MicroRNAs

NOVEL TARGETS FOR CARDIAC REGENERATIVE MEDICINE

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(met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER 1

INTRODUCTION

CLINICAL BACKGROUND

Over 7 million people die of ischemic heart disease worldwide each year, which will increase to an estimated 9 million by 2020[1]. This makes ischemic heart disease one of the current and future leading causes of morbidity and mortality worldwide. The major risk factors currently associated with ischemic heart disease are high blood pressure, high blood cholesterol, tobacco smoking, unhealthy diet, physical inactivity, diabetes, and advancing age[2]. The increased incidence of several of these risk factors, especially in developing countries, has led to a steep increase in mortality rate as a result of cardiovascular disease. This, together with the immense economic costs relative to other diagnostic groups, leaves a tremendous burden on society. Scientific research will undoubtedly lead to new therapeutic strategies to cope with this global epidemic of ischemic heart disease. Nevertheless, it is of vital importance to develop and implement new policies to reduce specific cardiac risk factors. That being said, development of regenerative therapeutic strategies to halt the progression of ischemic heart disease to advanced heart failure is one of the most urgent medical needs of this century and has therefore become a holy grail of modern-day cardiovascular research.

MYOCARDIAL INFARCTION

The main contributor to ischemic heart disease is myocardial infarction (MI), which is commonly caused by the erosion or rupture of a vulnerable atherosclerotic plaque leading to coronary artery occlusion and subsequent interruption of blood supply which initiates a cascade of events[3,4]. The acute deprivation of oxygen causes cardiomyocyte death by apoptosis and necrosis, which will trigger an inflammatory response contributing to the removal of cell debris by proteolysis and phagocytosis[5,6]. The damaged tissue is then invaded by cardiac fibroblasts that prevent rupture by increasing the tensile strength via production of extracellular matrix in the infarct area and border zone[3,7]. New blood vessels are formed in the border zone by angiogenesis and vasculogenesis, and pre-existing collateral arteries are enlarged by arteriogenesis, resulting in partial restoration of blood flow[4]. Because adult cardiomyocytes have a very low intrinsic proliferation rate and are unable to replace the substantial number of lost cardiomyocytes, a collagen-rich scar is formed. While scarring results in preservation of the cardiac integrity, the non-contractile scar impedes restoration of cardiac output. To compensate for the loss of function, the heart will undergo ventricular remodeling and cardiomyocyte hypertrophy, ultimately leading to severely impaired cardiac function and heart failure[8].

The prognosis of MI and resulting chronic heart failure remains extremely poor despite continues advancements in pharmaceutical and technological interventions. Certainly, today's treatment options have greatly reduced the number of patients that acutely die from MI, but therapeutic regimes for chronic heart failure are palliative, and currently, heart transplantation, which dates from 1967, is the only available therapy that addresses the underlying problem of heart failure, which is cardiomyocyte-loss. However, heart transplantation is not a common treatment option as it is limited by a severe shortage of donor hearts, which has led to the use of ventricular assist devices. Cardiomyocytes occupy approximately 75% of normal myocardial tissue volume and constitute 20 to 40% of total cardiac cells[9]. During MI, approximately 1 billion cardiomyocytes alone die[10], highlighting the need for methodologies that induce extensive myocardial regeneration.

INTRINSIC CAPACITY FOR REGENERATION

Cardiac regeneration requires the replacement of lost cardiomyocytes and the formation of new blood vessels, to prevent the maladaptive cascade of scarring, remodeling and consequent cardiac dysfunction. Unfortunately, and in contrast to the natural restoration of the microvasculature[11], cardiomyocyte regeneration is far too limited to compensate for the cell death after MI. Cardiomyocytes withdraw from the cell cycle shortly after birth, when myocardial growth shifts from a hyperplastic to a hypertrophic phase, characterized by the formation of binucleated cardiomyocytes[12]. This shift gave rise to the concept that in the adult, cardiomyocytes are incapable of proliferating, hence, terminally differentiated. This notion suggests that the heart has little or no capacity to generate new cardiomyocytes after birth, which is supported by the persistence of scar tissue following MI. In lower vertebrates, such as zebrafish, surgical removal of 20% of the ventricle resulted in complete cardiac regeneration devoid of scar tissue[13], occurring through the dedifferentiation and proliferation of cardiomyocytes[14]. In mice, similar heart regeneration has been demonstrated, although only up till 7 days after parturition[15]. Even though the majority of adult cardiomyocytes do not proliferate, some evidence has emerged indicating that the adult human heart does hold a limited capacity for regeneration. To date, the most objective evidence on human adult cardiomyocyte renewal was shown by a well-devised study which has taken advantage of the integration of carbon-14, generated by nuclear bomb tests during the Cold War, into DNA, to demonstrate that human adult cardiomyocytes renew. They found that renewal occurred with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75, indicating that a mature

