



# Cardiac regenerative medicine: At the crossroad of microRNA function and biotechnology



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## ABSTRACT

There is an urgent need to develop new therapeutic strategies to stimulate cardiac repair after damage, such as myocardial infarction. Already for more than a century scientist are intrigued by studying the regenerative capacity of the heart. While moving away from the old classification of the heart as a post-mitotic organ, and being inspired by the stem cell research in other scientific fields, mainly three different strategies arose in order to develop regenerative medicine, namely; the use of cardiac stem cells, reprogramming of fibroblasts into cardiomyocytes or direct stimulation of endogenous cardiomyocyte proliferation. MicroRNAs, known to play a role in orchestrating cell fate processes such as proliferation, differentiation and reprogramming, gained a lot of attention in this context the latest years. Indeed, several research groups have independently demonstrated that microRNA-based therapy shows promising results to induce heart tissue regeneration and improve cardiac pump function after myocardial injury. Nowadays, a whole new biotechnology field has been unveiled to investigate the possibilities for efficient, safe and specific delivery of microRNAs towards the heart.

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## 1. Introduction

Heart failure remains the primary cause of hospitalization and mortality in Europe, United States and in the developed nations [1,2]. Currently, the only therapeutic option for end-stage heart failure is transplantation, but since the number of heart failure patients is rising dramatically, there is a lack of hearts available for this procedure. Therefore, there is a dire need to develop a therapeutic approach coping with the damage after cardiac injury.

Already within a time-frame of a few hours after ischemic damage, a loss of 25% of the total amount of cardiomyocytes present in the left ventricle could be observed [3]. Aiming at maintenance of the ventricular structural integrity, a reparative process is initiated to rebuild the damaged myocardium, which unfortunately only results in formation of a scar [4]. Since this scar is non-functional, cardiac muscle contractility will decrease and heart failure will develop. Taken this into account, regeneration of the heart muscle is a main goal for many researchers within the field and during the last decade new approaches have been developed to identify the (cell) source responsible for the newly formed

cardiomyocytes in the adult heart. The applications range from *ex vivo* cell therapy strategies based on the direct delivery of regenerative cells isolated from different sources (e.g. induced pluripotent stem cells, embryonic stem cells, bone marrow, liposuction or heart biopsies), tissue engineering implantations of functional patches or *in vivo* stimulation of resident cell sources by delivery of reprogramming factors or inducers of cardiomyocyte cell-cycle re-entry [5–11].

From the molecular point of view, many scientists have turned their focus on the role of microRNAs (miRNAs, miRs) in order to unravel unknown and overlapping signaling cascades necessary for cardiac regeneration [12,13]. MiRNAs, small non-coding RNA molecules of about 18–22 nucleotides long and highly conserved among species, have been shown to fine-tune gene regulatory networks via post-transcriptional regulation of different mRNAs, functioning in similar pathways. This molecular biological approach, combined with the development of smart technology for specific and controlled delivery will open the door to bring a novel and efficient therapeutic strategy to the heart failure patient.

## 2. The regenerative capacity of the heart muscle: an historical perspective

Already since the 19th century scientists have been intrigued by

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the regenerative capacity of the human heart. An intense and controversial discussion about whether increase of the heart mass in the postnatal phase was due to hypertrophic growth or cardiomyocyte proliferation has been going on for more than 100 years. Back in the late 1800s, Goldenberg was one of the first to report that although longitudinal splitting of cardiomyocytes might take place, growth of the heart muscle was mostly due to hypertrophy of muscle fibers [14]. In that period, other scientists confirmed there was a lack of evidence of myocardial mitotic figures and thus they concluded that the increase of the heart mass after birth was largely achieved by hypertrophic growth rather than hyperplasia. In the early 1900s, researchers start to investigate the relationship between the amount of muscle fibers and nuclei. In this context, human hearts were studied and a comparison between normal, hypertrophic and atrophic status was made. Based on these findings, Karsner et al. published that enlargement of the heart in the hypertrophic state was due to hypertrophy of the muscle fibers without an actual increase in the number of fibers [15]. On the other hand, decreased heart size in atrophy was caused by both a reduction in muscle fiber size and fiber number, while the number of nuclei was clearly increased. Back then it was concluded that there might have been an attempt for the heart muscle to regenerate, however no mitotic figures were observed [15]. Interestingly, when comparing the myocardium of children versus adults, it became clear that mitotic figures in the hearts of children were present, while this was difficult to observe in the adult hearts [16]. These data indicated that cardiomyocytes were able to proliferate during the early postnatal phase but lose their capacity to proliferate at later ages. In the 60s and 70s, evaluation of DNA synthesis in cardiomyocyte nuclei was shown to be negligible and people started to classify the human heart as a post-mitotic organ, incapable to self-regenerate [17,18]. However, the atomic weapon testing during the Cold War followed by termination of these tests in 1963 led to the idea to use the measure of carbon-14 (<sup>14</sup>C) in genomic DNA of human cardiomyocytes to determine when these cells were born. Since the concentration of <sup>14</sup>C in the human body mirrors that in the atmosphere at any given time, and given the fact that <sup>14</sup>C incorporates in our DNA, these measurements could be used to retrospectively birth date cells in humans. The nuclear bombing tests led to a drastic increase in <sup>14</sup>C concentrations in the atmosphere, and using mass spectrometry on extracted DNA coming from humans born before this atmospheric <sup>14</sup>C peak, it became clear that cardiomyocyte DNA was synthesized even many years after birth. This geopolitical pulse-chase experiment led to the conclusion that in young adults, cardiomyocyte renewal was about 1% per year, while in elderly this was about 0.45% [19]. At that time, the self-repair capacity of the human heart after injury was not investigated yet, however given the fact that coronary artery disease leads to heart failure in patients, it was clear that even if the cardiomyocytes were able to proliferate, this was not sufficient to compensate the tremendous loss of cardiomyocytes after ischemic injury.

