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Review

Direct cardiac reprogramming: Toward the era of multi-omics analysis

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ABSTRACT

Limited regenerative capacity of adult cardiomyocytes precludes heart repair and regeneration after cardiac injury. Direct cardiac reprograming that converts scar-forming cardiac fibroblasts (CFs) into functional induced-cardiomyocytes (iCMs) offers promising potential to restore heart structure and heart function. Significant advances have been achieved in iCM reprogramming using genetic and epigenetic regulators, small molecules, and delivery strategies. Recent researches on the heterogeneity and reprogramming trajectories elucidated novel mechanisms of iCM reprogramming at single cell level. Here, we review recent progress in iCM reprogramming with a focus on multi-omics (transcriptomic, epigenomic and proteomic) researches to investigate the cellular and molecular machinery governing cell fate conversion. We also highlight the future potential using multi-omics approaches to dissect iCMs conversion for clinal applications.

1. Introduction

Heart disease is the leading cause of death worldwide. Cardiac injury leads to irreversible loss of cardiomyocytes (CMs) that fail to regenerate in adult mammalian heart. In parallel, resident cardiac fibroblasts (CFs) activate, proliferate, and form the scar tissue, leading to adverse remodeling and eventually heart failure. Heart transplantation holds great promise for heart failure patients, yet this treatment is largely limited by donor resources. It remains challenging to replenish the lost CMs to repair the injured heart(Aguirre et al., 2013; Khush et al., 2019; Shiba et al., 2012).

CFs are one of the most prevalent cell types in heart, accounting for more than 50% of the total number of cardiac cells (Baudino et al., 2006; Camelliti et al., 2005). Under physiological conditions, CFs are major sources of extracellular matrix and provide supporting structure for other cardiac cells. Upon injury, CFs become highly proliferative and actively remodel the heart. The existing pool of CFs could serve as an endogenous source for generating new CMs if they could be directly transformed to muscle cells. Recent researches have demonstrated that specific transcription factors (TFs), microRNAs (miRNAs) and small molecules could directly convert fibroblast into induced cardiomyocytes (iCMs) without going through the pluripotent stage(Dal-Pra et al., 2017; Fu and Srivastava, 2015; Hiroyuki and Nobuaki, 2014; Ieda et al., 2010; Kwon et al., 2009; Liu et al., 2016; Zhao et al., 2015a). At the molecular level, iCMs generated *in vitro* express cardiac-specific genes involved in sarcomere assembly, cell junction and ion channels required for contractile function of CMs. Interestingly, comparative transcriptomic analysis indicates that iCMs show features more similar to adult CMs comparing to induced pluripotent cell (iPSC) derived CMs, including relatively hyperdynamic epigenetic state, using fatty acid oxidative phosphorylation as major metabolic pathway, and cell cycle arrest(Zhou et al., 2017c). At the functional level, iCMs exhibit spontaneous Ca^{2+} flux, electrical activity, and beating *in vitro*. Importantly, iCMs generated *in vivo* contract synchronously with endogenous CMs and electrically coupled with surrounding myocardium (Ieda et al., 2010; Qian et al., 2012). In animal model of myocardial infarction, direct cardiac reprogramming converts CFs into iCMs in situ and greatly improves heart function, revealing the great promise of this strategy for future regenerative medicine(Qian et al., 2012; Song et al., 2012).

Cell fate conversion is tightly controlled by key regulators, including genetic factors, epigenetic modifiers, and mediators in signaling pathways. To investigate the cellular and molecular mechanisms underlying iCM generation, multiple assays dissecting global transcriptome, epigenomic landscapes and protein abundance have been applied (Hashimoto et al., 2019; Mohamed et al., 2016; Sauls et al., 2018; Stone et al., 2019). With the development of single cell sequencing technology,

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cellular heterogeneity and the reprogramming trajectory of iCM have now been illustrated at single cell level, leading to identification of detrimental factors and pathways that control the iCM generation(Liu et al., 2017; Stone et al., 2019; Wang et al., 2022a; Zhou et al., 2019). In this review, we present current progress of direct cardiac reprogramming *in vitro* and *in vivo*, with a specific focus on researches using multiple omics approaches to dissect the reprogramming process. We further discussed future potential and challenges using new multi-omics technologies to improve our understanding of reprogramming machinery.