human heart consists of approximately 50% postnatal-generated cardiomyocytes[16]. Remarkably, others have claimed this turnover to be 10 to over 40-fold higher based on other assumptions[17,18]. Importantly, while human adult cardiomyocyte renewal does occur, it is unclear if the new cardiomyocytes originate from a differentiated cardiomyocyte or a progenitor cell, or possibly both. In mice, a limited number of new cardiomyocytes are formed after MI, which are not derived from existing cardiomyocytes but rather originate from a progenitor cell population[19,20], similar to the developing heart[21]. These data indicate that cardiac regeneration after MI primarily occurs via progenitor cell differentiation, rather than by cardiomyocytes re-entering the cell cycle, as predominates in zebrafish[14].

The fact that the heart is not terminally differentiated does not change the fact that cardiomyocyte renewal is insufficient to restore heart function after MI. However, it has paved the way for the development of new therapeutic strategies that exploit this limited intrinsic regenerative potential to improve cardiac regeneration. An elegant option for regenerating new heart tissue after MI is to stimulate the formation of new cardiomyocytes and vasculature, by activating endogenous players present in the surviving myocardium. Though, there are currently few clues on how to unlock this intrinsic regenerative capacity of the human heart.

CELL-BASED THERAPY

In contrast to the limited knowledge on how to enhance regeneration from within the heart, much effort has been spent to develop cell therapies for reconstructing the myocardium by stem/progenitor cell injections, thereby replacing dead cardiomyocytes and blood vessels. A broad range of different cell-types has already been explored for cell replacement therapy in damaged hearts, and are expertly reviewed elsewhere[22,23].

Pre-clinical and clinical tests using adult stem cell therapy following MI, have shown that transplantation of non-cardiac stem cells such as skeletal myoblasts and different bone marrow-derived cells resulted in a minor improvement in left ventricular ejection fraction[24,25], but also the induction of arrhythmias[26]. Additionally, evidence has emerged indicating that the functional improvement may be due to paracrine effects that lead to enhanced vascularization[27-29], which is also supported by the fact that these transplanted cell populations have no or a dubious capacity to differentiate into functional cardiomyocytes[30-35]. The use of human embryonic stem cells (ESCs), which indisputably do possess the capacity to differentiate into the required cardiac cell types, like cardiomyocytes, endothelial cells and vascular

smooth muscle cells[36,37], is hindered by ethical concerns, immune rejection[38], partial or heterogeneous differentiation[39], and teratoma formation[38,40]. A possible solution to the ethical concerns of ESCs has come from the young, but rapidly progressing field of cellular reprogramming. In 2007, it was shown that ES-like cells, termed induced pluripotent stem cells (iPSCs), can be generated by reprogramming of human adult fibroblasts[41,42], which do not have the ethical restrictions. More recent studies have implemented this reprogramming strategy to indirectly or directly generate functional cardiomyocyte-like cells *in vitro*, or *in vivo*, thereby providing a possible autologous option for cardiac regeneration [43-48]. Despite this, these cells are currently not suited for cardiac cell therapy as recent evidence has indicated problems with efficiency[49], epigenetic and genetic abnormalities, teratoma formation[50], and immune rejection[51].

One of the most appealing and promising sources for cell replacement therapy is the adult heart itself. In 2003, the first adult murine cardiac progenitor cells were described[52,53], leading to the discovery of the first adult human cardiac progenitor cells a few years later[54-57], which opened up the possibility of autologous cell-based cardiac repair. These cardiac progenitor cells are isolated by their ability to form self-adherent clusters, termed cardiospheres, the presence of the transcription factor islet1, the expression of the surface antigen c-Kit, or the stem cell antigen-1-like protein (Sca-1-like). However, whether these human cardiac-derived progenitor cells, which will be collectively referred to as cardiac progenitor cells (CPCs), are indeed distinct cell populations, remains unclear. CPCs provide an excellent cell source for the regeneration of cardiac tissue, as they can differentiate into cardiomyocytes, endothelial cells and smooth muscle cells[55,57-59]. In addition, CPC transplantation therapy in experimental animal models of MI have shown modest, but promising improvements in cardiac function[54,56,60,61]. The differentiation capacity of CPCs, and the promising results of transplantation therapy in various rodent MI models[61-63], suggests that CPCs provide a better source for cell therapy compared to other investigated cell types. These results have led to the initiation of the first phase 1 clinical trials using cardiosphere-derived and c-Kit+ adult cardiac progenitor cells: ALCADIA (NCT00981006), SCIPIO (NCT00474461), and CADUCEUS (NCT00893360). Preliminary results from the SCIPIO (16 patients) and CADUCEUS (17 patients) trials have shown that the cardiac cell injections were safe. Both trials showed a reduction in myocardial scar mass, whereas only the SCIPIO trial reported an improvement in left ventricular ejection fraction[64-66]. Currently, CPCs are becoming the most important cell population for cardiac regeneration, but larger studies will be needed to prove the safety and efficacy of these treatment strategies, and actual regeneration of the damaged myocardium remains to be seen.