### 3. MicroRNAs: directors of cell fate

Development of regenerative medicine for the heart muscle could be addressed via several approaches targeting cell fate processes such as proliferation, differentiation and reprogramming. Interestingly, miRNA-dependent regulatory networks seem to be able to affect all the biological processes implicated in cardiac regeneration (Fig. 1). For example, miR-1 knockout in *Drosophila* has been shown to alter myofiber structure and muscle growth, causing larval paralysis and death [20]. Furthermore, the muscle-specific microRNAs, miR-1 and miR-133, appear to be potent repressors of non-muscle gene expression and cell fate in mouse and

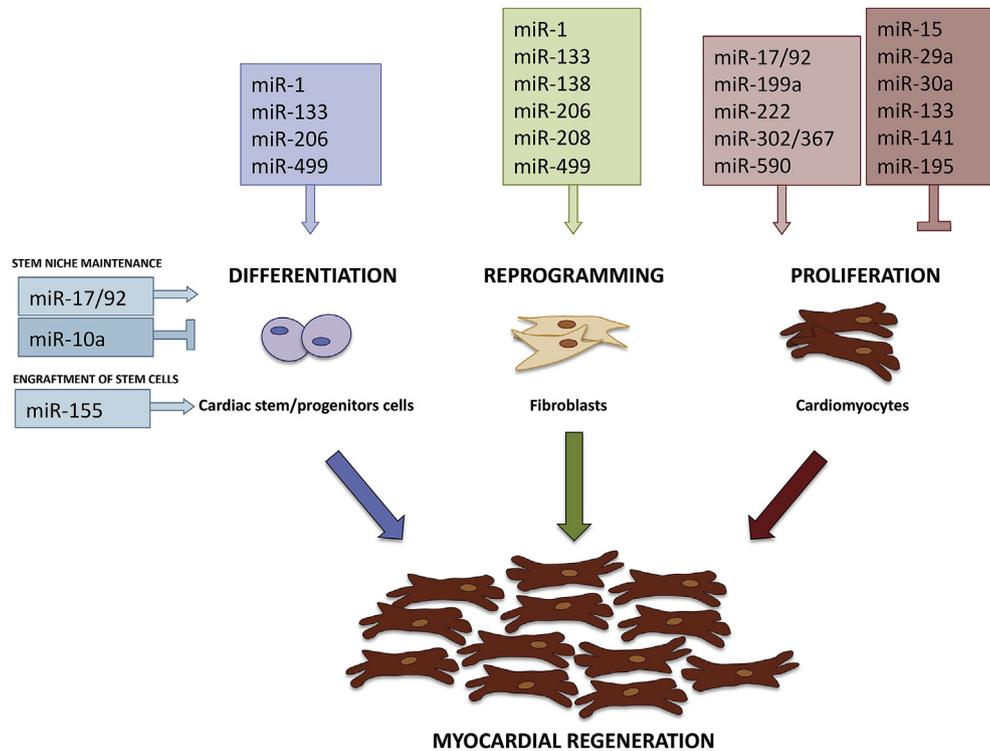
human pluripotent embryonic stem (ES) cells differentiation. Under the control of cardiogenic and myogenic transcription regulators, such as the serum response factor (SRF), miR-1 and miR-133 promote mesodermal differentiation from ES cells but act partially in opposition on further differentiation to the cardiac lineage [21].

Furthermore, researchers have been describing the molecular characterization of the postnatal maturing cardiomyocytes, isolated from mice at postnatal day 1 up to 1 year old [22]. Zhang et al. reported decreased expression of several cyclins, cyclin-dependent kinases (CDKs) and positive cell cycle modulators and increased expression on CDK inhibitors and negative cell cycle modulators. These data were associated with the upregulation of miR-29a, miR-30a and miR-141. Moreover, downregulation of these miRNAs in neonatal rat cardiomyocytes promoted cell cycle re-entry, which was partly explained by the induction of Cyclin A2 (CCNA2) expression. Cao et al. reported similar observations in rats [23]. Next, also miR-133 has been demonstrated to play a role in cardiomyocyte cell cycling. The levels of miR-133 are strongly downregulated during the process of heart regeneration in zebrafish. Upon miR-133 overexpression, several cell cycling genes are downregulated and the reparative capacity of the heart muscle is reduced. In mice, the double knockout (dKO) of miR-133a-1 and miR-133a-2 causes lethal ventricular-septal defects in approximately half of double-mutant embryos or neonates. Double-mutant mice that did survive up to adulthood developed severe dilated cardiomyopathy and heart failure due to ectopic smooth muscle genes expression and aberrant cardiomyocyte proliferation [24]. The direct downstream targets of miR-133a, namely SRF and Cyclin D2 (CCND2), showed to be upregulated in these dKO mice, which could (at least partly) explain the observed phenotype. These findings reveal an essential role for miR-133a-1 and miR-133a-2 in orchestrating cardiac development and cardiomyocyte cell fate and point to these miRNAs as critical components of an SRF-dependent myogenic transcriptional circuit. Whether manipulation of these miRNAs, involved in cell differentiation and cardiomyocyte cell cycling, could help to regenerate the adult injured heart needs further investigation. However, the indications that these miRNAs play a role in cardiac cell fate determination, suggests their involvement in biological processes important for cardiac regeneration. In light of these findings, it became clear that it would be a merit to develop tools to target miRNAs for tissue regeneration purposes. Consequently, the last decade there has been a considerable increase in the number of patent applications filed. Although most miRNA-based therapeutics are still in pre-clinical stadium for various diseases, miravirsin, a miR-122 agonist inhibiting hepatitis C virus infection (HCV), seems to be promising and is the first miRNA-based therapeutics to reach clinical phase IIb [25]. Miravirsin dosing in chronic hepatitis C patients results in decreased microRNA-122 levels without affecting other microRNAs in plasma [26].

Here we further review the different approaches to generate cardiac regenerative applications in which the role of microRNAs have been studied, and showing *in vivo* proof of improved cardiac pump function after myocardial injury.

### 4. The role of cardiac stem cells in cardiac regeneration

While moving away from the old classification of the heart as a post-mitotic organ, and being inspired by the stem cell research in other scientific fields, a new challenge in the field arose to identify the stem cell population present in the heart. The concept that stem cells could be a source for cardiomyocyte renewal arose from initial research which demonstrated that administration of bone-marrow-derived c-Kit-positive (c-Kit<sup>+</sup>) hematopoietic stem cells led to restoration of the heart muscle after a myocardial infarction (MI) [27].



**Fig. 1. The role of miRNAs in three strategies leading to myocardial regeneration, namely** by regulating cardiac stem/progenitors cells differentiation, reprogramming of fibroblasts into a cardiomyocyte phenotype and stimulation of pre-existing cardiomyocyte proliferation.

Next, several investigators came up with different approaches to identify resident Cardiac Stem Cells/Progenitor Cells (CSCs, CPCs) in the postnatal heart. These methods varied from the ability to form multicellular spheres or to efflux a fluorescent dye towards the expression of different surface markers, such as cardiac KIT Proto-Oncogene Receptor Tyrosine Kinase (c-Kit), Stem cells antigen-1 (Sca-1), Multi-drug resistance gene 1 (Mdr-1) and islet-1 (Isl-1) [28–31]. In mouse hearts, Sca-1-positive (Sca-1+) CPCs have been demonstrated to be implicated in cardiomyocyte formation and showed almost 100% co-expression with c-Kit [32–34]. However, In contrast to mouse hearts, Sca-1+ CPCs are not present in large mammals and humans. As a consequence, these data are not translatable to humans and so far CPCs showing expression of the tyrosine kinase receptor c-Kit are the most widely studied and characterized. During development, cardiomyocytes have been shown to express c-Kit, however in the adult human, c-Kit is only expressed in telocytes, thymic epithelium and mature circulating cells (e.g. hematopoietic cells and mast cells) [35]. Nevertheless, in the perivascular compartment of the adult heart, a particular small round shaped cell type has been identified to express c-Kit as well. Moreover, in the adult failing heart these c-Kit expressing cells seem to increase in abundance [36].