2. Direct cardiac reprogramming to generate cardiomyocytes

The discovery of induced pluripotent stem cells (iPSCs) generated from other cell lineage using four core transcription factors (Oct4, Sox2, Klf4 and c-Myc) inspires the conceptual idea of transforming one somatic cell type to another(Takahashi and Yamanaka, 2006). Recent findings have indicated that direct lineage conversion can be achieved by using transcription factors, microRNAs and small molecules that rebuild a new regulatory network controlling cell fate change(Aydin and Mazzoni, 2019). Induced neurons, hepatocytes, and pancreatic cells have been quickly generated and become promising to generate functional target cell types. Direct reprogramming of iCMs was firstly achieved by Srivastava group, starting with the pool of 14 transcription factors that tightly control heart development. Three master regulators of cardiac development, Gata4, Mef2c and TBx5 (GMT) were identified to convert fibroblasts into iCMs(Ieda et al., 2010) (Fig. 1). Since then, the field has progressed rapidly and the molecular mechanisms underlying this fate conversion have been extensively explored *in vitro* and *in vivo*.

2.1. iCM reprogramming by ectopic expression of transcription factors

Initial report has demonstrated that GMT factors are capable of inducing iCMs generation from CFs, resulting in global myocyte gene expression, sarcomere assembly, electrophysiological properties and epigenetic changes(Ieda et al., 2010). Addition of Hand2 to GMT (GHMT) or addition of Nkx2.5 to GHMT(HNGMT) could improve quantity and quality of iCMs (Table 1)(Addis et al., 2013; Song et al., 2012; Zhang et al., 2019). Recently, addition of Akt1, Znf281 and PHF7 to GMT or GHMT cocktails has been shown to further increase iCM reprogramming efficiency by 4–10 folds(Garry et al., 2021; Zhou et al., 2015, 2017a). Importantly, iCMs produced by direct reprogramming resembles ventricular CMs and transcriptionally more mature than iPSC derived CMs(Zhou et al., 2017d).

Another intriguing question is whether GMT reprogramming factors could be replaced by other factors. It has been reported that lentiviral infection of Tbx5, Mef2c and Myocd (3F-Myocd) directly reprogrammed mouse embryonic fibroblasts (MEFs) into iCMs(Protze et al., 2012). Very interestingly, inhibition of epigenetic factor Bmi1 can activate endogenous Gata4 expression and thus generate iCMs with Mef2c and Tbx5(Zhou et al., 2016).

Taking advantage of genetic lineage tracing strategy, Qian et al. and Song et al. have demonstrated that retroviral delivery of GMT and GHMT converted resident CFs in the infarct zone into iCMs after coronary ligation, thus reducing scare size and improving heart function in terms of cardiac ejection fraction, stroke volume and cardiac output(Qian et al., 2012; Song et al., 2012). Notably, the reprogramming efficiency *in vivo* was relatively higher than that *in vitro*, suggesting environmental niches may play critical roles in iCM conversion(Qian et al., 2012). Polycistronic expression of GMT and addition of small molecules could further enhance heart repair Interestingly, haplodeficiency of Beclin1 further reduced scar size and improved MGT-based recovery of heart function 4 weeks post myocardial injury(Wang et al., 2020a).

2.2. Direct iCM reprogramming using microRNAs

MicroRNAs(miRNAs) are single-stranded functional RNA molecules approximately 20 24-nucleotides in length. Jayawardena and co-workers identified the combination of muscle-specific miRNAs, including miR-1, miR-133, miR208 and miR-499, induced cardiomyocyte-like cells *in vitro*. miRNAs-induced iCMs, display well-organized sarcomere structure, spontaneous calcium transient and contraction. Injection of these miRNAs into infarcted heart leads to direct conversion of CFs to iCMs at injured area(Jayawardena et al., 2012).

Addition of miRNAs to transcription factor cocktails has been shown to improve cardiac reprogramming. Application of miR-133a(miR-133) to GMT or GMT plus Mesp1 and Myocd improved cardiac reprogramming in adult mouse CFs and human fibroblasts(Muraoka et al., 2014). MiR-133 enhances generation of functional iCMs through direct repression of Snail, a master regulator of epithelial-to-mesenchymal transition. In addition, miRNA-590 can replace Hand2 and Myocd during conversion of human and porcine fibroblasts to iCMs(Eulalio et al., 2012; Singh et al., 2016).

2.3. Human iCM reprogramming

Conversion of human fibroblasts to iCMs is more challenging than generation of murine iCMs. Early studies have shown that GMT and GMTH cocktails failed to effectively generate human iCMs (hiCMs) (Table 2) (Fu et al., 2013; Nam et al., 2013; Wada et al., 2013).

