–395].

Chemical inhibition of Bmi1 using PTC 209, in conjunction with the all-chemical cocktail CRFVPT, further demonstrated the benefits of Bmi1 repression in both mouse embryonic fibroblasts (MEFs) and adult cardiac fibroblasts (CFs) (Testa et al. 2020).

To explore factors enabling cardiac reprogramming in adult tail-tip fibroblasts (TTFs), Zhou and colleagues performed a high-throughput screen, overexpressing over 1,000 cDNAs encoding TFs, epigenetic factors and nuclear receptors during reprogramming with AGHMT (Zhou et al. 2017). The histone reader PHF7 emerged as a top hit from the screen. Despite not being expressed in the postnatal heart, PHF7, when combined with Mef2c and Tbx5, induced reprogramming of TTFs to iCMs even in the absence of Gata4 (Garry et al. 2021). PHF7 interacts with cardiac TFs, binds to multivalent cardiac enhancers and enhances chromatin accessibility and cardiac TF binding at these sites,

leading to broad changes in cardiac gene expression. This study highlights the potential of histone readers and epigenetic remodelers, such as PHF7, to recognize barriers and facilitate chromatin remodeling in directing cell fate conversion. While our understanding of the mechanisms dictating chromatin accessibility and epigenetic modification during reprogramming is still in its early stages, the application of next-generation sequencing and single-cell omics technologies holds promise for deeper exploration of these processes.

Epigenetic regulation is crucial in directing cellular plasticity during reprogramming, with histone modifications dictating chromatin accessibility to transcription factors. Studies have highlighted the role of chromatin remodeling factors like Bmi1 in modulating cardiac gene expression, offering potential therapeutic targets. Additionally, histone readers like PHF7 have emerged as key players, facilitating chromatin remodeling and enhancing reprogramming efficiency. Future research, aided by advanced sequencing technologies, promises deeper insights into these mechanisms, paving the way for more effective cell fate conversion strategies.

1.7 Inflammation as a barrier to Direct Cardiac Reprogramming

Recent findings have shown that inflammation, especially in aged fibroblasts, serves as a barrier to direct cardiac reprogramming (Muraoka et al. 2019). By means of high-throughput screening of 8400 chemical libraries, it was discovered that diclofenac sodium, a nonsteroidal anti-inflammatory medicine, when combined with GMT or GHMT in adult fibroblasts, improves the efficiency and maturation of cardiac reprogramming. Mechanistically, cyclooxygenase-2 (COX-2) is expressed in postnatal and adult TTFs in an age-dependent manner, compared to embryonic fibroblasts. (Muraoka et al. 2019). Activation of inflammatory and fibroblast programs in postnatal and adult TTFs is silenced by diclofenac, which also improves cardiac reprogramming by blocking signaling linked to COX-2, prostaglandin E2 receptor 4, cyclic AMP/protein kinase A and interleukin-1β. This is in line with findings from prior investigations on cardiac reprogramming that included objective screening of human transcription factors. For instance, Zhou et al. discovered that Znf281 caused cardiac reprogramming by influencing the expression of genes related to the heart through interactions with the transcription factor Gata4 and by inhibiting the production of genes linked to the inflammatory response (Zhou et al. 2017) (Figure 7).

Recent studies reveal inflammation, particularly in aged fibroblasts poses a challenge to direct cardiac reprogramming. However, combining diclofenac sodium, an anti-inflammatory drug, with reprogramming factors like GMT or GHMT in adult fibroblasts enhances reprogramming efficiency and maturation. By suppressing inflammatory pathways, diclofenac sodium offers a promising avenue for improving cardiac reprogramming in regenerative medicine applications.