In meanwhile around 2004, subsequent studies demonstrated that c-Kit + hematopoietic stem cells possessed essentially no ability to make cardiomyocytes, calling into question the earlier reports that these cells would lead to restoration of the heart muscle after injury [37,38]. At that time, opinions were divided and while some research groups reported these c-Kit + cells as CPCs, others point out that it are mast cells that reside in clusters in the perivascular space and that increase in number in the adult failing heart [39]. In 2007, using double-transgenic mice to track the fate of adult cardiomyocytes in a 'pulse-chase' fashion, Hsieh et al. reported that after injury, in areas bordering a MI, about 15% of newly formed cardiomyocytes were derived from stem cells or precursor

cells [40]. Whether these stem cells or CPCs do express c-Kit was not investigated. Later on, in 2014, van Berlo et al. generated two genetic approaches in mice to examine whether endogenous c-Kit + cells contribute to cardiomyocyte differentiation during development, with ageing or after injury in the adult heart. These c-Kit lineage tracing experiments showed that endogenous c-Kit + cells did only produce new cardiomyocytes within the heart at a percentage of 0.03 or less [41]. In line, one year later, work of Sultana et al. demonstrated that c-Kit expression rarely co-localizes with the expression of the cardiac progenitor marker Nkx2.5, or the myocardial marker cardiac troponin T [42]. Based on their observations, both research groups independently drew the conclusion that c-Kit predominantly labels a cardiac endothelial cell population in developing and adult hearts [41,42]. Recently, Liu et al. independently addressed the same issue using a *Kit-CreER* mouse line for inducible lineage tracing [43]. This instant lineage tracing approach identifies Kit-expressing cardiomyocytes that are labeled during tamoxifen treatment. In combination with long-term lineage tracing experiments, the authors concluded that the large majority of long-term labeled cardiomyocytes in MI hearts are derived from pre-existing cardiomyocytes, expressing Kit [43]. Thus, the latest work within this field does not support the interpretation that most of the labeled cardiomyocytes in lineage tracing are formed *de novo* from c-Kit + CSCs.

Besides the debate whether or not c-Kit + cells exist in the adult heart, investigators have tested the potential of c-Kit + CSCs transplantation into the injured myocardium. In this context, the clinical trials CADUCEUS, SCPIO and ALCADIA, which are based on the isolation, expansion and transplantation of differently derived regenerative cardiomyocytes, gave promising results, as reviewed by Yacoub and Terrovitis [44]. From a molecular point of view, exogenous treatment of high-mobility group box 1 protein has been shown to boost the activation, proliferation and differentiation of c-Kit + CSCs into a cardiomyocyte-like phenotype and

improved cardiac function in infarcted mouse hearts [45]. This effect of myocardial regeneration was linked to the increased levels of metalloproteinases MMP-2 and MMP-9 and an increased expression of miR-206 [46]. In line, one of the validated targets of miR-206 is Tissue Inhibitor of Metalloproteinase-3 (TIMP-3). The downregulation of this inhibitor may enhance the activity of the MMPs promoting the cardiac regeneration by the migration of the active CSCs into the scar. Furthermore, it has been demonstrated that overexpression of miR-499 induces *in vitro* commitment of human c-Kit + CSCs to mature functional cardiomyocytes [47]. MiR-499 was suggested to play a role in c-Kit + CSCs differentiation by affecting the formation of gap-junctions via direct downregulation of SRY (sex determining region Y)-box 6 (Sox6) and regulator of differentiation 1 (Rod1). Indeed, Sox6 and Rod1 have previously been reported to be involved in the modulation of cell differentiation [48,49]. *In vivo*, using a MI rat model, the transplantation of c-Kit + CSCs overexpressing miR-499 showed a restoration of myocardial mass and contractile function related to an enhanced myocyte differentiation. These findings were in line with the work of Sluijter et al. [50], which demonstrated the differentiation of human fetal Sca-1+ CPCs into beating cardiomyocytes, showing a strong upregulation of miR-1 and miR-499. Transient transfection of miR-1 and miR-499 in human CPCs indeed led to a reduction of the proliferation rate for about 25% and 15%, respectively, likely via the repression of Histone deacetylase 4 and Sox6 proteins. Similar work on miR-10a has shown that when human CPCs are transfected with miR-10a, a reduction of 5-ethynyl-2'-deoxyuridine (EdU) incorporation into the cardiomyocytes nuclear DNA during active DNA synthesis, was observed. This effect was abolished with a co-transfection of GATA Binding Protein 6 (Gata6), a direct downstream target of miR-10a [51]. On the other hand, miR-155 has been shown to be able to inhibit the differentiation of Sca-1+ CSCs, by directly targeting  $\beta$ -arrestin2 (Arb2). However, these data could not be confirmed *in vivo*. In fact, in a MI mouse model, transplanted Arb2-KO-CSCs did not show any protective effect [52]. Nevertheless, a different study suggests miR-155 as a useful tool to improve the engraftment for cells *in vivo*. This suggestion was based on the fact that miR-155 could repress necrotic death of CPCs by targeting the receptor interacting protein 1 (RIP1), independently from Akt (Protein kinase B) pro-survival pathway activation [53]. Also, the miR-17/92 cluster (harboring miR-17, -18, -19a, -19b, -20a, -92a) has been demonstrated to play an important role in regulating the proliferation rate of c-Kit + CPCs. Indeed, overexpression of the miR-17/92 cluster both *in vitro* and *in vivo* induced an increase in proliferation rate of mouse CPCs with two fold. This effect was shown to be associated with an increased expression of the cell cycle regulator retinoblastoma-like 2 (Rb12/p130) protein [54].

Taken together, driven by the clinical need, scientists have explored the possibility of identifying and using CPCs as therapeutic strategy to repair the injured heart. Most of the studies and clinical trials have been focusing on cell culture and transplantation of these cells, however there rules a huge controversy within this research field since a good characterization of these cells and their role *in vivo* remains unclear.