Adding Esrrg and Mesp1 to GMT (5F) can reprogram human fibroblasts into hiCMs, albeit at low frequency (around 15.8% αMHC-mcherry+) (Fu et al., 2013). Adding Mesp1 and Myocd to GMT (GMTMM) (5F) generated cardiomyocyte-like cells from human fibroblasts with 5.9% reprogramming efficiency. Co-culture with rat neonatal CMs can induce 5% of these hiCMs to contract synchronously with surrounding CMs. Nam and colleagues found that combination of Gata4, Hand2, Tbx5, Myocd, miR-1 and miR-133 (6F) can induce spontaneously

Table 1
Combinations for murine cardiac reprogramming.

Reprogramming combinations	Starting cells	Markers and Efficiency
Gata4, Mef2c, Tbx5 (GMT) (Ieda et al., 2010)	CF, TTF	αMHC+ 25%; cTnT+ 8%(CF), cTnT+ 4%(TTF)
Gata4, Hand2, Mef2c, Tbx5 (GHMT) (Song et al., 2012)	adult TTF, CF	αMHC+15%(CF), αMHC+18%(TTF)
Hand2, Nxk2.5, Gata4, Mef2c, Tbx5 (HNGMT) (Addis et al., 2013)	MEF, CF	GCaMP5 activity+ 1.6%
GHMT, Akt1 (AGHMT) (Zhou et al., 2015)	CF, TTF	~0.7% of beating cells (CF), ~0.5% of beating cells
Mef2c, Tbx5, Myocd (Protze et al., 2012)	MEF, TTF, CF	cTnT+ 12% (96% of α-MHC)
miR-1, miR-133, miR208, miR-499 (Jayawardena et al., 2012)	CF	~1% of beating cells
GMT, miR-133 (Muraoka et al., 2014)	CF	αMHC+~32%, cTnT+~12%

Table 2
Combinations for human cardiac reprogramming.

Reprogramming combinations	Starting cells	Markers and Efficiency
GMT, Esrrg, Mesp1 (Fu et al., 2013)	H9F	αMHC-mcherry+15.8%
GMT, Mesp1, Myocd (GMTMM) (Wada et al., 2013)	CF	cTnT+~5%
Gata4, Hand2, Tbx5, Myocd, miR-1, miR-133 (Nam et al., 2013)	HFF	cTnT+~19%
GHMT, PHF7 (Garry et al., 2021)	adult CF	αMHC + cTnT+~3%
GMT, Tead1(GMTd) (Singh et al., 2021)	CF	cTnT+~7%; αActinin+6%

CF: cardiac fibroblast; TTF: tail-tip fibroblast; MEF: mouse embryonic fibroblast; HFF: neonatal human foreskin fibroblasts; H9F: H9 human ESC-derived fibroblasts; cTnT: troponin T; αMHC: alpha-myosin heavy chain.

beating hiCMs(Nam et al., 2013). Optimal stoichiometry of GMT has been shown to improve murine reprogramming by 5–10 folds(Wang et al., 2015). Inspired by this notion, Yang et al. applied polycistronic hMGT construct and obtained approximately 40% cTnT + hiCMs together with miR133 using H9F fibroblast and primary hCFs (Zhou et al., 2019). More recently, overexpression of Tead1 plus GMT resulted in 4-fold increase in the number of aActinin + hiCMs(Singh et al., 2021). However, it is difficult to observe spontaneously beating iCMs, although some groups did find that hiCMs generated Ca^{2+} transients and action potentials, it seems that significant effort would be required to overcome the human reprogramming barriers.