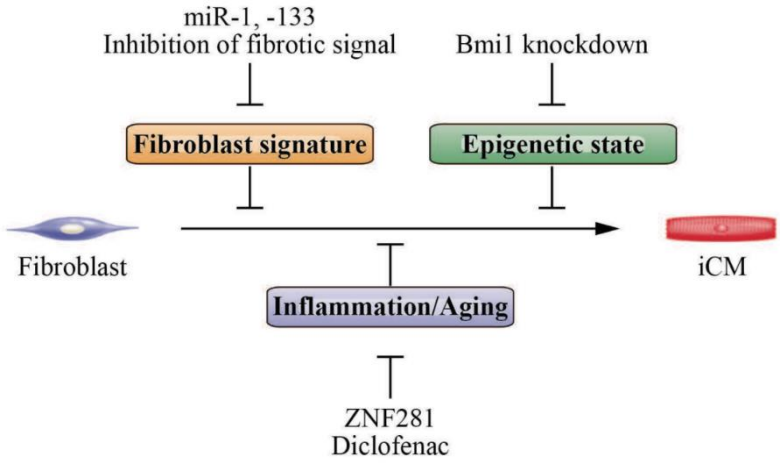

Figure 7. Schematic representation of barriers in direct cardiac reprogramming and proposed solutions based on molecular insights.
[Image extracted from Sadahiro, T., & Ieda, M. (2020). Direct Cardiac Reprogramming for Cardiovascular Regeneration and Differentiation. The Keio journal of medicine, 69(3), 49–58.]

1.8 In Vivo Direct Cardiac Reprogramming

Leveraging the existing CF population as an endogenous source for generating new CMs through direct transformation is a promising approach to restore the damaged myocardium following injury (Figure 8). CFs constitute more than 50% of all cardiac cells and serve as a crucial source of extracellular matrix under normal conditions, providing structural support to other cardiac cells. In response to injury, CFs become highly proliferative and form scar tissue, resulting in adverse remodeling and eventual heart failure.

In 2009 Takeuchi and Bruneau made a remarkable discovery. They found that the overexpression of Gata4, Tbx5 and the chromatin remodeling protein Baf60c in noncardiogenic mesoderm had the capacity to induce spontaneously contracting cardiomyocytes in 50% of transfected mouse embryos. Robust beating induced cardiomyocytes (iCMs) could be generated in vivo with the co-expression of Gata4, Tbx5 and Baf60c, which were not previously identified as promoters of in vitro cardiac reprogramming (Ieda et al. 2010), (Takeuchi and Bruneau 2009), suggests that elements of the natural microenvironment may foster the reprogramming process. Consequently, one might expect significantly higher conversion efficiencies for direct cardiac reprogramming in vivo compared to in vitro. To explore this hypothesis, Qian et al. (2012) employed retroviral delivery of GMT factors to peri-infarct regions in 2-month-old male mice following coronary artery ligation. They observed a heightened efficiency of iCM conversion compared to in vitro GMT reprogramming. Cre-based lineage tracing studies were utilized to ascertain the origin of retrovirally infected iCMs. In the reprogrammed infarct area, a subset of cells displaying

sarcomere structures and cardiomyocyte-like morphology exhibited positivity for both fibroblast and cardiomyocyte lineage markers (Fsp1-Cre and Periostin-Cre). Crucially, lineage tracing using αMHC-MerCreMer mice ruled out the possibility of cell-cell fusion accounting for the observed iCMs. These in vivo-generated iCMs shared many characteristics with native cardiomyocytes: they were binucleate, displayed well-assembled sarcomeres and demonstrated expression of cardiac-specific genes. Additionally, single-cell analysis of iCM electrophysiology revealed that half of the iCMs exhibited action potentials resembling those of ventricular cardiomyocytes upon electrical stimulation. Remarkably, these iCMs were electrically coupled with endogenous iCMs, a critical feature for potential future clinical applications. Importantly, in vivo GMT delivery to infarcted mouse myocardium also resulted in reduced scar formation and improved cardiac function, as assessed by MRI and echocardiography. In addition to this study on in vivo cardiac reprogramming, other independent research groups have made modifications to GMT factor reprogramming techniques. Song et al. (2012) found that the addition of Hand2 to the GMT factors efficiently induced the expression of cardiac markers in vivo. The resulting iCMs closely resembled endogenous cardiomyocytes in terms of morphology, with rod-shaped structures and well-organized sarcomeres, as well as functionality, with many iCMs exhibiting Ca^{2+} transients and action potentials. Similar to GMT factor reprogramming, the GHMT factors were capable of reducing scar size and improving pump function in infarcted mouse hearts. In another example of in vivo GMT factor reprogramming, Inagawa et al. (2012) reported results consistent with those of Qian et al. (2012). However, the novelty of this study lay in alternative experimental methods aimed at enhancing reprogramming efficiency. Instead of using multiple retroviral deliveries of GMT factors as in the Qian study, Inagawa et al. (2012) employed a single-promoter polycistronic retrovirus containing GMT with intervening sequences of 2A self-cleaving peptides, allowing for more uniform GMT expression. Additionally, immunosuppression of mice treated with retrovirus helped preventing immune-mediated destruction of transduced cells. While this study lacked functional characterization of iCMs, these novel experimental modifications significantly improved the generation of iCMs in vivo. With the goal of achieving more homogeneous gene expression of reprogramming factors, Mathison et al. (2014) also generated a single-promoter polycistronic vector encoding GMT and 2A self-cleaving peptides. Compared to multiple viral deliveries of GMT, delivering polycistronic GMT to infarcted rat hearts resulted in a 2-fold increase in iCM generation as well as enhanced ventricular function.