## 5. Direct reprogramming of fibroblasts into cardiomyocytes

Another possible method to boost generation of new cardiomyocytes is the reprogramming of the non-cardiomyocyte cell types present in the heart, namely fibroblasts, endothelial cell and vascular smooth muscle cells. Of these cell types, fibroblasts appear to be the most ideal candidates for this direct reprogramming approach. Cardiac fibroblasts are fully differentiated somatic cells, representing the largest proportion of the total cell population in the human heart, ranging from 40% up to 60% [55,56]. Furthermore

this cell type provides a supportive structure, secrete signals, and contribute to scar formation upon cardiac damage [57].

Two decades ago, it was demonstrated that fibroblasts could be stimulated to transdifferentiate into skeletal muscle cells by overexpressing the myogenic transcription factor, MyoD. However, back then a similar transcription factor to promote cardiac transdifferentiation was not found. Later on, the discovery of induction of pluripotent stem (iPS) cells suggested that a specific combination of defined factors, rather than a single factor would be necessary to reactivate a regulatory gene network of the desired cell type. In this context, an experiment was designed to systematically screen 14 different transcription factors simultaneously in order to reprogram cardiac-derived fibroblasts into cardiomyocytes. 13 of these factors were selected based on the severe developmental cardiac defects and embryonic lethality that was developed upon mutation of these factors. Additionally Mesp1 was selected based on a previous publication showing cardiac transdifferentiation capacities in *Xenopus* [58].

Out of the selection of these 14 factors, a cocktail of three transcription factors was shown to be essential for transdifferentiating fibroblasts into cardiomyocytes. A cocktail of GATA Binding Protein (Gata4), Myocyte Enhancer Factor 2c (Mef2c), and T-Box 5 (Tbx5) reprogrammed postnatal fibroblasts directly into differentiated, spontaneously contracting cardiomyocyte-like cells. Furthermore, also *in vivo* transplantation of fibroblasts, transduced for one day with these three factors, into mouse hearts differentiated into cardiomyocyte-like cells [57]. Inclusion of the basic helix–loop–helix transcription factor Hand2 within this cocktail further increased reprogramming efficiency. Introduction of either transcription factor cocktail into cardiac fibroblasts of mice following MI resulted in the formation of cardiomyocytes associated with an improved cardiac function and diminished fibrosis [59,60]. In the meantime, Efe et al. showed conventional reprogramming towards cardiogenesis through overexpression of Octamer-binding transcription factor 4 (Oct4), Sox2, Krueppel-Like Factor 4 (Klf4) and proto-oncogene c-Myc. Within 4 days of transgenic expression of these factors, mouse embryonic fibroblasts (MEFs) showed to be reprogrammed to spontaneously contracting patches of differentiated cardiomyocytes over a period of 11–12 days [61]. The same group also reported that small molecules could enable cardiac transdifferentiation of mouse fibroblasts with only one transcription factor Oct4 [62]. One of the advantages of these reprogramming approaches is that it seems to avoid an intermediate pluripotent stage, theoretically obviating a potential tumorigenic effect. However, the limitations related to an *ex vivo* treatment persist, namely the timing from the cell isolation, *ex vivo* expansion until the final cell delivery may affect the success of this kind of treatments [63].

Nevertheless, based on the potential of miRNAs in regulating gene regulatory networks during development, researchers began to investigate their role in redirecting cell fate [64–67]. In this context Jayawardena and coworkers were the first to develop a strategy in order to identify miRNAs capable of reprogramming cardiac fibroblasts into a cardiomyocyte lineage [68]. Candidate miRNAs were selected based on previous reports demonstrating their roles in cardiac muscle development and differentiation [24,69–72]. By using a combined transfection of synthetic, chemically modified mimics of the selected miRNAs the authors showed an induction of direct differentiation of mouse adult fibroblast to cardiomyocytes-like cells. Top candidates identified included miR-1 alone; miRs-1, -133, -206; miRs-1, -133, -208; miRs-133, -206, -208; miR-1, -138; and miRs-1, -138, -208. This was further demonstrated *in vivo* using transient transfection of the microRNAs in neonatal cardiac fibroblasts isolated from double-transgenic mice carrying both the Fibroblast-specific protein-1

(Fsp1)-driven Cre recombinase gene and a floxed tdTomato reporter (Fsp1-Cre/tdTomato). The fibroblastic origin of miRNA-induced cardiomyocyte-like cells was shown by a co-localization of the tdTomato marker with heart muscle-specific markers; cardiac Troponin I or  $\alpha$ -actinin. Interestingly, miRNA-mediated reprogramming was enhanced 10-fold on Janus kinase (JAK) inhibitor 1 treatment. Furthermore, administration of lentivirus encoding miR-1 or a combination of 4 lentiviruses encoding the miRNA combo (miR-1, -133, -208, -499) into ischemic mouse myocardium resulted in evidence of direct conversion of cardiac fibroblasts to cardiomyocytes *in situ*. Genetic tracing analysis using Fsp1Cre-traced fibroblasts from both cardiac and non-cardiac cell sources strongly suggests that induced cells are most likely of fibroblastic origin. Moreover the authors could associate these results with an improvement of cardiac function on up to 3 months after MI [73]. Simultaneously, another research group highlighted the importance of MyoD in the reprogramming of fibroblasts into a cardiomyocyte-like phenotype. They demonstrated more effective induction of reprogramming with MyoD in combination with Mef2c and Tbx5, compared with any other combination of three factors from 10 candidate, including the ones previously described in literature. The cocktail of Mef2c, Tbx5 and MyoD expressed cardiac contractile proteins, had cardiac-like potassium and sodium currents and action potentials could be elicited [74]. In 2013, Nam and colleagues investigated whether human adult fibroblasts could be reprogrammed into cardiac-like myocytes by the cardiac transcription factors and muscle-specific miRNAs previously reported to reprogramme mouse fibroblasts into cardiomyocytes. The authors showed that four human cardiac transcription factors, including Gata4, Heart And Neural Crest Derivatives Expressed 2 (Hand2), T-box5, and MyoD, combined with miR-1 and miR-133, activated cardiac marker expression in neonatal and adult human fibroblasts. After maintenance in culture up to 11 weeks, human fibroblasts reprogrammed with this cocktail displayed sarcomere-like structures and calcium transients, and a small subset of such cells exhibited spontaneous contractility [75].

To avoid genetic insertion and carcinogenicity by viral based methods, Fu et al. tested a small-molecule combination CRFVPTZ (C, CHIR99021; R, RepSox; F, Forskolin; V, VPA; P, Parnate; T, TTNPB; and Z, DZnep), previously developed by Hou et al. [76]. Using this chemical cocktail it was possible to generate automatically beating cardiomyocyte-like cells from mouse fibroblasts. These chemical-induced cardiomyocyte-like cells express cardiomyocyte-specific markers, exhibit sarcomeric organization, and possess typical cardiac calcium flux and electrophysiological features [77]. Bypassing the use of viral-derived factors for *in vivo* cardiac trans-differentiation with pharmacological agents could open a new avenue towards regenerative medicine for heart failure patients. Although research in this field has made tremendous progress during the latest years, further optimization of this process and the eventual generation of more mature and homogeneous populations of the generated cardiomyocyte-like cells is necessary. In order to be able to do this, further research unraveling the underlying molecular mechanisms of all these strategies would be indispensable.