2.4. Enhancement of iCM conversion by small molecules

Upon GMT basic combination, the addition of small molecules improves iCM reprogramming. Mohamed and his colleagues found that simultaneous inhibition of TGF-β and WNT signaling improved the efficiency, quality, and speed of direct cardiac reprogramming *in vitro* and *in vivo*. (Mohamed et al., 2017). Other groups demonstrated that pharmacological inhibition of Rock, Notch, C–C chemokines, p38 mitogen-activated protein kinase and phosphoinositol 3-kinase/AKT pathways can also increase reprogramming efficiency(Abad et al., 2017; Guo et al., 2019; Mohamed et al., 2017; Wang et al., 2020a; Yamakawa et al., 2015; Zhao et al., 2015a, 2015b). Taken together, these results suggest that inhibition of the profibrotic pathway contributes to the progression of iCM reprogramming. The anti-inflammatory response was found to be upregulated during iCM reprogramming(Zhou et al., 2017b). Subsequently, diclofenac sodium (diclofenac), a non-steroidal anti-inflammatory drug, greatly enhanced cardiac reprogramming in combination with GMT or GMTH via inhibition of the PGE2/EP4 pathway.(Muraoka et al., 2019). Epigenetic repressors targeting Ezh2, G9a and Mll1 complex also enhance iCM generation(Hiroyuki and Nobuaki, 2014; Liu et al., 2016). Interestingly, small molecules containing fibroblast growth factor (FGF) 2, FGF10, and vascular endothelial growth factor (VEGF), termed FFV, also improve iCM conversion with high quality under defined serum-free conditions (Yamakawa et al., 2015). FFV increased a global cardiac program through p38 MAPK and PI3K/Akt signaling pathways. Intriguingly, FFV can replace Gata4 and enable cardiac reprogramming with Mef2c and Tbx5.

2.5. Non-genomic integration methods for direct reprogramming

Safe delivery of reprogramming factors into the injured heart is critical for clinic translation of direct reprogramming. So far, most studies used retroviral and/or lentiviral construct to deliver reprogramming factors, which potentially integrate into the host genome and cause malfunctions. Recent researches have begun to seek other bio-safe approaches.

Sendai virus (SeV) vector, a single negative strand RNA virus with advantages of efficient transduction and non-integration, was used to induce pluripotent stem cell (iPSC) (Haase et al., 2017; Macarthur et al., 2012; Tan et al., 2018). SeV vectors expressing cardiac reprogramming factors efficiently reprogrammed fibroblasts into integration-free iCMs (Miyamoto et al., 2018). Compared with retroviral vectors (pMX-GMT), SeV-GMT efficiently and rapidly reprogramed mouse fibroblasts into functional iCMs in 7 days. The number of iCMs induced from MEFs by SeV-GMT was 100 times more than that induced by the retrovirus, and the beating time of iCMs could be shortened from 25 days to 10 days. *In vivo*, SeV-GMT was shown to improve cardiac function and reduce scar area after MI, indicating its further broad application in the field.

Non-biological carriers, such as nanoparticles, offer a promising perspective to delivering cargos into cells while maintaining target specificity and biological activity of cargos. A recent research has demonstrated that packing modified mRNAs against fibroblast activation protein (FAP) in lipid nanoparticles (LNPs) successfully produced CAR-T cells that are capable of eliminating fibrosis in the heart(Rurik et al., 2022). Cationic gold nanoparticles (AuNPs) loaded with GMT factors induced iCMs from MEFs (Chang et al., 2019). Upon myocardial injury, injection of AuNP-GMT to infarcted heart resulted in effective recovery of cardiac function and decreased size of scar area. Recently, a non-viral and transgene-free approach using extracellular vesicles derived from ESCs have been used to induce fibroblasts into functional CMs(Kim et al., 2022), indicating rising interest in developing biologically safe strategy to guide heart repair.

3. Multi-omics dissection of iCM reprogramming

During acquisition of myocyte cell fate, the starting fibroblast experience epigenetic alternations, transcriptional remodeling, protein turnover, and eventually turn into functional iCMs. Comprehensive understanding of this process requires application multi-omics approaches to dissect molecular intricacy at multiple levels (Fig. 2) (Liu et al., 2017; Sauls et al., 2018; Stone et al., 2019). Advances in single cell technologies allow for deciphering the iCMs heterogeneity and reprogramming trajectories at single cell resolution, and thus greatly improved our knowledge of cardiac reprogramming (Wang and Navin, 2015; Welch et al., 2019).

3.1. Epigenetic landscape of iCMs

Epigenetic regulation orchestrates cellular plasticity and cell fate conversion (Wamstad et al., 2012). Early studies using bisulfite genomic sequencing indicated the reduced DNA methylation levels in promoter region of cardiac genes such as Nppa and Myh6. In parallel, chromatin Immunoprecipitation sequencing (ChIP-seq) showed that trimethylation of histone H3 at lysine 27 (H3K27me3, repressive marker of gene expression) was significantly reduced, while trimethylation of lysine 4 of histone H3 (H3K4me3, active marker of gene expression) was dramatically augmented at promoters of cardiac genes, indicating dramatic epigenetic changes for iCMs conversion (Ieda et al., 2010).

