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## Micromanaging cardiac regeneration: Targeted delivery of microRNAs for cardiac repair and regeneration

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### Abstract

The loss of cardiomyocytes during injury and disease can result in heart failure and sudden death, while the adult heart has a limited capacity for endogenous regeneration and repair. Current stem cell-based regenerative medicine approaches modestly improve cardiomyocyte survival, but offer neglectable cardiomyogenesis. This has prompted the need for methodological developments that create *de novo* cardiomyocytes. Current insights in cardiac development on the processes and regulatory mechanisms in embryonic cardiomyocyte differentiation provide a basis to therapeutically induce these pathways to generate new cardiomyocytes. Here, we discuss the current knowledge on embryonic cardiomyocyte differentiation and the implementation of this knowledge in state-of-the-art protocols to the direct reprogramming of cardiac fibroblasts into *de novo* cardiomyocytes *in vitro* and *in vivo* with an emphasis on microRNA-mediated reprogramming. Additionally, we discuss current advances on state-of-the-art targeted drug delivery systems that can be employed to deliver these microRNAs to the damaged cardiac tissue. Together, the advances in our understanding of cardiac development, recent advances in microRNA-based therapeutics, and innovative drug delivery systems, highlight exciting opportunities for effective therapies for myocardial infarction and heart failure.

**Key words:** Cardiac repair; Cellular plasticity; Targeted drug delivery; MicroRNA; Reprogramming

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**Core tip:** Cardiac fibroblast reprogramming into cardiomyocytes holds great promise for future cardiac regenerative medicine therapies. Here, we discuss current advances in the state-of-the-art protocols for the direct reprogramming of cardiac fibroblasts into *de novo* cardiomyocytes *in vitro* and *in vivo* with an emphasis on

microRNA-mediated reprogramming. Additionally, we discuss current advances on the state-of-the-art targeted drug delivery systems that can be employed to deliver these microRNAs to the damaged cardiac tissue.

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## INTRODUCTION

Ischemic cardiac disease is characterized by a chronic or acute reduction in myocardial perfusion and affects over 120 million people globally of which approximately 4% suffer from myocardial infarction (MI) annually<sup>[1,2]</sup>. MI is the process of cell death occurring after occlusion of a coronary vessel that supplies blood to a specific area of the heart and results in a massive loss (up to 11 billion cells) of viable muscle cells<sup>[3]</sup>. This loss of cardiac tissue may in turn lead to functional cardiac impairments and, if large enough, severe contractile dysfunction with an inability of the heart to maintain organ perfusion resulting in sudden death.

Although the recognition of MI and the success rates of primary angioplasty have greatly improved in the past decades, treatment of MI is commenced after the cardiac damage response has already started. Cell death, either by apoptosis or necrosis, is the initial response of cardiomyocytes to the decreased oxygen supply and commences already 4 h after MI<sup>[4,5]</sup>. Cardiomyocyte cell death is followed by the influx of inflammatory cells that phagocytize the dead cells, resulting in thinning of the ventricle wall. Cytokines secreted by these inflammatory cells recruit myofibroblasts that secrete collagens and replace the lost cardiomyocytes<sup>[6,7]</sup>. This remodeling process culminates in the formation of a scar tissue that preserves the ventricle integrity, but possesses little contractile function which hampers cardiac function. At this stage, chronic heart failure is likely to develop as the cardiac tissue is unable to regain its normal function<sup>[8,9]</sup>. Current treatment options consist of appropriate diet and lifestyle changes and medicinal in the use of diuretics, ACE inhibitors and AT receptor blockers, in an attempt to alleviate the heart from the warring strains it encounters. However, although these interventions have a pronounced effect on increasing the patients lifespan, they do not treat the underlying pathology, which is the loss of cardiomyocyte mass<sup>[10-12]</sup>.

So, if the morbidity following MI is due to the massive loss of cardiomyocytes, would it not be logical to therapeutically induce cardiomyocyte proliferation to compensate for the lost myocytes?

Although most cardiomyocytes form terminally

differentiated binucleated cells that withdraw from the cell cycle<sup>[13,14]</sup>, limiting the myocardial regenerative capacity, some evidence exists for postnatal cardiomyocyte proliferation. Retrospective birth dating of human cardiomyocytes using carbon-14 in the DNA of cardiomyocytes demonstrated that human cardiomyocytes have a turnover rate of approximately 0.45%-1% per year<sup>[15]</sup>. During normal human wound healing, cell cycle activation occurs which compensates for the loss of tissue<sup>[16,17]</sup>. Indeed, a small number of cardiomyocytes enters the cell division cycle following myocardial infarction<sup>[18]</sup>, however the level of proliferation is insufficient to regenerate the lost tissue.

The observation that the postnatal heart retains some proliferative capacity has inspired therapeutic approaches that aim to enhance the endogenous cardiomyocyte proliferation for regeneration. Indeed, forced expression of cell cycle activators such as Cyclin A2 and D2 promotes the proliferation of postnatal cardiomyocytes and limits damage following MI<sup>[19,20]</sup>. Additionally, regenerative medicine approaches using a wide variety of growth factors (*i.e.*, ERBB2<sup>[21]</sup>, FGF1<sup>[22,23]</sup>, HGF<sup>[24,25]</sup>, IGF1<sup>[25]</sup>, NRG1<sup>[22,26,27]</sup>, MYDGF<sup>[28]</sup>, and POSTN<sup>[29]</sup>, reviewed in<sup>[30,31]</sup>) induce cardiomyocyte proliferation after MI, albeit relatively ineffectively.

The relative ineffectiveness of cardiomitogenic therapies using growth factors in restoring cardiomyocyte numbers following myocardial infarction warrants the need to increase cardiomyocyte numbers from exogenous sources. The effectiveness of adult stem and progenitor cells of various origins (*i.e.*, bone marrow-derived cells [Mesenchymal stem cells (MSC)<sup>[32]</sup> and endothelial progenitor cells (EPC/ECFC)<sup>[33]</sup>], adipose tissue-derived regenerative cells (ADRC)<sup>[34]</sup> and cardiac-derived progenitor cells (CPC)<sup>[35]</sup> to induce cardiac regeneration has been assessed in numerous clinical studies (reviewed in<sup>[36-39]</sup>). In general, intramyocardial transplantation of adult stem and progenitor cells in the post-infarct myocardium induces neoangiogenesis and promotes cardiomyocyte survival<sup>[40]</sup> and thereby reduces the infarct size and improves cardiac function long term<sup>[39]</sup>. Although these effects are beneficial to the survival of the myocardium, retention of therapeutic cells at the site of cardiomyocyte death is highly limited<sup>[41,42]</sup> and their cardiomyogenic effects are neglectable<sup>[43,44]</sup>. Hence, the regenerative effectiveness of transplantation of adult stem and progenitor cells is under debate<sup>[43,45]</sup>.