## 6. Endogenous cardiomyocytes as target for the development of regenerative medicine

Shortly after birth, the majority of human cardiomyocytes shift from a proliferate state towards a terminally differentiated phenotype, unable to re-enter the cell cycle. Consequently, adult mammalian cardiomyocytes possess a restricted proliferative capacity, insufficient to rebuild the ventricular wall and restore cardiac function after injury [19]. However, since the mid 50s a

number of reports indicated that a few replicating cardiomyocytes could be detected after cardiac injury. These were the first suggestions that cardiomyocytes by themselves underwent an attempt to regenerate the damaged myocardium [78–81]. Although these reports were published by independent research groups, the scientific community kept on being skeptic about the fact that certain cardiomyocytes are still able to proliferate during adult life. The latest years, research methods have improved and by combining genetic fate-mapping and stable isotope labeling techniques, researchers again showed that the dominant source of cardiomyocyte replacement during myocardial homeostasis, and even more after myocardial injury, is represented by pre-existing cardiomyocytes [82]. Furthermore, it was demonstrated that pre-existing cardiomyocytes preferably in a hypoxic state, which is a typical feature of proliferating cells in diverse organs, displayed a significant contribution in the generation of new cardiomyocytes in the adult heart. This finding confirms that the heart contains a small population of cardiomyocytes, which keep their prenatal proliferative nature, even during adulthood [83]. The recognition of endogenous cardiomyocytes as a source for new cardiomyocytes, even at a very low percentage, raises the possibility of stimulating this process for developing cardiac regenerative therapies.

In contrast to mammals, lower vertebrates like the teleost fish are able to intrinsically regenerate the heart muscle after induction of myocardial injury. Jopling et al., as well as Kikucki et al., reported pre-existing cardiomyocytes as major source of the newly formed cardiomyocytes during the regenerative process in zebrafish. This was demonstrated by enhanced green fluorescent protein (EGFP) labeling of the pre-existing cardiomyocytes through an inducible Cre recombinase system. 30 days after ventricular sectioning, the majority of the newly formed cardiomyocytes showed to be EGFP positive [84,85]. Moreover, in 2007, a technology was developed to designate -90 color labels to murine neurons [86]. This technology, termed brainbow, facilitated the visualization of adjacent neurons and their connections in the brain with high resolution. This technology enabled the potential to assign many colors to different cells in a population, and thus investigate cell proliferation and lineage decisions. Using this brainbow technology to study the zebrafish heart, it was demonstrated that 14 days after injury, cortical muscle regeneration is the primary component of the newly formed wall, whereas the primordial muscle was regenerated much later (30 day after injury). Interestingly, these events occur in a temporally reversed manner compared to initial morphogenesis during cardiac development [87].

In 2008, the regenerative capacity of the mammalian fetal heart was investigated by Drenckhahn et al. [88]. They introduced a cardiomyocyte-lethal mutant gene in the X chromosome, which became conditionally expressed at embryonic day 12.5 (E12.5) in half of the cardiomyocytes in female embryos (due to random X inactivation). Fetal hearts that had undergone this genetic ablation were able to restore approximately 50% of lost cardiomyocyte mass. These data indicate that the embryonic environment facilitates cardiomyocyte repopulation of the heart [88]. This repopulation capacity of the heart muscle ability was even reported to be maintained during the early postnatal phase. After partial surgical resection of the apex of the heart in 1-day old mice, the cardiac muscle regenerates itself by boosting cardiomyocyte cell cycle re-entry [89]. Echocardiographic analysis, measuring systolic cardiac function of these regenerated hearts showed to be normal up to 2 months after surgery. Nevertheless, the regenerative capacity of the heart muscle seems to be inexorably lost by 7 days of age.

Interestingly, it has been suggested by Porrello et al. that upregulation of the miR-15 family during the postnatal period may be a possible regulatory mechanism directing cardiomyocyte cell cycle withdrawal and binucleation [90]. In line, a microarray analysis

revealed that miR-195, a miR-15 family member, was highly upregulated in mouse cardiac ventricles at postnatal day 10 versus postnatal day 1 [90]. Furthermore, knocking down the entire miR-15 family in neonatal mice showed a pro-proliferative boost of cardiomyocytes, associated with a higher expression of the cell cycle gene checkpoint kinase 1 (Check1). Check1 was identified as a direct downstream target of miR-195. Recently, it was reported that the expression of miR-195 can be modulated by the Long Non-coding RNA UC.283 + A, which targets the lower stem region of pri-miR-195 transcript, preventing the cleavage by Drosha both *in vitro* and *in vivo* [91]. However, the exact role of UC.283 + A in cardiomyocyte proliferation is unknown so far. Other studies also indicated that singular miR-15 knock down in a both a mouse and pig model for cardiac ischemia-reperfusion injury showed a reduction in infarct size and attenuation of cardiac remodeling, resulting in a better heart function [92].

In 2012, Eulalio et al. performed a high-throughput functional screening in neonatal rat cardiomyocytes, and reported that 204 miRNAs, of the 875 miRNAs tested, were able to boost cardiomyocyte proliferation *in vitro*, showing at least a 2-fold increase in nuclear EdU incorporation in stimulated cardiomyocytes compared to controls [93]. 40 of these miRNAs were confirmed to have pro-proliferative capacities in murine neonatal cardiomyocytes as well. In order to demonstrate true karyokinesis and cytokinesis occurred upon miRNA stimulation, histone H3 phosphorylation and Aurora B kinase localization were analysed and validated the screening results for the top 10 pro-proliferative miRNAs in both rodent models. Among these miRNAs, the top two candidates, namely miR-199a and miR-590, were validated in an *in vivo* model for mouse MI. Indeed, treatment with either miR-199a or miR-590 boosted post-natal cardiomyocyte proliferation and consequently preserved contractile function and decreased the levels of fibrosis after MI. The direct downstream targets identified for both microRNAs were Homer Scaffolding Protein 1 (Homer1), a regulator of the calcium signaling, and in HOP Homeobox (Hopx), a suppressor of the embryonic cardiomyocyte proliferation. Additionally, the chloride intracellular channel Clc5 showed to be a direct target of miR-590.