Epigenetic factors play pivotal role during iCMs reprogramming. Bmi1, an essential component of the polycomb repressive complex 1 (PRC1), was the first epigenetic factor identified to inhibit iCMs conversion (Zhou et al., 2016). Mechanistically, Bmi1 binds to cardiac loci marked by monoubiquitination of histone H2A at lysine 119 (H2AK119ub) and thus suppresses target gene expression including Gata4 and Tbx20. Knockdown Bmi1 enhanced endogenous Gata4 expression and generated iCMs together with ectopic expression of Mef2c and Tbx5. Recently, overexpression of histone reader PHF7 was found to improve iCMs generation. PHF7 binds to cardiac super enhancer and interacts with SWI/SNF complex to increase chromatin accessibility. Interestingly, PHF7 plus Mef2c and Tbx5 can achieve effective cardiac reprogramming without Gata4(Garry et al., 2021). Other researches targeting H3K4 methyltransferase Mll1(Liu et al., 2016), BAF60c (cardiac-specific subunit of ATP-dependent chromatin remodeling SWI/SNF complexes)(Nicolas et al., 2013) and chromatin modulators Bcor and Ruvbl1 significantly increase iCM reprogramming(Zhou et al., 2018).

Our previous researches have indicated the importance of stoichiometry of reprogramming factors GMT(Wang et al., 2015). How these factors changed global chromatin structure and gene expression remained elusive. Stone and colleagues have demonstrated that GMT factors induced rapid epigenomic and transcriptomic changes during the first 24–48 h of reprogramming (Stone et al., 2019). Using integrative analysis of ChIP-seq, assay for transposase-accessible chromatin sequencing (ATAC-seq) and RNA-seq, they showed that GMT factors could either cooperatively or independently modulate chromatin accessibility. Especially, most accessibility changes were associated with Mef2c and Tbx5 binding (Stone et al., 2019). Genome-wide research from Olson group showed that reprogramming factor GMT, together with Hand2 and Akt1, synergistically activate cardiac enhancers and silence fibroblast enhancers. Gene regulatory networks (GRN) analysis showed that EGF receptor signaling was directly inhibited by reprogramming