OPTIMIZING CELL-BASED THERAPY

The identification of adult CPCs, which can easily be obtained from small cardiac biopsies, has certainly increased the feasibility of cardiac regeneration after MI. However, one of the major obstacles to the success of cell-based therapy is the extremely limited survival and engraftment of transplanted cells. Only up to 7% of injected cells were found back in the myocardium 24 hours after transplantation, and long-term engraftment is even lower[22,67]. These low percentages are a direct indication that the observed functional effects may be greatly enhanced if a higher percentage of cells survive and engraft in the damaged myocardium, which signifies the need for novel approaches that potentiate cardiac repair. Technological advances in cell delivery strategies may help in improving the efficiency of cell delivery. However, the extent of cardiac regeneration is dependent on a combination of biological factors. The cornerstone of restoring heart function is cardiomyocyte renewal. New cardiomyocytes need to integrate and couple electrically and metabolically to the surviving tissue, and beat in synchrony to prevent arrhythmias. This requires CPC differentiation into adult-like cardiomyocytes that can integrate into the three-dimensional structure of the myocardium. Cell engraftment may be enhanced by limiting cell migration. However, to be able to engraft and differentiate, the cells need to survive in the hostile environment after MI. A possible solution to cell death is to prepare the cells so they are able to escape apoptosis and necrosis, evoked by the ischemic and inflamed nature of the infarcted area. Survival may also be enhanced by blood vessel formation, and subsequent restoration of oxygen and nutrient availability. During MI, restoration of blood flow is essential for the survival of the myocardial tissue surrounding the necrotic infarct, and to a great extent for the following repair process. Vascularization may be improved by stimulating the intrinsic regenerative capacity for microvascular formation and arteriogenesis, or by the formation of new blood vessels through CPC differentiation.

It is clear that many challenges need to be tackled before we can proceed with the development of a true cardiac regenerative therapy. To date, most cellular and molecular mechanisms involved in CPC differentiation or the intrinsic regeneration of the injured adult heart remain far from understood. This, together with the limited functional improvements gained by existing regenerative strategies has sent scientists back to the drawing board. New innovative methods are needed to improve CPC differentiation and transplantation efficiency or to potentiate the intrinsic regenerative capacity of the heart.