Thus, MI results in a massive loss of cardiomyocytes that are replaced by scar tissue. Endogenous repair mechanisms, such as cardiomyocyte proliferation, are insufficient to efficiently regenerate the lost myocardial tissue and therapeutic approaches to induce cardiomyocyte proliferation using growth factors are ineffective. Current regenerative medicine therapies using stem and progenitor cells improve cardiomyocyte survival, but pose neglectable cardiomyogenesis. This warrants the development of new therapeutic strategies that focus on increasing the number of viable cardiomyocytes at the infarct site, reviewed below.

## CELLULAR PLASTICITY AS THE NEW THERAPEUTIC OPPORTUNITY

### **Induced pluripotent stem cells and cardiomyogenesis**

In 2006, Takahashi *et al.*<sup>[46]</sup> challenged the dogma of terminal cell differentiation. Probing the effects of transcription factors that are pivotal to embryonic stem cell maintenance in terminally differentiated skin fibroblasts, four transcription factors (*i.e.*, Oct4, Sox2, Klf4 and c-Myc) were identified that could convert skin fibroblasts into a more primitive pluripotent stem cell resembling embryonic stem cells<sup>[46,47]</sup>. These data exemplify that cell fate is not fixed, but is determined by the available transcription factors and can be altered by the addition of alternative transcription factors. The obtained induced-pluripotent stem cells (iPSC) introduced a new era in regenerative medicine wherein cellular reprogramming is used to treat disease.

iPSC have been used in preclinical models of MI repair<sup>[48-51]</sup>. Transplantation of iPSC directly into the infarcted myocardium improves cardiac function [*e.g.*, left ventricle ejection fraction (LVEF), fractional shortening, and contractility] and reduces infarct size<sup>[48-50]</sup>. Although transplanted iPSC contribute to cardiac repair, a major impediment to their clinical use in human patients lies in the inefficiency of transplanted iPSC to form cardiomyocytes (0.5%-2%)<sup>[49]</sup>, their tumorigenicity<sup>[52]</sup>, and their limited retention in the infarcted tissue. Yet, proof-of-concept that iPSC can differentiate into functional cardiomyocytes has tantalized researchers in studying cardiac embryology as iPSC differentiation into functional cardiomyocytes is merely a reiteration of embryology.

Embryonic cardiogenesis (Figure 1A) begins from the mesoderm that arises from the primitive streak during gastrulation. Gene regulation and cell movement that control cardiogenesis are spatially and temporally stringently regulated (reviewed in<sup>[53]</sup>). Bone morphogenetic protein (BMP)-4, activin A and fibroblast growth factor (FGF)-2 induce mesoderm specification<sup>[54-56]</sup> from pluripotent progenitors in the primitive streak by inducing Wnt3a expression, whereas Notch signaling inhibits the transition from mesodermal precursors into cardiac mesoderm<sup>[57]</sup>. MESP1, the most early expressed marker of the cardiac lineage<sup>[58,59]</sup>, is expressed by all cardiac precursors that arise from the cardiac mesoderm and drives further cardiac specification by the Dkk1-mediated repression of Wnt signaling<sup>[60]</sup>, resulting in the formation of specialized cardiac progenitor cells. This pool of cardiac precursors gives rise to the endocardium, the first heart field (from which the atria, left ventricle and nodal conduction system are formed) and the second heart field (from which the right ventricle and outflow tract are formed)<sup>[61]</sup>. Specification of cardiac precursors into cells of the first and second heart field is regulated by the complex interplay of transcription factors downstream of MESP1<sup>[62,63]</sup>. Herein, GATA4, MEF2c, HAND2 and NKX2.5 represent common transcription factors to all cardiac precursors, whereas the expression of TBX5 is restricted to the first heart field<sup>[64]</sup> and ISL1 and TBX1

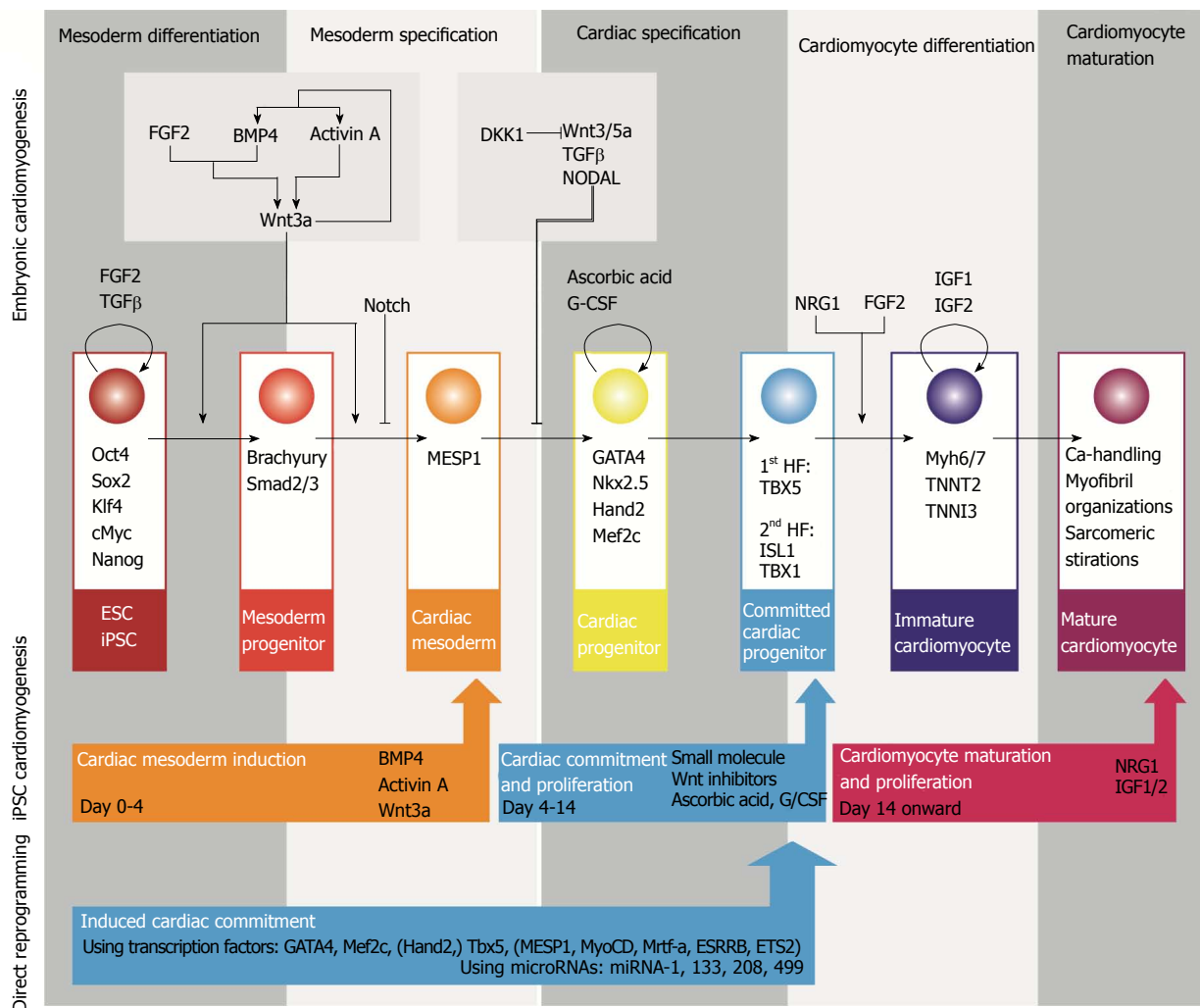
are restricted to the second heart field<sup>[65,66]</sup>. Once formed, cardiac cells of the first and second heart field proliferate in response to endocardial-derived Neuregulin (NRG1) and epicardial-derived retinoic acid and FGF2<sup>[67,68]</sup>.