The past few years, more and more scientists reported the involvement of miRNAs in boosting cardiomyocyte proliferation, resulting in improved cardiac contractile function after injury. For example, the miR-17/92 cluster, consisting of miR-17, miR-18a, miR-19a/b, miR-20a and miR-92a, was also discovered to be a regulator of native cardiomyocyte proliferation [94]. *In vivo* knockout of this cluster specifically in the cardiomyocytes showed a reduction in number of cardiomyocyte, which was compensated by an increase in cell size. In line, overexpression of the entire cluster was able to boost cardiomyocyte proliferation *in vivo*, as well as in the embryonic phase, the postnatal period and in adult hearts. The tumor suppressor Phosphatase and tensin homolog (PTEN) showed to be altered both in the miR-17-92 transgenic and knockout mice. As a reported direct downstream target of miR19a/b, the PTEN overexpression abolished the pro-proliferative effect of miR19a/b completely [95].

Also the miR-302/367 cluster showed pro-proliferative effects in cardiomyocytes [96]. The miR-302/367 cluster decreases in expression while progressing from the embryonic phase towards the adult phase. However, upon overexpression of this cluster in the developing heart, cardiomyocyte proliferation and cardiomegaly is induced. Macrophage Stimulating 1 (Mst1), Large tumor suppressor 2 (Lats2), MOB Kinase Activator 1B (Mob1b) were identified to be putative targets of miR302/367, indicating a role of this microRNA cluster in regulation of the Hippo pathway, an important signaling cascade that regulates organ size by cell proliferation and death. Furthermore, transient overexpression of the

miR-302/367 cluster, and in particular miR-302, promotes cardiac regeneration leading to improved cardiac contractile function after MI.

An interesting report on the role of miR-222 in cardiomyocytes has been published by Liu et al. [97]. The expression of miR-222 appears to be induced during physiological hypertrophy in two different mice model of exercise, while this was not observed during pathological cardiac remodeling. Furthermore, miR-222 inhibition completely blocked the physiological exercise-induced cardiomyocyte remodeling, and also reducing the expression of proliferation markers. Furthermore, by inducing ischemia-reperfusion in hearts of cardiac-specific miR-222 overexpression mice, the authors demonstrated improved cardiac contractile function and a 70% reduction on scar formation compared to wild-type mice. This protective effect, associated with the increase of cardiomyocyte proliferation and reduced apoptosis, was linked to the direct inhibitory effect of miR-222 on cyclin-dependent kinase inhibitor 1B (p27), homeodomain-interacting protein kinase 1 (HIPK1) and homeobox containing 1 (HMBOX1).

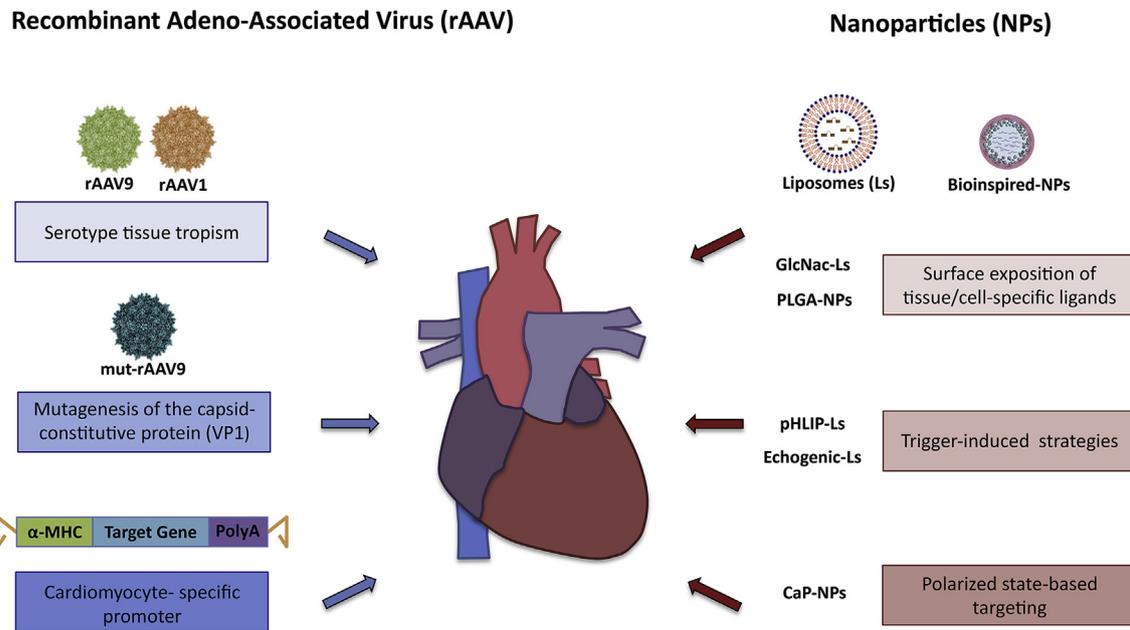
## 7. Delivery of therapeutics to heart failure patients

Stimulation of endogenous heart regeneration might lead to severe side effects, such as tumor formation in other organs. Therefore one must not oversee the importance of developing a strategy that lead to very specific targeting and thus directing the therapeutics need to turn their action. Concerning the essential role of microRNAs in recovering cardiac dysfunction, several efforts have been made in order to apply *in vivo* strategies to modulate miRNA expression [93,98]. To inhibit microRNA expression, scientists developed antisense oligonucleotides (ASOs) and modified microRNA mimics, such as plasmid or lentiviral vectors, have been shown to carry microRNA sequences designed to deliver microRNAs to cells and tissues *in vivo*. The use of anti-microRNA oligonucleotides (AMOs), their modifications, perspectives and challenges has been extensively reviewed by Philippen et al. [99]. On the contrary, *in vivo* applications that overexpress and deliver miRNAs are still a challenge for the field. For example, The use of miRNA mimics is limited by their intolerance to extensive modifications [98]. Recombinant adeno-associated virus vectors and nanoparticles applications, with their limitations and difficulties, are nowadays two alternative and promising delivery systems that are finding their way in the present and future molecular medicine (Fig. 2).

## 8. Recombinant Adeno-Associated viral vectors

The recombinant adeno-associated virus (rAAV) is a product of the adeno-associated virus (AAV), a nonpathogenic human virus that possesses a weak immunogenicity and does not induce inflammatory events (reviewed by Zacchigna et al., 2014 [100]). As a safe and efficient gene delivery vector, AAV has been used in a wide range of biotechnological applications during the last decades. In contrast to several other viral vectors, the rAAV are unable to integrate into the host-cell genome, and *in vivo*, rAAV is able to infect non-cycling cells, such as the majority of adult cardiomyocytes, with high efficiency.