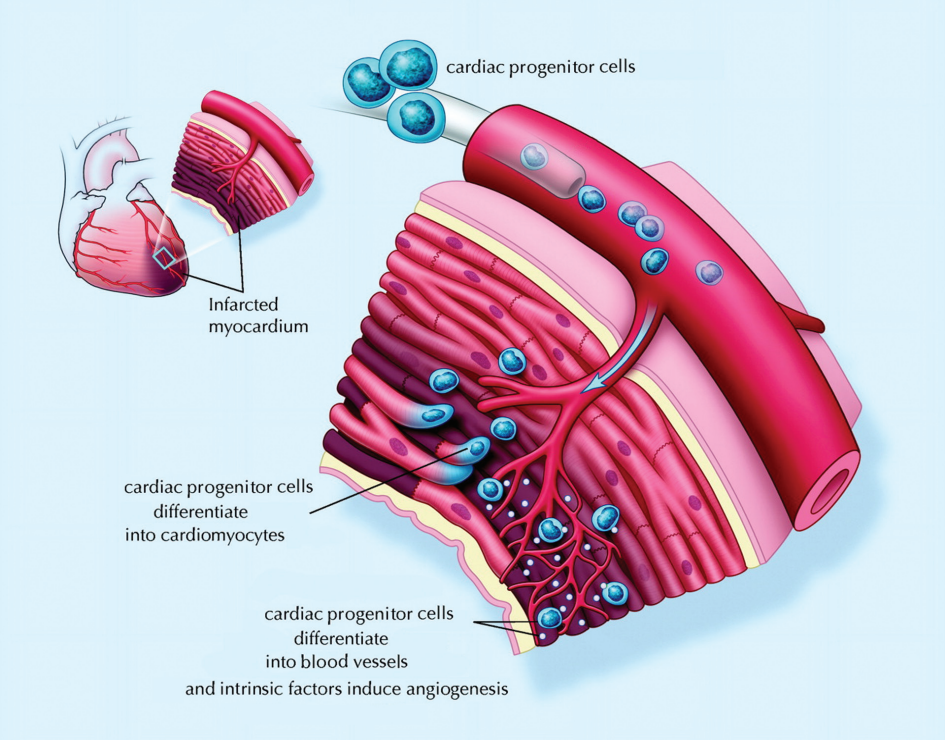

Figure 1 Cardiac regeneration by CPC transplantation and intrinsic regeneration. Adapted from [68]

MicroRNAs

Recent research has suggested that one of the factors crucial to the success of cardiac regenerative medicine might have appeared in the form of small non-coding endogenous regulatory RNAs, termed microRNAs (miRNAs). The first miRNA, lin-4, was identified in 1993[69], before the discovery of the mechanism of RNA interference[70], which was awarded the 2006 Nobel Prize for physiology and medicine. Remarkably, miRNAs were not recognized as a distinct class of biological regulators with conserved functions until 2001, when these small non-coding regulatory RNAs were first termed miRNAs[71-73]. The evidence indicating that these miRNAs were members of a whole new class of abundant, highly conserved post-transcriptional regulators of gene expression triggered an exciting, and still ongoing age of research into the physiological and pathological roles of miRNAs.

MiRNAs are non-coding single-stranded RNAs of approximately 22 nucleotides (nt) that inhibit the expression of specific messenger RNA (mRNA) targets through Watson-Crick base pairing between the miRNA 'seed region'; positions 2 to 8 from

the 5′, and sequences in the 3′ untranslated region (UTR) of their target mRNAs[74]. MiRNAs are generally transcribed by RNA polymerase II into primary transcripts termed pri-miRNAs. Pri-miRNAs are characterized by the presence of one or more imperfect complementary hairpin structures with a stem of approximately 33 base-pairs[74]. Approximately half of known miRNAs are clustered in the genome and transcribed as large primary transcripts containing more than one miRNA, whereas