Indeed, reiteration of key steps in cardiogenesis by supplying iPSC with stage-specific pivotal signaling molecules efficiently differentiates iPSC into the cardiac lineage. Differentiation protocols rely on progressive sequential inductive signals using growth factors (Figure 1B). Monolayers of iPSC are stimulated with BMP4, Activin A and Wnt3a in the first 4 d of differentiation to induce cardiac mesoderm formation<sup>[69-72]</sup>. Inhibition of Wnt signaling using small molecule inhibitors after day 4 of differentiation advances mesodermal precursors to cardiac progenitors and reiterates the actions of Dkk1-mediated inhibition of Wnt signaling during embryology<sup>[69,70]</sup>. The addition of ascorbic acid<sup>[73]</sup> or G-CSF<sup>[74]</sup> at this stage enhances cardiomyocyte formation by stimulating proliferation of cardiac progenitor cells (Figure 1B). Culture of the obtained cardiac progenitor cells in the presence of NRG1 or IGF1 allows further maturation of cardiac progenitor cells into immature cardiac cells from the first and second heart field<sup>[75]</sup>. Modifications to this general protocol include embedding in extracellular matrix<sup>[76]</sup>, mechanical<sup>[77]</sup> and electrical<sup>[78]</sup> stimulation of the immature cardiomyocytes. These modifications may influence the maturity of the iPSC-derived cardiomyocytes but do not increase the differentiation efficiency.

### **Direct reprogramming of cardiac fibroblasts into cardiomyocytes**

In equivalence to the iPSC generation, where pluripotency-associated transcription factors are expressed in terminally differentiated cells, direct conversion of fibroblasts into the cardiac lineage has been attempted<sup>[79-83]</sup>. Although no single master regulator of cardiomyogenesis has been identified to date, in analogy to the pioneering iPSC work of Yamanaka, Ieda *et al.*<sup>[79]</sup> used a reductionist approach to test fourteen different transcription factors to induce cardiomyogenic gene expression in fibroblasts, and found that the combination of cardiac-specific transcription factors GATA4, Mef2c and Tbx5 successfully reprograms murine cardiac fibroblasts directly into immature cardiomyocytes (Figure 1C)<sup>[79]</sup>. Although the efficiency of fibroblast reprogramming is rather low, with only about 30% of transduced cells display spontaneous contraction (about 6% of the total fibroblast population)<sup>[79,84]</sup>, the proof-of-concept that cardiac fibroblasts can be converted into cardiomyocytes by retroviral expression of GATA4, Mef2c and Tbx5 paved the way for *in vivo* delivery of these transcription factors.

Cardiac fibroblasts account for the majority of cells in the heart<sup>[85]</sup> and are therefore considered a viable cell population for reprogramming and restoration of cardiac function. Lineage tracing models<sup>[86,87]</sup>, wherein the cardiac fibroblasts are genetically tagged with a marker protein, were subjected to cardiac damage (either coronary ligation<sup>[86,87]</sup> or cryoinjury<sup>[84]</sup>) and treated with GATA4, Mef2c and TBX5 retroviruses. Up to three months



**Figure 1 Schematic of factors involved in cardiomyocyte differentiation in embryology, embryonic stem cell/induced-pluripotent stem cells and cardiac fibroblast reprogramming.** Factors that influence the progression through the five steps in cardiomyocyte differentiation and maturation: mesoderm differentiation, mesoderm specification, cardiac specification, cardiomyocyte differentiation and cardiomyocyte maturation in: A: Embryonic cardiomyocyte differentiation; B: Cardiomyocyte differentiation from ESC and iPSC using exogenous (growth) factors; C: In direct reprogramming of cardiac fibroblasts into cardiomyocytes. Transcription factors associated with each of the seven cell types during cardiomyocyte differentiation are presented in the boxes below. ESC: Embryonic stem cell; iPSC: Induced-pluripotent stem cells.

after treatment, cardiac transcription factor delivery to the heart reduces infarct sizes and attenuates cardiac dysfunction<sup>[84,86,87]</sup>, providing therapeutic proof-of-concept for *in vivo* cellular reprogramming, although efficiencies differ widely (1%-30%) between studies. Surprisingly, *in vivo* reprogrammed cardiomyocytes develop more characteristics (e.g., binucleation, assembled sarcomeres) of native cardiomyocytes as compared to their *in vitro* counterparts<sup>[87]</sup>. This improvement in reprogramming may be derived from microenvironmental clues, exposure to native extracellular matrix or mechanical forces during reprogramming and could provide clues for further improvements to the reprogramming protocols.

Additionally, it must be noted that reprogramming of cardiac fibroblasts into cardiomyocytes is efficient in mice, however the conversion of human fibroblasts into the cardiac lineage proves more difficult<sup>[80-83]</sup>. The expression of GATA4, Mef2c and TBX5 in human cardiac fibroblasts is insufficient for cardiac induction. The addition of MESP1 and Myocardin (MyoCD)<sup>[80]</sup>, MyoCD and MyoCD-related

transcription factor-A (Mrtf-a)<sup>[81]</sup>, MESP1 and estrogen-related receptor beta (ESRRB)<sup>[82]</sup>, or MESP1 and ETS2 (Figure 1C)<sup>[83]</sup> all increase reprogramming efficiency of human cardiac myocytes and underscore the need for further research in this area before a definite transcription factor cocktail can be put to the test in human trials.

Moreover, additional major impediments need to be addressed prior to clinical translation. Although issues such as tumorigenicity and retention encountered with iPSC and stem cell therapeutics, may be minimized by the direct conversion of cardiac fibroblasts into cardiomyocytes, heterogeneity in reprogramming efficacy, leading to the formation of immature cardiomyocytes that do not properly couple to adjacent cardiomyocytes, may cause fatal arrhythmias. Furthermore, current strategies rely on the use of viruses integrating randomly in the genome of cells that undergo reprogramming, which may elicit tumorigenic events. It is evident that *in vivo* reprogramming protocols without the use of viruses are essential before clinical translation can commence.