Introducing another dimension to transcription-factor-mediated cardiac reprogramming, several other research groups have explored the impact of small molecule addition to the GMT cocktail of factors. Mathison et al. (2012) incorporated the expression of three major VEGF isoforms into GMT factor expression in infarcted rat hearts. This approach, compared to treatment with GMT alone, resulted in reduced fibrosis and a 4-fold increase in ejection fraction. Supporting the role of additional compounds in enhancing GMT factor

reprogramming, Srivastava et al. (2012) demonstrated that adding a fibroblast activator, thymosin $\beta 4$, to the GMT reprogramming cocktail also increased the number of reprogrammed iCMs.

Alternative methods to transcription factor reprogramming have also been explored. Jayawardena et al. (2012) reprogrammed mouse fibroblasts into cardiomyocyte-like cells in vivo using a combination of miR-1, -133, -208 and -499. Later, it was shown that treatment with a JAK inhibitor and miR-1 alone was sufficient to reprogram fibroblasts into cardiomyocyte-like cells. In 2015, Jayawardena et al. demonstrated that delivering this miR combination to peri-infarct regions of mouse myocardium resulted in cardiomyocyte-like cells with mature features, including sarcomere structure, excitation-contraction coupling and action potentials similar to those of mature ventricular cardiomyocytes. Serial echocardiography also revealed progressive improvements in left ventricular function starting 1 to 2 months after surgery, with enhanced function at the 3-month follow-up.

Cao et al. (2016) successfully employed the cocktail of nine small molecules (CHIR99021, A83-01, BIX01294, AS8351, SC1, OAC2, Y27632, SU16F and JNJ10198409) to guide cardiac reprogramming of human fibroblasts in vitro, followed by transplantation into injured murine hearts. This cocktail included epigenetic modifiers such as BIX01294 (a methyltransferase inhibitor) and AS8351 (a histone demethylase inhibitor), SC1 (an ERK2 and Ras-GAP inhibitor), OAC2 (an Oct4 activator), SU16F (a PDGFRβ inhibitor) and JNJ10198409 (a PDGF receptor tyrosine kinase inhibitor).

The direct transformation of cardiac fibroblasts (CFs) into cardiomyocytes (CMs) offers a promising approach to repair damaged myocardium, leveraging the endogenous CF population. CFs, which predominate in the heart, undergo proliferation and scar formation in response to injury, contributing to adverse remodeling. Through in vivo experiments, researchers have shown that specific combinations of transcription factors, such as Gata4, Tbx5, and Baf60c, can induce the transformation of noncardiogenic mesoderm into functional CMs. These findings suggest that the heart's microenvironment may enhance the reprogramming process, leading to more efficient and mature CM generation compared to in vitro methods. Additionally, innovative approaches, such as small molecule cocktails and microRNA combinations, have demonstrated significant success in driving cardiac reprogramming both in vitro and in vivo. These advancements hold promise for future therapeutic strategies in cardiac regenerative medicine.