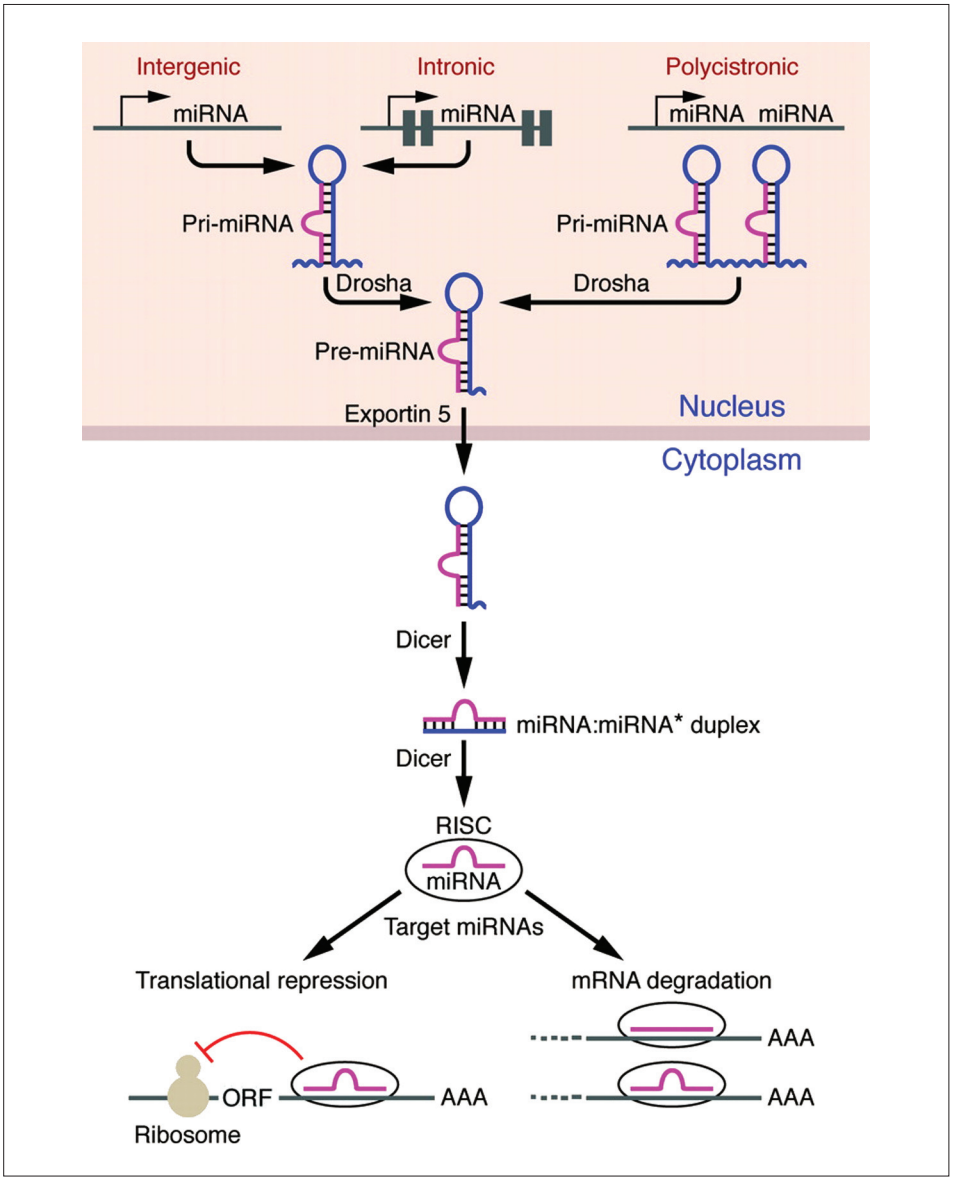

Figure 2 miRNA biogenesis. See text for details. Adapted from[86]

the rest are expressed as individual transcripts from intergenic or intronic locations. After transcription, pri-miRNAs are processed in the nucleus into approximately 70 nt precursor miRNAs (pre-miRNAs) by the double-stranded RNA-specific type III endoribonuclease Drosha, and the double-stranded RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8)[75,76]. The nuclear export factor exportin 5 binds correctly processed pre-miRNAs and transports them to the cytoplasm[77] where they are processed by the endoribonuclease III Dicer into a miRNA duplex of approximately 22 base-pairs[78]. Once cleaved by Dicer, the duplex unwinds and one strand of the duplex, named the mature miRNA or guide strand, is selected by the argonaute protein and loaded into the RNA-induced silencing complex (RISC)[79] which, depending on the degree of complementarity between the miRNA 'seed region' and its mRNA target sequence[80], results in translational blockage or mRNA degradation[81,82].

The use of deep-sequencing technologies has greatly accelerated miRNA discovery and to date, over 1500 mature miRNAs have been identified in the human genome, many of which are highly conserved among species[83]. MiRNA gene regulatory networks are highly complex, as each specific miRNA can target up to several hundred distinct molecules of mRNA, and each mRNA can in its turn be targeted by many different miRNAs, which individually may have multiple target sites in the specific mRNA[84,85]. MiRNAs provide broad and robust transcriptional regulation that can be governed by individual miRNAs or the combined action of multiple miRNAs. The action of an individual miRNA can lead to a cumulative reduction in expression of multiple components of one specific functional network, and several miRNAs may cooperatively target various mRNAs whose protein products are part of the same molecular pathway. MiRNAs may target both positive and negative regulatory components, providing well-balanced pathways. It has also been shown that intronic miRNAs may regulate the same cellular processes as the protein encoded by the host gene. Undoubtedly, miRNA gene regulation is sophisticated and highly orchestrated, and has an impact on many, if not all biological processes in humans. Above all, evidence is accumulating suggesting particularly important roles for miRNAs in disease, including cardiovascular disease.

MIRNAs IN CARDIOVASCULAR DISEASE AND REPAIR

Although the gene mutations, proteins pathways, and cellular mechanisms responsible for various cardiovascular disorders have been explored at length, it has only recently become clear that miRNAs fulfill vital roles in cardiovascular development and disease.