**MicroRNAs in cardiomyocytes reprogramming**

The use of microRNAs in reprogramming strategies may overcome some of the limitations encountered in reprogramming fibroblasts into cardiomyocytes using viruses, since chemically synthesized microRNA mimics are easily transfected into cells and exhibit low toxicity in animal models<sup>[88]</sup>. MicroRNAs are endogenous small (about 21-23 nucleotides in length) non-coding RNAs that function as repressors of gene translation<sup>[89,90]</sup>. Endogenously, microRNAs are encoded in the genome either in extronic regions that form microRNA gene clusters or intronically in both protein-coding and non-coding genes. Regardless of their genomic location, microRNA transcription is initiated by the RNA Polymerase II, resulting in the generation of a pri-microRNA<sup>[91]</sup>. Pri-microRNAs are processed into pre-microRNAs by the RNA-processing complex formed by Drosha and DGCR8 and exported from the nucleus by Exportin 5<sup>[92-94]</sup>. In the cytosol, pre-microRNAs undergo a second processing step, performed by the cytoplasmic endonuclease Dicer, which forms the mature microRNA duplex<sup>[95]</sup>. Next, one strand of the microRNA duplex is loaded in to the RNAi-induced silencing complex (RISC)<sup>[96]</sup> that utilizes the microRNA to identify and silence its target genes<sup>[97,98]</sup> (extensively reviewed in<sup>[90,99]</sup>). The effects of microRNAs on cardiomyogenesis might be powerful, as a single microRNA may target multiple signaling pathways simultaneously, a phenomenon known as multiplicity of microRNA targets<sup>[100]</sup>. Indeed, mice lacking the enzyme Dicer, which is essential to process microRNA precursors into their mature form<sup>[90]</sup>, die at day E12.5 from cardiac failure<sup>[101]</sup>.

Advances on iPSC and embryonic stem cell (ESC) differentiation into cardiomyocytes (described in sections "Induced pluripotent stem cells and cardiomyogenesis" and "Direct reprogramming of cardiac fibroblasts into cardiomyocytes") allowed Fu *et al.*<sup>[102]</sup> and Wilson *et al.*<sup>[103]</sup> to identify microRNAs essential to cardiomyogenesis. ESCs were differentiated using exogenous growth factors into beating cardiomyocytes and their "microRNA-ome" were analyzed on array platforms. Next, these microRNA signatures were compared to genuine fetal and adult cardiomyocytes and adult cardiac fibroblasts. MicroRNAs that are differentially expressed in ESC-derived cardiomyocytes and native ESC and that are not expressed by cardiac fibroblasts were identified as cardiomyogenic microRNAs or "cardiomiRs". Although the two "cardiomiR" screens show limited overlap (46%) when considering all differentially expressed microRNAs between native ESC and ESC-derived cardiomyocytes, the overlap is greatly increased when only microRNAs with increased abundance are compared (85%). This comparison allowed the identification of 7 "cardiomiRs" whose expression is increased during cardiomyogenesis (Table 1)<sup>[102,103]</sup>.

MicroRNA-1 and microRNA-133 are pivotal regulators of muscle differentiation<sup>[104]</sup> and loss of microRNA-1 or microRNA-133 results in embryonic lethality due

to several cardiac failures, including defective morphogenesis, electrical conduction and cardiomyocyte proliferation<sup>[101,105]</sup>. MicroRNA-1 and microRNA-133 are polycistronically transcribed from a duplicated locus in the human genome on chromosomes 18 and 20. MicroRNA-1 and microRNA-133 expression is under control of SRF and promotes cardiac mesoderm formation from naive ESCs<sup>[101,106]</sup>.

MicroRNA-1 is highly conserved among mammals and its expression in ESC shifts their gene expression profile toward that of cardiomyocytes<sup>[107,108]</sup>. The induction of the cardiomyogenic phenotype is mediated through several cooperative actions of microRNA-1. Inhibition of Notch signaling by microRNA-1-mediated direct repression of Dll1<sup>[106]</sup> and its downstream effector Hes1<sup>[109]</sup>, liberates the expression of the cardiac transcription factors GATA4, Nkx2.5 and Myogenin, whereas repression of the histone deacetylase HDAC4<sup>[104]</sup> liberates the cardiac transcription factor Mef2c (Figure 2). Additionally, repression of Hand2<sup>[110]</sup> and the smooth muscle transcription factor Myocardin<sup>[111]</sup> by microRNA-1 facilitate cardiomyocyte maturation through the repression of proliferation of mesenchymal progenitors and smooth muscle gene expression, respectively. Interestingly, the sole expression of microRNA-1 in cardiac fibroblasts is sufficient to induce cardiac reprogramming<sup>[112]</sup>.

MicroRNA-133 aids in cardiomyogenesis, however, in contrast to microRNA-1, its sole expression is insufficient to differentiate ESC into spontaneously contracting cells<sup>[106]</sup>. MicroRNA-133 promotes the actions of microRNA-1 through the suppression of smooth muscle specific genes in the myogenic precursors, thereby facilitating cardiomyocyte maturation. The direct repression of SRF<sup>[104,105]</sup> and the mesenchymal transcription factor Snai1<sup>[113]</sup> during cardiac differentiation of ESC or reprogramming of cardiac fibroblasts into cardiomyocytes reduces smooth muscle and fibroblast associated genes, which allows for the maturation of cardiomyocytes (Figure 2).

The cardiac myosin genes, which facilitate cardiac contraction, house three additional cardiomiRs, namely microRNA-499 and the microRNAs-208a and b that are encoded by the Myh7b and Myh6/7, respectively<sup>[114]</sup>. MicroRNA-499 facilitates expression of the cardiogenic transcription factor Mef2c<sup>[103]</sup> through a Wnt/ $\beta$ -Catenin-mediated mechanism (Figure 2)<sup>[115]</sup>, which remains to be elucidated but appears to involve repression of the transcription factor Sox6 and the transcription inhibitor Regulator of differentiation (Rod)-1<sup>[116]</sup>.

MicroRNA-208a and microRNA-208b are involved in cardiomyocyte maturation and orchestrate the expression of myosin fibers in the heart. In the adult heart, the abundance of myosin fibers are alpha fibers (or fast fibers) whereas in the developing heart the majority of myosin fibers are beta fibers (or slow fibers). The gene encoding alpha-MHC encodes a cardiac-specific microRNA (microRNA-208a) that targets the repressors of beta-MHC Sox6, Pur $\beta$  and SP3<sup>[114,117]</sup>. MicroRNA-208a-mediated repression of these inhibitors thus facilitates the expression