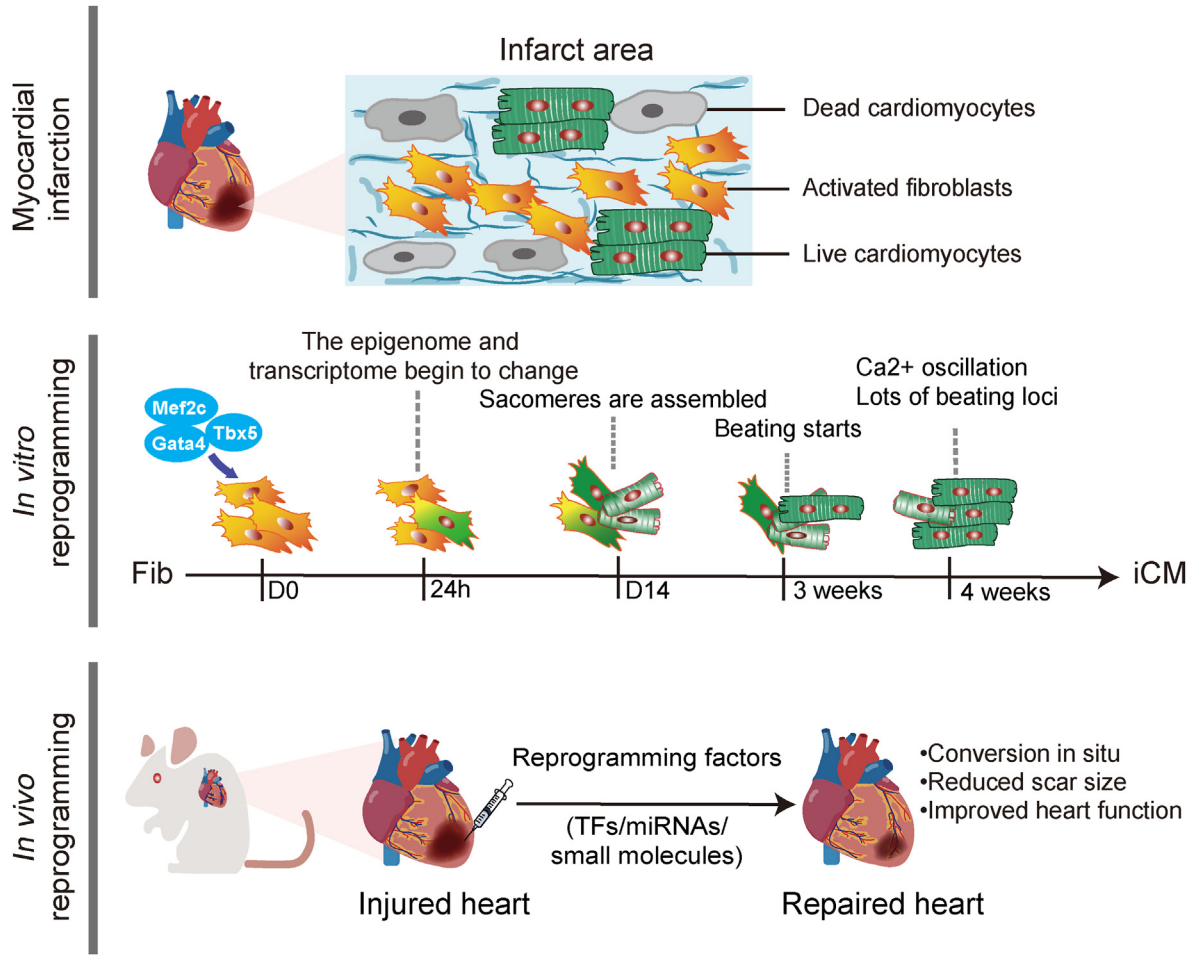

Fig. 1. A model of direct reprogramming of cardiac fibroblasts into cardiomyocytes. After myocardial infarction, cardiomyocytes die and fibroblasts proliferate to form a scar in the infarcted area. Direct conversion of cardiac fibroblasts (CFs) into induced cardiomyocytes (iCMs) can be achieved both *in vitro* and *in vivo* with reprogramming factors, including transcription factors (TFs), microRNAs (miRNAs) and small molecules. Myocardial infarction area decreases and heart function improves after reprogramming factors delivery.

TFs. Consequently, inhibition of EGF and JAK2 signaling could enhance iCM reprogramming efficiency(Hashimoto et al., 2019).

Single-cell ATAC-seq (scATAC-seq) is emerging as a powerful tool for dissecting epigenomic heterogeneity at the single-cell level, revealing cis regions and identifying potential interaction transcription factors. Combining pseudotime trajectories obtained from scRNA-seq and scATAC-seq, Wang et al. identified active TFs including Smad3, Smad4, Fos and Tcf21 as reprogramming barriers(Wang et al., 2022a). Strikingly, Smad3 functions as an inhibitor at early stage of reprograming while a booster at later stage. Further analysis of epigenomic trajectory indicates that cis-regulatory elements, such as promoters and enhancers, dynamically control cardiac gene expression along iCMs generation(Wang et al., 2022a).

3.2. *Transcriptomic reconstruction of reprogramming trajectory*

Direct cardiac reprogramming converts fibroblasts to iCMs without going through pluripotent and progenitor stage. How this process was conducted remained largely unknown until the discoveries from scRNA-seq researches. Recently, scRNA-seq performed with Fluidim C1 system revealed the heterogeneity of murine iCMs. The reprogramming culture contains cells at different reprogramming states, including Fib (untransduced fibroblast), iFib (intermediate fibroblast with low GMT expression), piCM (pre-iCM that express both fibroblast and CM features), and fully reprogramed iCMs. The trajectory built by SLICER suggested a reprograming route from iFib to piCM, and to iCM (Liu et al., 2017). Further analysis of global gene expression changes during reprogramming revealed an unexpected down-regulation of factors involved in mRNA processing and splicing. Silencing splicing factor Ptbp1was found to improve the efficiency of iCM reprogramming by changing the splicing pattern of genes related to CM lineage and function. Reprogramming trajectory from another group also unveiled a route from early iCMs to late iCMs, with additional branches characterized by either progression of cell cycle genes or activation of Mmp3(Stone et al., 2019).

To identify hiCM reprograming route, Zhou and colleagues performed scRNA-seq along reprogramming process. They found that hiCM reprogramming involves a decision point, or bifurcation event, for a cell to determine if it would step back to be a fibroblast or process into a myocyte (Zhou et al., 2019). Further analysis of reprogrammed iCMs using cell fate index algorithm indicated different reprogramming speed of human iCMs versus murine iCMs. Mouse fibroblasts progressed significantly faster toward CM fate, at a speed that is about 1.6 times faster compared to human cells. This study provided first evidence showing differences in fate acquisition between murine and human cardiac reprogramming at single-cell resolution, which help to understand the molecular mechanism of iCM reprogramming and has important implications for clinical translation of this strategy.