The rAAV vector consist of a DNA transgene cassette of 4.5 kb, surrounded by two inverted terminal repeats essential for the viral packaging, which are the solely maintained elements of the original viral genome. The single-strand DNA genome is packaged in an icosahedral capsid built of 60 proteins [101]. Depending on the cluster of amino acid residues exposed on the surface, their topology and tridimensional structure, over more than 100 AAV variants have been isolated [102,103]. However, only around 10 of



**Fig. 2. Promising delivery strategies in cardiac molecular medicine.** 1) Recombinant Adeno-Associated Viral vectors (rAAV): Among the different serotypes tested both rAAV9 and rAAV1 showed a high cardiotropic effect; this specificity can be further increased by mutagenesis of the capsid-constitutive protein VP1, which has been demonstrated for AAV9 (mut-rAAV9); further specificity can be obtained by using a cardiomyocyte-specific promoter, such as alpha-myosin heavy chain ( $\alpha$ -MHC). 2) Nanoparticles (NPs): which could be Liposomes (Ls) or Bioinspired-NPs. Their cardiac-specific targeting can be improved by a) surface exposition of tissue/cell-specific ligands, which has been shown for e.g. *N*-acetylglucosamine-conjugated liposomes (GlcNAc-Ls) and poly *D,L*-lactide-co-glycolide nanoparticles (PLGA-NPs); b) Instant activation by environmental or body-external factors, such as pH insertion peptide liposomes (pHLIP-Ls) and Echogenic liposomes (Echogenic-Ls); or c) via polarized state-based targeting as demonstrated with calcium phosphate nanoparticles (CaP-NPs).

these variants fit into the accepted definition of new serotypes. Their genomic simplicity allows their use as a carrier for the over-expression of both protein-coding and non-coding genes, such as miRNAs, or siRNAs against specific genes in the case a knockdown effect is desired. The interaction between the capsid and receptors/co-receptors exposed on the cell surface give them differential cell-tropism. However, also the route of delivery can affect both the efficiency of infection and the tissue-specificity. This modulation becomes even more complex when comparing the efficiency and specificity of the AAV vectors in different species. So far, AAV serotype 9 has been identified to show the best cardiotropic effect [104,105]. As we mentioned before, miR-590 and miR-199a have been shown to be delivered to the mouse cardiac muscle via an rAAV9 vector, and show a functional effect on cardiac regeneration and heart function after MI [93]. Similarly, using rAAV9 transduction, miR-378 has been demonstrated to become overexpressed in the heart muscle, which attenuated hypertrophy, induced by thoracic aortic constriction [106]. Also, rAAV9-miR-669a treatment was reported to decrease cardiac dilatation in dystrophic mice [107]. Nevertheless, AAV serotype 1, 6 and 8 also report cardiomyocyte transduction, but with differential efficacy [108,109]. In Rhesus Macaques and in pigs, delivery of AAV6 has been shown to result in highly efficient and global gene transfer [110,111]. In human rAAV1 has been used to deliver Sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (Serca2a), via intracoronary administration. SERCA is a calcium transporter critical to maintaining calcium homeostasis, which is downregulated in heart failure. Thus, these therapies are designed to restore impaired calcium signaling in the failing heart. AAV1-SERCA therapy has been proven to be safe and is already in use in the following clinical trials; the Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID), the AAV1-CMV-Serca2a Gene Therapy Trial in HF (AGENT-HF) and the SERCA2a Gene Therapy in LAVD [112]. Although for the CUPID trial, rAAV1 was used, in 60% out of 1552 heart failure

patients the presence of high titers of neutralizing antibodies (Nab) against AAV2, which cross-reacted with AAV1, caused a problem in this trial [113]. Second- or third-generation AAV vectors designed using mosaics and directed evolution will become very important.

To circumvent this problem, Pleger et al. showed that retrograde coronary venous delivery of rAAV9-mediated therapy, named AAV9-S100A1, at a concentration of  $1.5 \times 10^{13}$  viral particles is cardiotropic in a pig model for MI [114]. This was confirmed by Fish et al. by showing successful intracoronary gene transfer of I-1c to the heart using rAAV9 [115]. In meantime, uniQure's gene therapy platform to restore S100A1 levels was started-up. UniQure already successfully brought the AAV1-based gene therapy Glybera<sup>®</sup> to the market [116]. Glybera<sup>®</sup> is designed to restore the lipoprotein lipase (LPL) enzyme activity required to enable the processing and clearance of fat-carrying chylomicron particles formed in the intestine after a fat-containing meal [117]. After this success, they will now use retrograde delivery and include the cardiac-specific myosin light chain promoter into their vector, to specifically deliver S100A1 to the heart muscle [114]. Indeed, alpha-myosin heavy chain ( $\alpha$ -MHC) has already shown to be effective to deliver transgene expression solely in the heart [118]. Unfortunately, the use of a specific promoter causes a decrease in the transcription level, and therefore biotechnologists are still dealing with a dilemma between targeted delivery and system efficiency. Thus investigators have been working on manipulating these promoters. In rats, cardiac-selective expression of a therapeutic transgene has been achieved by using only a fragment of the mouse alpha-cardiac actin gene enhancer, which was ligated to the elongation factor 1alpha promoter [110]. Furthermore, the engineering of synthetic capsid variants gives further possibilities towards tissue specific targeting. By random mutagenesis of the mainly capsid-constitutive protein (VP1) of rAAV9 Pulicherla et al. provided several functional rAAV9 variants (mut-rAAV9) which seem to be more cardiac- and musculoskeletal-tropic, while the liver is

effected less [119].

Given the progress scientists have been made during the latest years, the use of rAAV-based therapeutics for heart failure patients seems to become reality within a few years from now.

## 9. Nanoparticles (NP)

Another biotechnological development for drug delivery, which is worthwhile to mention in this context, is the use of nanotechnology. This technology, through controlling materials at nanoscale, has driven revolutionary developments in almost all fields. Nanoparticles (NPs) have been widely used for fast-diagnosis, molecule delivery, and tissue engineering. Nanoparticles are a class of nanostructures that can be composed and built by different kind of materials and motifs such as dendrimers, carbon nanotubes, polymers, micelle or liposomes [120].

A range of nanoparticle delivery systems have been investigated for the targeted delivery of miRNAs, in a variety of different disease models outside of the cardiovascular field, with varying degrees of success and translational potential, including Poly lactide-co-glycolide (PLGA) particles, dendrimers, lipid based systems and Polyethylenimine (PEI)-based delivery systems (reviewed by Zhang et al., Muthiah et al. and Christiakov et al. [121–123]). Due to their plasticity and the fact that nanoparticles are relatively easy to manipulate, the amount of research in this field to use nanoparticles as a possible drug delivery system in an *in vivo* context is increasing (reviewed by Ho et al., 2015 [124]). Moreover, their use has even paved a way for assisting stem cell therapy delivery to the ischemic heart (reviewed by Kai Zhu et al. [125]).