**Table 1** MicroRNAs involved in cardiomyocytes differentiation

microRNA	Targets	Effect on cardiomyogenesis (mechanism)	Used in reprogramming	Ref.
Increased during cardiomyogenesis				
1	Dll1 (Notch)	↑ CM Differentiation (↑ Nkx2.5 and Myogenin)	+	[102-104,106,109-111]
	Hes1 (Notch)	↑ CM Differentiation (↑ Nkx2.5 and GATA4)		
	Hand2	↓ CM Proliferation		
	HDAC4	↑ CM Differentiation (↑ Mef2c)		
30a-e	Myocardin	↑ CM Maturation (↓ SMC phenotype)	-	[102,103,120]
	Snai2	↑ CM Differentiation (↓ mesenchymal genes)		
	Smarcd2	↑ CM Differentiation (↓ mesenchymal genes)		
133a-b	Tnrc6a	↑ CM Maturation (↓ miR-206: ↓ SMC Phenotype)	+	[102-105,113]
	Snai1	↑ CM Differentiation (↓ mesenchymal genes)		
	SRF	↓ CM Proliferation		
181a-d	Cyclin D2	↓ CM Proliferation	-	[103,175]
	?	↑ CM Proliferation		
195	Cyclin D1	↓ CM Proliferation	-	[102,103,119,176]
	HMG A	↓ CM Differentiation (↓ Nkx2.5)		
208b	Myostatin	↑ CM Proliferation	+	[103,114,117,118]
	Sox6, Purβ	↑ CM Maturation (↑ beta-Myosin Heavy Chain)		
	THRAP1	↑ CM Maturation (↑ beta-Myosin Heavy Chain)		
499-5p	? (↑ Wnt)	↑ CM Differentiation (↑ Nkx2.5, Mef2c and GATA4)	+	[102,103,115]
Decreased during cardiomyogenesis				
31	?	?	-	[103]
34c-3p	?	?	-	[103]
151-3p	ATP2a2	↓ CM Maturation (↓ beta-Myosin Heavy Chain)	-	[103,177]
221	?	?	-	[103]
222	?	?	-	[103]

ATP2a2: Sarcoplasmic reticulum Ca<sup>2+</sup> ATPase 2; CM: Cardiomyocyte; Dll1: Delta-like 1; GATA4: GATA Binding Protein 4; Hand2: Heart and neural crest derivatives expressed 2; HDAC4: Histone deacetylase 4; Mef: Myocyte enhancer factor; miR: MicroRNA; Nkx2.5: NK2 homeobox 5; Purβ: Purine-rich element binding protein beta; Smarcd2: SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily d member 2; SMC: Smooth muscle cell; Snai: Snail family zinc finger; Sox6: Sex determining region Y-box 6; SRF: Serum response factor; THRAP1: Thyroid hormone receptor associated protein 1; Tnrc6a: Trinucleotide repeat-containing gene 6A; Wnt: Wingless-type MMTV integration site family.

of beta-MHC by the developing cardiomyocyte. Moreover, the beta-MHC gene (encoded by Myh7) contains the related microRNA-208b. Expression of beta-MHC, induced by microRNA-208a, thus induces the expression of microRNA-208b that provides a feed forward mechanism that maintains the expression of beta-MHC<sup>[114,117]</sup>. Additionally, microRNA-208 targets myostatin<sup>[118]</sup>, a known inhibitor of cardiac progenitor cell proliferation, which reduces the inhibitory effect of myostatin on cardiac progenitor cell propagation.

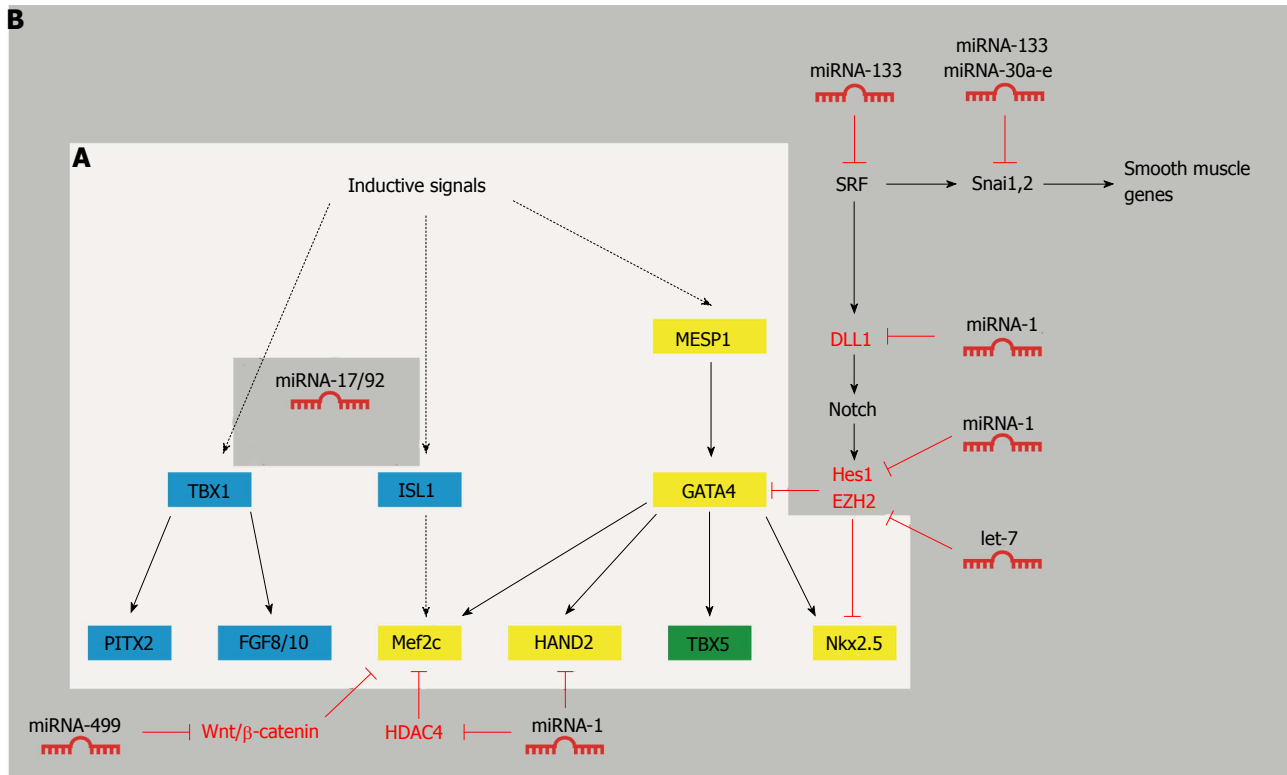
The other cardiomiRs, microRNA-30a-e, microRNA-181a and microRNA-195, are less well characterized. Overexpression of microRNA181a in ESC increased proliferation of differentiated cardiomyocytes through unidentified mechanisms<sup>[103]</sup>, whereas the expression of microRNA-195 decreases cardiomyocyte proliferation through the inhibition of cell cycle regulator cyclin D1<sup>[119]</sup>. MicroRNA-30a-e regulate cardiomyogenesis by targeting Snai2 and Smarcd2<sup>[120]</sup>, two known inducers of mesenchymal gene expression. Their inhibition by microRNA-30a-e thus favors maturation of the cardiac phenotype over the maintenance of the mesenchymal phenotype (Figure 2).

The non-cardiac restricted microRNAs let-7, microRNA-99, and the microRNA-17/92 cluster also facilitate cardiomyogenesis<sup>[121,122]</sup>. MicroRNA-99 facilitates the transition from mesenchymal precursor to cardiac pro-

genitor cells by the Smarca5-mediated repression of TGFβ signaling<sup>[121]</sup>. Additionally, let-7 induces the expression of cardiogenic transcription factors GATA4, Mef2c, Nkx2.5 and Tbx5 by the repression of EZH2, a histone methyltransferase that epigenetically silences these genes in mesenchymal precursors<sup>[121]</sup>. The microRNA-17/92 cluster subsequently facilitates ventricular myocyte generation from the first heart field. The microRNA-17/92 cluster targets Tbx1 and ISL1, the master transcription factors for second heart field development, thereby favoring differentiation of the first heart field (Figure 2)<sup>[122]</sup>.