3.3. *Proteomic profiling of early reprogramming events in iCMs*

Dynamic changes of cellular components at protein level during iCM

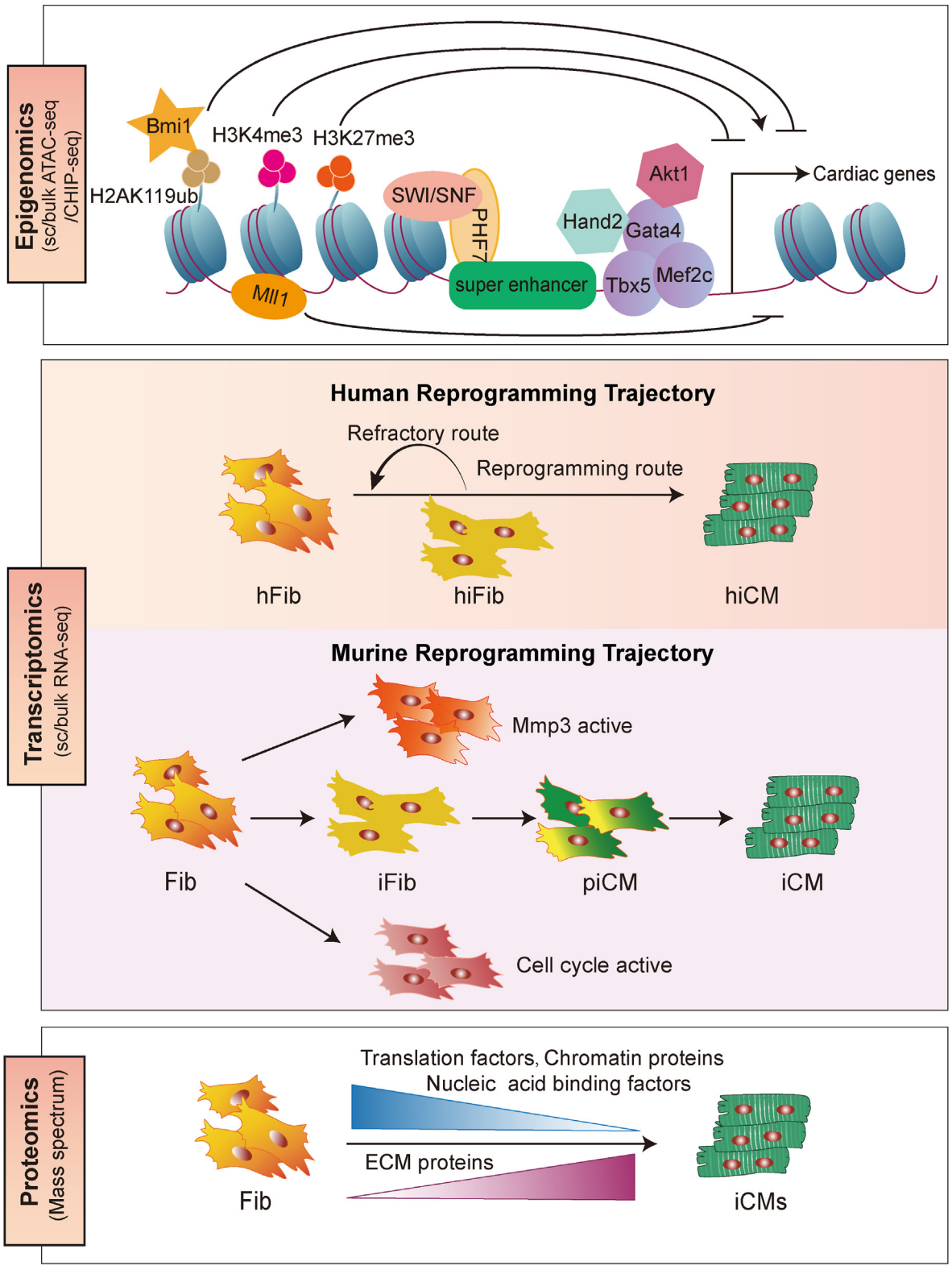

Fig. 2. Integrated analysis of multi-omics in iCM reprogramming. Multiple omics analysis, including epigenomics, transcriptomics and proteomics, have been applied in direct cardiac reprogramming studies. Epigenomic appoaches, including ChIP-seq and ATAC-seq have been used to dissect the epigentic modifications and changes of chromatin accessibility during reprogramming. Some representative epigenetic factors, such as Bmi1, PHF7 and Mll1 were included. The reprograming trajectories, revealed by sc-RNAseq plus bulk RNA-seq, were somehow different between human and murine reprogramming. Proteomics research explored the dynamic changes in protein abundance during iCM reprogramming.