The first evidence for an indispensable general role of miRNAs in normal mammalian development came from analysis of mice deficient of the miRNA processing enzyme Dicer, which resulted in embryonic lethality between E12.5 and E14.5[87]. This study showed that Dicer-deficiency resulted in severe defects in vascular development due to a lack of blood vessel formation. The apparent crucial role of miRNAs in angiogenesis was reinforced by data showing that conditional ablation of Dicer from endothelial or vascular smooth muscle cells resulted in defective blood vessel development[88,89]. The essential role of miRNAs in cardiogenesis was shown by cardiac progenitor (nkx2-5) conditional knockout of dicer. The developing embryos exhibited pericardial edema and a poorly developed ventricular myocardium, leading to cardiac failure and death before E12.5[90]. In addition, alpha myosin heavy chain (α-MHC) conditional deletion of dicer led to rapidly progressive dilated cardiomyopathy, heart failure, and postnatal lethality within 4 days after birth[91]. In adult mice, inducible, α-MHC conditional dicer gene deletion provoked spontaneous and severe cardiac remodeling with high mortality rates[92]. Moreover, cardiomyocyte-specific deletion of dgcr8, which is specifically required for miRNA processing, resulted in dilated cardiomyopathy and premature lethality[93]. Clearly, the knockout of Dicer or Dgcr8 and subsequent loss of all miRNAs has lethal consequences. However, to date, not a single specific miRNA knockout has been found to result in complete embryonic lethality in mice. This suggests a significant redundancy in miRNA function and shows that embryonic lethality in Dicer or Dgcr8 knockouts reflects the cooperative functions of many miRNAs. Nevertheless, single miRNA knockouts have been shown to lead to a high degree of lethality caused by significant defects in vascular development, as was shown for miR-126[94,95], and defects in cardiac development, in the case of miR-1 and miR-133a[90,96].

The Dicer and Dcrg8 knockout studies have established the direct role of miRNAs in normal heart and vascular development and function, and suggested that miRNAs are likely to be involved in cardiac disease. This led to several miRNA profiling studies, which used microarray platforms to measure individual miRNA expression levels in the healthy heart versus heart failure, showing a significant number of differentially regulated miRNAs[97-101], of which only eighteen account for more than 90% of all miRNAs present in the heart[93]. The identification of the most prominent heart failure associated miRNAs led to a tremendous upsurge in research into the roles of these specific miRNAs in various cardiovascular disease processes. To date, nearly all of the differentially expressed miRNAs have been investigated in various experimental models of heart failure, and researchers are slowly piecing together a picture of how miRNAs regulate the different processes associated with the progression towards heart failure. From 2006 onwards, several key studies, using various *in vivo* models of heart failure, have provided key insight on the actions of a collection of miRNAs in

the different processes associated with MI and the progression to heart failure. These studies have shown the important roles of miR-195, 208a, 133, 23a, 199b, and 499 in cardiac hypertrophy[102-108], miR-29, 133a, and 30 in cardiac fibrosis[109-111], miR-499, 24, and 15b in cardiomyocyte survival[112-114], miR-126, 92a, 210, and 24 in cardiac angiogenesis[95,115-117], miR-143 and 145 in vessel remodeling[118,119], and miR-208a in cardiac metabolic control[120]. These studies show that miRNA function becomes especially pronounced under conditions of stress, and underlines the important role of miRNAs in disease. In addition, some of these studies indicate that miRNAs may be used to enhance intrinsic repair processes after MI.

While the number of miRNAs implicated in cardiac function under stress is rapidly expanding, very little is known regarding miRNA regulation of stem cell, and in particular CPC differentiation and function. Increasing our knowledge in this area may eventually help to optimize cell-based therapies for cardiac regeneration. Next to implicating miRNAs in cardiac development and disease, dicer and dgcr8 knockout studies have also identified miRNAs as critical regulators of ESC self-renewal and differentiation[121-123]. However, to date only a handful of miRNAs have been implicated in regulating cardiovascular lineage differentiation. The muscle-related miR-1 and 133 were found to regulate cardiac muscle cell fate decisions[124-126]. MiR-145 was shown to promote SMC differentiation from fibroblast, neural crest stem cells[127], and ESCs[128]. MiR-1 and 10a regulate ESC-SMC differentiation[129,130], and miR-99b, 181a, and 181b were shown to induce endothelial cell differentiation from ESCs[131].

Taken together, these studies show that miRNAs are important regulators in cardiovascular disease and stem cell renewal and differentiation. This suggests that modulation of specific miRNA expression may be used to stimulate CPC differentiation and function *in vitro* and *in vivo*, which may enhance the success of cardiac regenerative therapies. In addition, miRNAs may provide interesting novel therapeutic targets to promote cardiac repair processes after MI, especially because miRNAs can easily be up- or downregulated *in vivo* through so-called miRNA mimics or antagomiRs. However, before these novel therapies may be developed, we first need to identify specific miRNAs that regulate cardiovascular lineage differentiation and function of CPCs, and further increase our understanding of the role of specific miRNAs in regulating endogenous cardiac repair responses.