Notably, Jayawardena *et al.*<sup>[112]</sup> used the most abundantly expressed cardiomiRs, *i.e.*, microRNA-1, 133, 208 and 499, to reprogram cardiac fibroblasts directly into cardiomyocytes. Transient expression of these four microRNAs *in vitro* generated mature cardiomyocytes that spontaneous beat, albeit at low efficiency (1.5%-7.7% of all fibroblasts). The reprogramming efficiency could be increased to about 28% by the addition of a Janus Kinase inhibitor. Moreover, the four microRNAs reprogram cardiac fibroblasts *in vivo* in a mouse model of MI, providing therapeutic proof-of-concept for the microRNA-mediated reprogramming of fibroblasts to ameliorate damage following MI<sup>[112]</sup>.

Thus, advances in iPSC biology and cardiac reprogramming have identified exogenous growth factors and endogenous transcription factors that drive cardio-



**Figure 2** The complex web of transcription factors in cardiac specification and their regulation by microRNAs. A: Crosstalk between transcription factors involved in the formation of the first and second heart field (light grey box). MESP1, GATA4, Mef2c, HAND2 and Nkx2.5 are central transcription factors in the first and second heart field (yellow). TBX5 is only expressed in the first heart field (green). ISL1 and TBX1 are expressed in the second heart field (blue); B: MicroRNA-mediated regulation of cardiac transcription factors during cardiomyocyte differentiation (dark grey box).

myogenesis, and have provided novel therapeutic approaches for the amelioration of damage from MI by the therapeutic expression of cardiac transcription factors. Moreover, these recent advances have provided a platform to study cardiogenesis in more detail. MicroRNAs can similarly induce fibroblast reprogramming into cardiomyocytes and can be delivered to the cardiac tissue without the use of randomly integrating viruses, and may thus improve safety of reprogramming in a clinical context. The question that remains is how to deliver these microRNAs safely and efficiently to the site of damage and cell type of choice to perform their function. This question is addressed in the next section.

## TARGETING MICRORNAS FOR CARDIAC REGENERATION

MicroRNA-mediated reprogramming of cardiac fibroblasts *in vivo* requires advanced delivery strategies. In the section below, we will describe general and targeted drug delivery strategies and discuss possibilities to specifically target microRNAs to cardiac fibroblasts.

A range of chemical modifications to enhance cellular uptake of microRNAs have been developed recently. Additionally, particulate drug delivery systems, including liposomes, polymeric micelles, polymeric vesicles, polymeric nanoparticles (NPs), and dendrimers have been investigated

for targeted delivery of drugs<sup>[123]</sup> including microRNAs in a variety of disease models outside the cardiac field and with varying degrees of success. Current advances in targeted drug delivery from these fields provide a solid basis for the burgeoning field of cardiac drug delivery.

In general, the prime reasons for targeted drug delivery is the modulation of the drug's pharmacokinetics, the avoidance of toxicity of the drug in non-diseased tissue or cells and to alter the apparent physicochemical characteristics of a drug by making use of a carrier. An ideal drug delivery vehicle needs to be non-toxic, biocompatible, non-immunogenic and biodegradable<sup>[123]</sup>. Particle sizes of the drug delivery system have a preferred size between 10 and 200 nm. The lower limit is determined by the glomerular permselectivity in the kidney that captures particles below 10 nm and rapidly clears them through renal filtration<sup>[124]</sup>, whereas the upper limit is set by clearance through the reticuloendothelial system and uptake by the spleen and liver<sup>[125]</sup>. Additionally, surface charge and chemistry are key parameters in the design of drug delivery systems. Systems with a positive surface charge may electrostatically interact with the cell membrane or its associated negatively-charged proteoglycans and subsequently internalized through endocytosis<sup>[126,127]</sup>. Negatively charged systems are preferentially recognized by monocytes/macrophages and internalized *via* the caveolar or clathrin endocytic



pathways<sup>[128-130]</sup>.

### Classes of drug targeting systems

Cardiac microRNA delivery poses huge challenges as unmodified microRNAs are rapidly degraded by systemic nucleases, secreted through renal filtration and phagocytosed by monocytes/macrophages, limiting their ability to reach their target cell<sup>[131,132]</sup>. A range of chemical modifications to enhance microRNA stability and cell permeability, including 2'-O-methyl modifications, locked nucleic acid chemistry, the conjunction of small molecules or cell penetrating peptides (Figure 3)<sup>[133]</sup> and peptide nucleic acids have been developed that increase therapeutic efficacy of microRNA therapies (reviewed in<sup>[131,132,134]</sup>), albeit they do not add cell or organ specificity. Hence, the development of targeted delivery systems for myocardial microRNA delivery is of the utmost importance.

As described above, various particulate drug delivery systems have been developed for cell and organ specific targeted delivery of drugs (Table 2). Liposomes<sup>[135]</sup>, the related polymerosomes<sup>[136]</sup> and polymeric micelles<sup>[137]</sup> are a system of lipids or polymers that self-assemble into spherical structures with an aqueous core that can hold the microRNA payload<sup>[123,138,139]</sup>. Single or multiple types of lipids and polymers can be combined to generate liposomes, polymerosomes and polymeric micelles, which allows for additional flexibility in designing the physical and chemical properties of the drug delivery vehicle<sup>[140]</sup>. Liposomes and polymerosomes are internalized *via* endocytosis and destined for lysosomal degradation<sup>[141]</sup>. Endosomal escape from the liposomal content occurs through pH-sensitive fusion of the liposome and the endosomal membrane, resulting in drug release in the cytoplasm<sup>[142]</sup>. Although liposomes have a long history in drug delivery in basic and clinical medicine with FDA approval, some concerns regarding their clinical applicability are reported, such as the immunogenicity and toxicity of certain cationic lipid particles<sup>[143,144]</sup>. Regardless, liposomes and polymerosomes are highly promising for future clinical microRNA delivery.

Microbubbles (Table 2) are a second class of drug delivery systems that can be used for microRNA delivery *in vivo* and represent a specialized form of liposome that is sensitive to external clues, such as high powered ultrasound (described below). Microbubbles are gas-filled lipid spheres of various diameters (10-1000 nm)<sup>[145,146]</sup>. Cationic microbubbles can form complexes with anionic drugs, such as microRNAs, by electrostatic interaction<sup>[147,148]</sup>. The sensitivity of microbubbles to ultrasound, which destroys the microbubble, delivers the payload directly to its environment<sup>[145,147]</sup>. Hence, for efficient targeting of microRNAs into the tissue, additional modifications to the microRNA (described above) may be necessary to increase cellular uptake by the target cells<sup>[131,132]</sup>.