conversion remain elusive. Sauls et al. performed proteomics profiling and revealed the temporal and global changes in protein abundance that occur during initial phases of iCM reprogramming(Sauls et al., 2018). Protein turnover in reprogramming occurs at 48 h post-infection, with upregulation of extracellular matrix (ECM) proteins and decreased expression of chromatin associated proteins (Sauls et al., 2018). This proteomic research indicates the importance of ECM for cell fate conversion. Consistently, Kurtus et al. found that changing the stiffness of ECM to an extent comparable to cardiac tissue improves reprogramming efficiency. Interestingly, soft ECM inhibits fibrotic programs and suppresses signaling pathways involving integrin and YAP/TAZ(Kurotsu et al., 2020). So far, how existing cytoplasmic protein of fibroblast get removed and the consequence of it on reprogramming remain unknown. Recent study indicated the potential activation of autophagy during iCM conversion(Wang et al., 2020a), however, more proteomics researches will be needed to further improve our understanding of iCM conversion at post translational level.

4. Conclusions and perspectives

Substantial progress has been made in the field of heart regeneration using direct reprogramming in recent years. Especially, rapid advances in multi-omics technologies have refined our understanding of iCM reprogramming with unprecedented precision and resolution. As we delineate the molecular mechanisms underlying iCMs fate determination largely *in vitro*, additional work investigating reprogramming *in vivo* using multi-omics approaches is encouraging.

First, the characteristics of starting fibroblast for iCM reprogramming *in vivo* remain unknown. Accumulating researches demonstrated the heterogeneity of cardiac fibroblasts under physiological and pathological conditions(Farbehi et al., 2019; Gladka et al., 2018; Skelly et al., 2018; Wang et al., 2022b). Discovery of reparative cardiac fibroblast upon myocardial infarction (Ruiz-Villalba et al., 2020) inspires us to hypothesize the existence of specialized fibroblast susceptible to reprogramming. Further researches combing single cell multi-oimcs with sophisticated lineage tracing strategy potentially unravel the origin of iCM reprogramming(Wang et al., 2020b).

Second, iCMs generated *in vivo* display different reprogramming quality(Ma et al., 2015; Qian et al., 2012; Song et al., 2012). Questions on the heterogeneity and reprograming trajectory *in vivo* remain unknown. One of the major challenges using regular scRNA-seq platform to study iCMs generated in situ lays on the large nozzle size of ventricular CM/iCMs which is around 100–150 μm in length. This could be solved by using other high throughput platforms such as ICELL8 single cell system, which allows separation of individual live CMs in traceable nanowells regardless of cell shape and cell size(Wang et al., 2020b). Meanwhile, transcriptome from nuclei can provide additional profile to infer the cell identity and developmental trajectory. Recent development of single-nucleus RNA sequencing (snRNA-seq) revealed cellular heterogeneity of murine cardiac cells including CMs(Vidal et al., 2019; Zhang et al., 2019). Combination of snRNA-seq and scRNA-seq further identified subsets of human cardiac cells with diverse developmental origins and cell-to-cell interactions (Litvinukova et al., 2020). Collectively, application of single cell multi-omics technologies would benefit better understanding on iCM fate switching *in vivo*.

Finally, it has been suggested that microenvironments similar to *in vivo* conditions may improve direct cardiac reprogramming(Li et al., 2016; Qian et al., 2012; Sia et al., 2016). Due to the complex environment *in vivo*, it is challenging to study how iCMs integrate with neighboring cells, as well as the dynamic changes of surrounding cardiac cells during reprogramming. Recent studies using spatial transcriptomics together with other sc-seq approaches have mapped spatial organization of cardiac cells and cellular lineages during cardiogenesis and heart regeneration from different species (Asp et al., 2019; Cui et al., 2021; Mantri et al., 2021). Further application of spatial transcriptomics will greatly expand our knowledge in cardiac reprogramming *in vivo*.

While many challenges remain, the opportunities and prospects for direct cardiac reprogramming are enormous. With the rapid advances in multi-omics technologies, understanding of the basic biology of direct cardiac reprogramming will be significant, and will ultimately lay a foundation for the translation of direct reprogramming to clinic application.

Declaration of competing interest

The authors declare no competing interests.

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