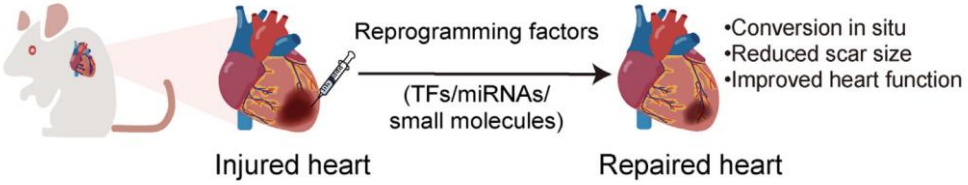

Figure 8. In vivo direct reprogramming of cardiac fibroblasts into cardiomyocytes. Following myocardial infarction, cardiomyocytes undergo cell death and fibroblasts proliferate to form a scar in the infarcted area. The direct conversion of cardiac fibroblasts (CFs) into induced cardiomyocytes (iCMs) is achievable through the administration of reprogramming factors, which include transcription factors (TFs), microRNAs (miRNAs) and small molecules. [Image extracted from Engel, J. L., & Ardehali, R. (2018). Direct Cardiac Reprogramming: Progress and Promise. Stem cells international, 2018, 1435746.]

1.9 Looking to the future

In contrast to transcription factors (TFs) and microRNAs (miRNAs), small molecules represent an appealing therapeutic option, particularly due to their safer profile. They avoid the integration concerns associated with retroviral and lentiviral vectors into host chromosomes. Additionally, they offer a range of advantages, including improved cell delivery efficiency, nonimmunogenic properties and cost-effectiveness. Moreover, the flexibility to adjust concentrations and combinations of small molecules provides a convenient way to modulate the reprogramming process. These tiny compounds can be administered orally, intravenously or via field injection (Figure 9). However, achieving successful chemical reprogramming requires a comprehensive understanding of protocols, encompassing factors such as dosage, administration duration, routes of delivery and other critical considerations.

Although Chemically induced Direct Cardiac Reprogramming is promising, further research is required before this technology can be fully translated for application in regenerative medicine.

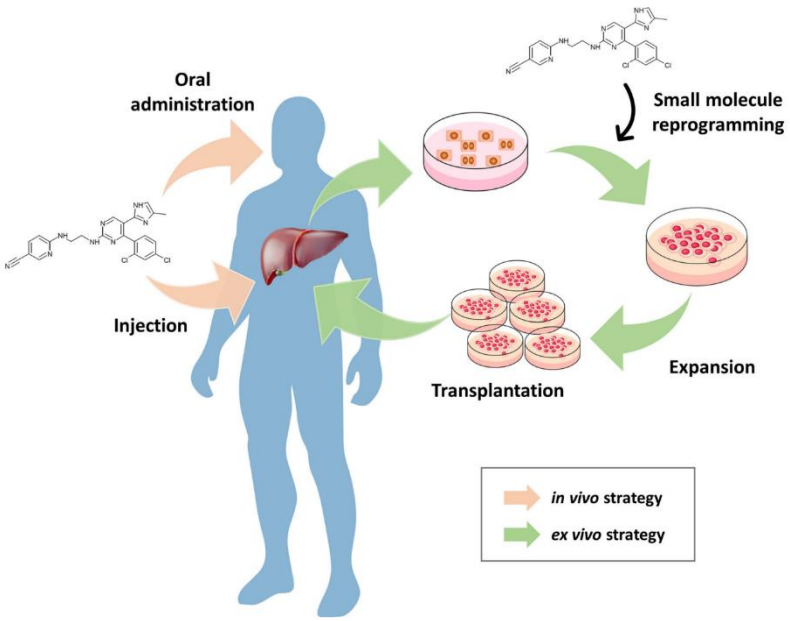

Figure 9. Small molecules will play a key role in the future of customized medicine.
By small chemicals in 'in vitro' reprogramming, an ex vivo method can produce the required cells, which are subsequently amplified and transplanted into patients (green arrow). The small-molecule combination could be administered directly to patients by injection or oral means (pink arrow). [Image extracted from Kim, Y., Jeong, J., & Choi, D. (2020). Small-molecule-mediated reprogramming: a silver lining for regenerative medicine. Experimental & molecular medicine, 52(2), 213–226.

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