Nanoparticles and nanospheres (Table 2) are a third class of drug delivery vehicles that consist of lipids or block co-polymers, respectively<sup>[149,150]</sup>. Nanoparticles and nanospheres are commonly produced using emulsion or

precipitation techniques which form solid structures typically 10-100 nm in size<sup>[139,151]</sup>. Changing the composition of the block co-polymers that build up the nanoparticle allows tuning drug delivery rates<sup>[128]</sup>, as drug delivery occurs through diffusion of the drug through the solid nanoparticle or *via* biodegradation of the particle<sup>[139,150,151]</sup>. The solid nature of nanoparticles confers great stability advantages *in vivo* and provides slow-release properties. Therefore, nanoparticles are more efficient in delivering proteinaceous and small molecule drugs than microRNAs, as cellular uptake and degradation properties are inferior to the delivery efficiency of liposomes and polymeric micelles.

Dendrimers (Table 2), represent the last class of drug delivery systems are highly branched macromolecules with a controlled repeated branching around a central core that forms a small (1-10 nm), spherical and highly dense nanocarrier that holds many cavities that may contain drugs<sup>[152-155]</sup>. Targeting efficacy and extravasation of dendrimers can be controlled by their size, molecular weight and the functional groups present on their surface<sup>[153,156]</sup>.

### Passive drug targeting

Targeting of drug delivery systems can be achieved *via* two general concepts, namely passive or active targeting. Passive targeting is based on the so-called enhanced permeability and retention effect (EPR)<sup>[157]</sup>. At sites of inflammation, the integrity of the endothelial lining is often compromised, resulting in a defective or leaky vasculature. Circulating drug delivery systems are able to pass these leaky vessels and can thus enter the inflamed tissue. Hence, colloidal drug delivery systems passively accumulate at sites of inflammation, such as the infarcted heart<sup>[158,159]</sup>. An important prerequisite for passive targeting is a relatively long (hours-days) circulation time of the drug delivery system since extravasation occurs only by chance. Additionally, if passive drug delivery is to be used to target cardiac fibroblasts, detection by monocytes/macrophages needs to be avoided in order to reduce rapid clearance of the drug carriers from the cardiac tissue by these phagocytic cells.

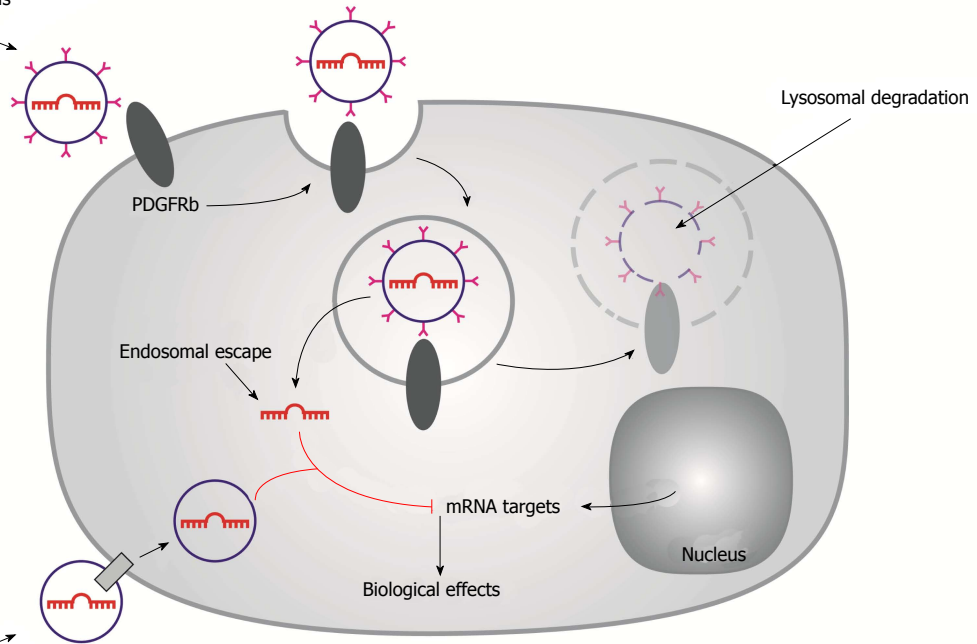
### Active drug targeting

Active targeting drug delivery systems are equipped with specific targeting devices that recognize or have affinity for certain cells. Although the recent identification of biomarkers that are differentially expressed in the diseased cardiac tissue has advanced the development of experimental therapies that can be employed for the targeted delivery of microRNAs, there is a huge challenge for active-targeting strategies to find specific target molecules for a certain disease process and to test its effectiveness in drug delivery therapies.

Active drug targeting of microRNAs to cardiac fibroblasts may be achieved in two distinct manners, depending on the interaction of the targeting device and the cell. Either the drug delivery system can be internalized by the cell where it releases the microRNAs subsequently (epitope targeted drug delivery, Figure 3), or the drug delivery system can

**B**

Epitope-targeted intracellular delivery *via* antibody/peptide-mediated binding and receptor-mediated endocytosis

**A**

CPP-mediated intracellular delivery *via* electrostatic interactions or by macropinocytosis

**Figure 3 Schematic of passive and active targeted drug delivery systems for microRNA delivery.** A: Passive targeting by cell-penetrating peptide-coated nanoparticles are internalized by receptor-mediated endocytosis; B: Active targeting by PDGFRb-targeted liposomes. Liposomes interact with cell surface receptors (PDGFRb) and internalized *via* receptor-mediated endocytosis. The endocytotic vesicles fuse to form early endosomes which ultimately become part of the lysosomes, where proteins and nucleic acids are degraded by acid hydrolases. To achieve target gene silencing, microRNAs need to be released from the liposome and escape from the endosomes into the cytoplasm, where the microRNA directs the cleavage of target mRNAs.

bind to the cell and act as a drug release depot that can be activated at the diseased site (inducible targeted drug delivery). Although targeted drug delivery approaches have been pursued cardiovascular disease, data on the delivery of microRNA to fibroblasts are scarce<sup>[160]</sup>.

Epitope targeting of drug delivery systems is a rapidly evolving field in cardiac drug delivery and was shown by Dasa *et al.*<sup>[161]</sup>, who used *in vivo* phage display methods to identify peptide sequences specific for cardiac endothelial cells, cardiomyocytes and myofibroblasts<sup>[161]</sup>. These peptide sequences were conjugated to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) liposomes using polyethylene glycol (PEG). The obtained peptide-PEG-DSPE was loaded with the small molecule inhibitor of PARP-1 activation AZ7379. Although the publication only shows proof-of-concept data in efficiently (> 90%) reducing PARP-1 activation in cardiomyocytes<sup>[161]</sup>, it is tempting to assume that the targeted delivery of small molecule inhibitors or microRNAs to cardiac fibroblasts would be equally efficient as antibody-functionalized liposomes are highly efficient in delivering non-coding RNAs to vascular cells<sup>[162]</sup>.