In the cancer field, nanoparticles are used to deliver therapeutics as well as to visualize within hours whether the treatment is effective or not [126,127]. Obviously, among all existing molecules, non-coding RNAs seem to be good candidates to be delivered via nanoparticles in order to treat a variety of pathologies. However, one must overcome issues as low miRNA transfection efficiency and cytotoxicity [128]. In this context, polyethylene glycol-polyethylenimine nanocomplexes have been designed as carriers to deliver miR-150 to chronic myeloid leukemia cells with relatively high efficiency [129]. However, in the field of heart disease, nanotechnology still has a long way to go. Lipids forming liposomes and bio-inspired nanoparticles are the most investigated nanoparticle varieties so far. In general, the main challenges are related to the loading efficacy, biodegradability, biocompatibility, retention of drug in the blood stream and specific release at the target site [130]. From this list, specific targeting as well as having a safe and optimized application is a fundamental need for heart regeneration therapeutics. Concerning the active delivery of the nanoparticles at a specific location, it has been described that after MI, an enhanced permeation and retention effect (EPR) takes place. This means that the vessels perfusing the left ventricles appear to be hyper-permeable, actually promoting the accumulation of lipidic particles in the border zone of the infarcted areas. Therefore, Tan et al. developed a particles-cell hybrid model to predict the nanoparticle dispersion related to blood dynamics [131]. They took in account the complexity of the blood composition (containing compounds such as deformable cells, proteins, platelets and plasma) and the blood stream in the capillaries, where the bigger compounds tend to flow in the core of the vessel. By this model the authors observed that the presence of a cell-free layer between the core and the wall of the vessel increases the dispersion of the nanoparticles toward the walls. Further studies demonstrate that the margination of the nanoparticles through the wall appears significantly influenced by the size and shape of the nanoparticles. In fact, the smaller and oblate particles are, the more they are localized towards the vessel wall when compared with bigger-sized and sphere-shaped

particles [132].

In case of a myocardial injury, the caliber of the arterioles is decreased, which is caused by occlusions or vessel spasms combined with enhanced extravasation. Therefore, it would be a merit to take into account the knowledge on the EPR effect for developing new therapeutics to target specifically the myocardium. This strategy could be even boosted by a local administration, for example during an angioplasty procedure.

Next, several researchers demonstrated the possibility to manipulate the surface of nanoparticles in order to expose different kinds of ligands that theoretically can give the nanoparticle a tissue-specific or cell-specific action [133–135]. Unfortunately, *in vivo* myocardial-specific targeting remains still an open challenge. In fact, the characterization of an exclusive surface marker for cardiomyocytes has not been discovered yet [136]. Nevertheless, the *in vitro* findings reported by Aso et al. combined with the *in vivo* work by Gray et al. already show interesting results in this context [137,138]. These scientists demonstrated an interaction and uptake of *N*-acetylglucosamine-conjugated liposomes (GlcNAc-Ls) by cardiomyocytes. Moreover, as proof of concept, *in vivo* administration of this type of nanoparticles loaded with an inhibitor of p38 led to decreased apoptosis, a reduced infarct area and improved cardiac function. More recently also the peri-infarct injection of insulin-like growth factor (IGF)-1-complexed poly D,L-lactide-co-glycolide (PLGA) nanoparticles (PLGA-IGF-1 NPs) prevented cardiomyocytes apoptosis, improved left ventricle ejection fraction and reduced infarct size 21 days after MI in mice [139].

Another issue is the development of a smart drug delivery system in order to release the nanoparticle content only at the target location under specific conditions. This type of nanoparticles technology works via localized auto/induced destabilization or upon the intervention of an external effector, causing an indirect site-specific release. For the trigger-induced strategies, biotechnologists are considering heat, magnetism and light as different type of triggers to make the nanoparticles releasing their content when present at the target side [140,141]. Unfortunately, no trustworthy application to target specifically the myocardium in this way has been found yet. However, knowing that the pH of the extracellular environment is lower in several pathological conditions, including myocardial ischemia [142,143], a promising application of pH (low) insertion peptide (pHLIP) was demonstrated by Sosunova et al. [144]. The technology of pHLIP targets cells within an acid extracellular environment (low pH) [145]. In two different models of myocardial ischemia, it was reported that pHLIP-liposomes are able to target predominantly the ischemic but not the uninjured areas of the myocardium [144].

Another attractive strategy is based on the localized destruction of nanoparticles through ultrasound high acoustic pressure. So far, this technique mainly has been applied to microbubbles in order to target the heart muscle. Microbubbles can image the heart via effectively reflecting sound waves when ultrasound pulses are present. Upon adjusting the acoustic setting of the ultrasound, they could burst and deliver their payload to a target region of the heart (reviewed by Dijkmans et al., 2004 [146]). However, microbubbles actually cannot be classified as nanoparticles because of their bigger size [147]. In the future, current available technology could be further optimized using echogenic liposomes (Els), which are more prone for extravessel targeting [148]. Interestingly, very recently Di Mauro et al. introduced a therapeutic system for the delivery of bioactive molecules to the heart, based on the polarized state of cardiomyocytes. By synthesizing negatively charged calcium phosphate bio-inspired nanoparticles (CaP-NPs), employing citrate as stabilizing agent, the authors could report an efficient delivery of synthetic microRNAs into cardiomyocytes both *in vitro* and *in vivo* [149]. In contrast to surface decoration or adsorption

approaches, this approach seems to increase the stability and functionality of miRNAs. These findings will open up new avenues for the potential application of nanomedicine in the context of heart muscle regeneration.

## 10. Concluding remarks

Since microRNAs have been shown to be able to direct cell fate processes, many miRNA-based therapeutic strategies have been proposed in the context of heart regeneration. Taken into account the debate about the existence of CSCs and the difficulties of generating a mature and homogenous cardiomyocyte population from reprogrammed fibroblasts, stimulation of endogenous cardiomyocyte proliferation seems to give the most promising future perspective so far. In this context, AAV9 vectors have been used to successfully deliver miRNAs into the cardiac muscle, where this type of therapeutics has shown a clear regenerative effect in the mouse heart. Although usage of AAV vectors already showed to be successful in pigs and humans as well, there is still a dire need for a more efficient, safe and cardiac-specific drug delivery system. Latest years, new biotechnological approaches have been developed e.g. by altering AAV vectors or by using bio-engineered nanoparticles. Further optimization of current technologies and obtaining better insight into molecular mechanisms underlying the successful therapeutics, would make a cardiac-specific drug delivery possible, efficient and safe for the future heart failure patient.

## Conflict of interest disclosures

A.R.: none, E.D.: none.

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