SCOPE OF THIS THESIS

The aim of this thesis was to investigate the role of miRNAs in various processes necessary to optimize cardiac repair.

Chapter 2 compares the miRNA expression profile of proliferating hCMPCs with hCMPCs differentiated into cardiomyocytes, and further describes the indispensable roles of miR-1 and miR-499 in inducing the cardiomyogenic differentiation of hCMPCs via targeting of Sox6, a transcription factor involved in cell fate determination. In **Chapter 3**, we show that miR-1 is also involved in the angiogenic differentiation of hCMPCs, and targets Spred1, a negative regulator of growth factor-induced ERK/mitogen-activated protein kinase activation. These first two chapters show that miRNAs can be used to direct and improve the differentiation of human cardiomyocyte progenitor cells (hCMPCs) into the three cardiovascular lineages. In **Chapter 4**, we present data showing that miR-155 inhibits necrotic cell death in hCMPCs, which was mediated by targeting RIP1, a protein required for programmed necrotic cell death. The same miRNA was investigated in **Chapter 5**, showing that miR-155 inhibits hCMPC migration by targeting MMP16, an enzyme involved in the breakdown of extracellular matrix. These two chapters show that miRNAs can also be used to increase hCMPC survival and regulate their migratory capacity, providing the possibility of improving cell survival and cell retention upon cell injection. In **Chapter 6**, we provide a detailed overview and small history of the various applications of small RNA technology *in vivo*, including the current methods for *in vivo* modulation of miRNA function. In **Chapter 7**, we used a highly effective and specific type of miRNA silencer, termed antagomiR, to explore the role of miR-214 in regulating angiogenesis in various *in vivo* models. We show that miR-214 negatively regulates angiogenesis by targeting the RNA-binding protein Quaking (QKI) and reducing pro-angiogenic growth factor secretion. This study indicates that miRNA modulation may be used to enhance vascularization and presents the opportunity to improve tissue regeneration after MI.
Finally, in **Chapter 8**, the results and conclusions of the studies described in this thesis are discussed, together with future directions.

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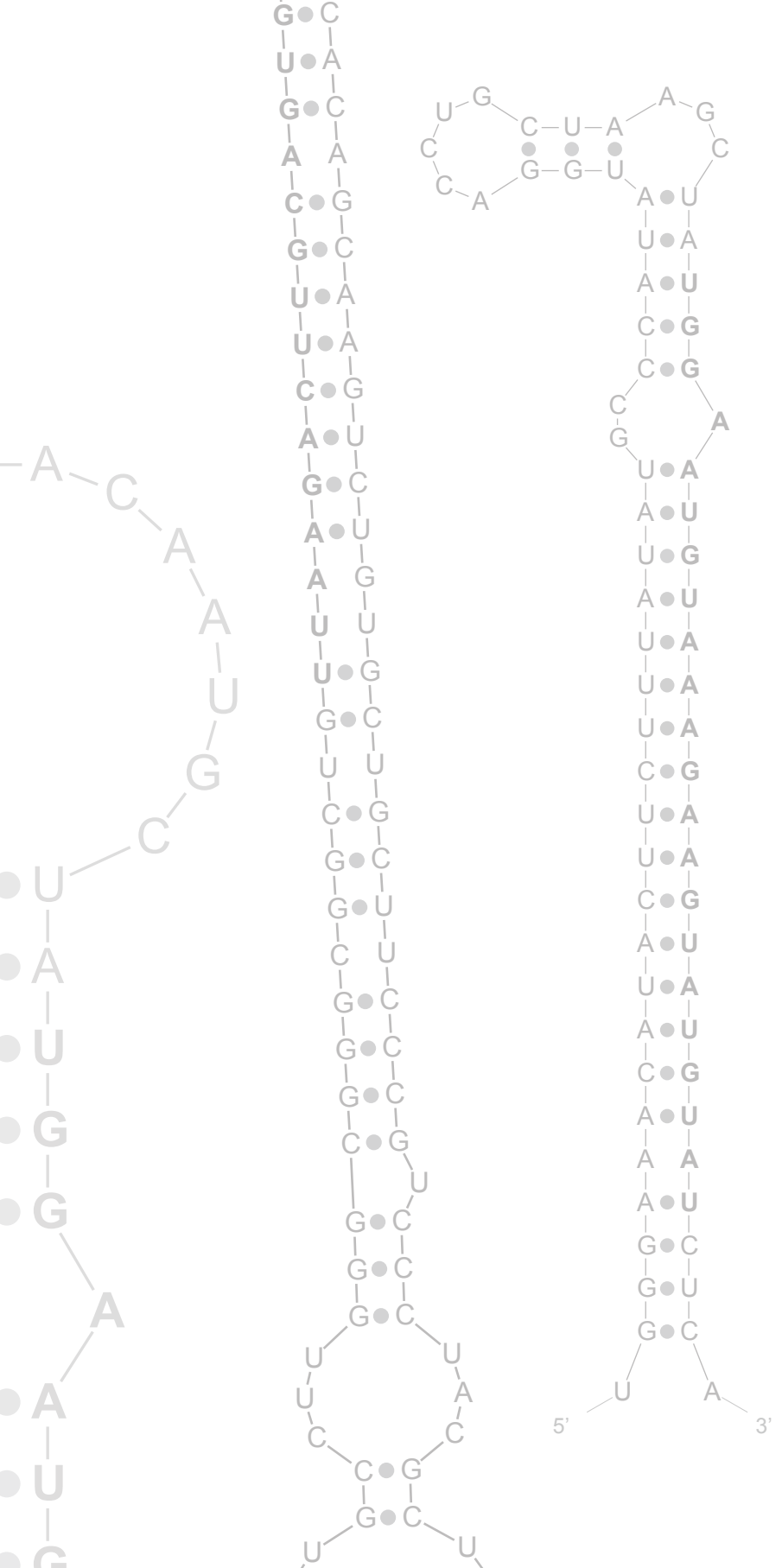
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CHAPTER 2