Inducible targeted drug delivery uses drug delivery systems that are sensitive to their environment, *e.g.*,

heat<sup>[163]</sup>, light<sup>[164]</sup>, pH<sup>[165]</sup> or ultrasound<sup>[145]</sup>, that will release their payload by the indicated external trigger if present at the disease site. Ultrasound-sensitive microbubbles (described in section "Classes of drug targeting systems") have been used for cardiac microRNA delivery with high efficiency, although reports on targeting of cardiac fibroblast remain scarce. Gill *et al.*<sup>[166]</sup> used liposomal ultrasound-sensitive microbubbles to deliver microRNA-133 into HL1 cardiomyocytes *in vitro*. Both encapsulated (inside the microbubble) and complexed (on the outer shell of the microbubble) microRNA formulations efficiently delivered the microRNA-133 mimic, without affecting cardiomyocyte viability, indicating that, although encapsulation increases the microRNA-carrying capacity of microRNAs, complexation strategies do not affect the ability of microbubbles to deliver microRNAs<sup>[166]</sup>. Using a similar approach, Liu *et al.*<sup>[167]</sup> delivered microRNA-21 mimics into the hearts of swine without inflicting cardiac damage. Myocardial microRNA-21 expression levels were efficiently elevated in hearts treated with the microRNA-microbubble complex that received ultrasound activation compared to control conditions. Interestingly, the transfection efficiency of microRNA-microbubble complexes that were administered by intracoronary

**Table 2** Characteristics of particulate drug delivery systems

Carrier	Size range (nm)	Preparation method	Advantages for drug delivery	Disadvantages for drug delivery	Ref.
Liposomes and polymerosomes	10-2000	Self-assembly in aqueous solutions	High drug-carrying capacity Good for hydrophobic and hydrophilic drugs Surface functionalization possible Simple preparation	Batch-to-batch variability Difficulties in sterilization	[123,135,138,141,143,150,161,178]
Microbubbles	10-1000	Various depending on type	Surface functionalization possible	Not good for hydrophobic drugs Low drug-carrying capacity	[145-148,166,168,179]
Polymeric micelles	10-100	Direct organization or controlled aggregation in solvent	Long blood circulation time Surface functionalization possible Simple preparation	Not good for hydrophobic drugs Low drug-carrying capacity	[123,136,137,155,158]
Nanoparticles and nanospheres	10-100	Nanoparticles: Polymerization of monomers by emulsion Nanospheres: Interfacial polymerization and phase inversion with polymeric emulsions	Shape, size and mechanical properties tunable Possibility for controlled release	Toxicity of residual chemicals from preparation process Limited cellular uptake and degradation	[123,126,128,139,150,151,155,180]
Dendrimeres	1-10	Convergent or divergent synthesis	High functionalized surface	Difficult preparation process Toxicity	[123,154,156]

injection was higher compared to systemic administration. These results indicate that the application site may affect therapeutic outcome and should be considered in clinical translation<sup>[167]</sup>. Kwekkeboom *et al.*<sup>[168]</sup> delivered microRNA mimics and anti-miRs to the cardiac endothelium using a combination of microbubbles and ultrasound activation. Notably, delivery of anti-miRs (cholesterol-conjugates anti-miRs<sup>[169]</sup>) had a higher transfection efficacy compared to control anti-miRs implying that cellular uptake of delivered microRNAs is still highly dependent on their physicochemical properties<sup>[168]</sup>.

The concept of cardiac fibroblast reprogramming into cardiomyocytes holds great therapeutic value for the treatment of MI and its associated cardiac failure. However, fibroblast reprogramming is a recent concept and although current studies have provided proof-of-concept, focus on its clinical translation is limited. A range of drug delivery systems are reported for the delivery of microRNAs outside the cardiac field (reviewed in<sup>[149,170]</sup>) that can easily be transposed onto the reprogramming paradigm. As this field evolves, clinically relevant delivery approaches and suitable targeting epitopes for fibroblast-specific drug delivery will be explored as will their clinical effectiveness.

## SUMMARY AND FUTURE PERSPECTIVES

Deciphering the signaling pathways that underlie cardiac development has led to new therapeutic strategies that trigger cardiac regeneration. Vast progress is made in promoting cardiomyocyte proliferation and in direct reprogramming of cardiac fibroblasts into cardiomyocytes, which offer new perspectives on the possibility to advance

from treating cardiac disease to curing cardiac disease. Additionally, advances in drug delivery have yielded a plethora of drug delivery systems that can selectively deliver therapeutic agents to relevant cell populations at the site of damage. However, many challenges remain to be addressed before clinical translation can commence.

During a MI, billions of cardiomyocytes are lost and although current reprogramming strategies using exogenous transcription factors or microRNAs have emerged as potential therapeutic strategies, they are vastly inefficient. Thus, to enhance cardiac regeneration it will be pivotal to develop procedures that increase the yield and efficiency of generating *de novo* cardiomyocytes. Advancing our mechanistic understanding of the reprogramming process, including the directed differentiation of subtypes of cardiomyocyte (*i.e.*, ventricular, atrial or nodal), is key to the success of this promising therapy, however when subtype specification occurs during development and how these processes are regulated remain elusive. Moreover, *in vivo* efficacy and safety in large animals needs to be addressed before clinical translation can commence.

Additionally, it has been reported that the delivery of immature or heterogeneous populations of cardiomyocyte derived from progenitor cells or iPSC can lead to arrhythmias<sup>[171,172]</sup>. Currently, reprogrammed cardiomyocytes are immature and phenotypical heterogeneous, which could contribute to arrhythmogenesis. Hence, it is crucial to promote maturation and integration of reprogrammed cardiomyocytes. Yet, our current understanding of these processes is limited and further research into these processes is highly warranted.

While an intense research focus has been on the

development of new drug delivery systems, efforts to identify epitopes that are differentially expressed in diseased cardiac tissue has received little attention, as the field of cardiac drug delivery is still in its infancy. The identification of target epitopes that discriminate between fibroblasts in the affected vs the healthy tissue is pivotal to clinical translation of targeted delivery of microRNAs using liposomes, polymeric micelles or microbubbles. In addition, the heart contains a large population of fibroblasts that are necessary for its normal function<sup>[173,174]</sup>. Therefore, it may be detrimental to the cardiac function to target all fibroblasts for reprogramming. Drug delivery systems may need to be comprised of multiple targeting mechanisms, *e.g.*, ultrasound sensitive and fibroblast targeted, if a sufficiently selective molecular targeting epitope cannot be identified that distinguishes fibroblasts in the scar tissue from those elsewhere in the heart.

In summary, MI results in a massive loss of cardiomyocytes that are replaced by scar tissue. Endogenous repair mechanisms are insufficient to efficiently regenerate the lost myocardial tissue and therapeutic approaches to induce cardiomyocyte proliferation using growth factors are relatively ineffective. Advances in our basic understanding of cardiomyogenesis obtained from embryology and iPSC biology has led to the identification of factors that drive cardiomyogenesis, and have provided a novel therapeutic approach for the amelioration of damage from MI through the therapeutic delivery of microRNAs that reprogram cardiac fibroblasts into cardiomyocytes. These microRNAs can be delivered to the cardiac fibroblasts using advanced drug delivery systems. Although there are many challenges ahead in advancing this emerging technology, the opportunities and potential clinical benefits are substantial and we are confident that the field will continue to push this technology further in the years to come.

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