MICRORNA-1 AND -499 REGULATE DIFFERENTIATION AND PROLIFERATION IN HUMAN-DERIVED CARDIOMYOCYTE PROGENITOR CELLS

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ABSTRACT

Objective

To improve regeneration of the injured myocardium, it is necessary to enhance the intrinsic capacity of the heart to regenerate itself and/or replace the damaged tissue by cell transplantation. Cardiomyocyte progenitor cells (CMPCs) are a promising cell population, easily expanded and efficiently differentiated into beating cardiomyocytes. Recently, several studies have demonstrated that microRNAs (miRNAs) are important for stem cell maintenance and differentiation via translational repression. We hypothesize that miRNAs are also involved in proliferation/differentiation of the human CMPCs *in vitro*.

Methods and Results

Human fetal CMPCs were isolated, cultured, and efficiently differentiated into beating cardiomyocytes. miRNA expression profiling demonstrated that muscle-specific miR-1 and miR-499 were highly upregulated in differentiated cells. Transient transfection of miR-1 and -499 in CMPC reduced proliferation rate by 25% and 15%, respectively, and enhanced differentiation into cardiomyocytes in human CMPCs and embryonic stem cells, likely via the repression of histone deacetylase 4 or Sox6. Histone deacetylase 4 and Sox6 protein levels were reduced, and small interference RNA (siRNA)-mediated knockdown of Sox6 strongly induced myogenic differentiation.

Conclusion

miRNAs regulate the proliferation of human CMPC and their differentiation into cardiomyocytes. By modulating miR-1 and -499 expression levels, human CMPC function can be altered and differentiation directed, thereby enhancing cardiomyogenic differentiation.

INTRODUCTION

The old paradigm that the heart is a terminally differentiated organ, lacking the capacity for self-renewal and regeneration of the damaged myocardium, is being challenged, because resident cardiac progenitor cells have been identified[1–3] that are able to differentiate into cardiomyocytes, as well as other cardiac cell types, such as endothelial cells or fibroblasts.[4–7] These progenitor cells can be isolated, expanded *in vitro*, and transplanted into the damaged rodent myocardium, thereby improving cardiac performance. Recently, we have isolated human cardiomyocyte progenitor

cells (hCMPCs) from fetal hearts and adult biopsies that can be expanded in culture and efficiently differentiated into beating cardiomyocytes, without the need for co-culture with neonatal cardiomyocytes.[8–10] We tested the functional relevance of these hCMPCs by transplanting them into ischemic murine cardiac tissue, which resulted in improved cardiac performance up to 3 months.[11] Human cells were still present, including in situ differentiated cardiomyocytes and vascular structures. Little is known about what drives proliferation and differentiation of hCMPCs *in vitro*, and their exact role and regulation *in vivo* are still unexplored.

MicroRNAs (miRNAs) were shown to be important for the posttranscriptional regulation of target genes and serve important regulatory functions in a range of biological processes, including maintenance of stem cell–ness and modulation of differentiation.[12] miRNAs are short (19 to 23 nucleotides), noncoding small regulatory RNAs that are loaded into the RNA-induced silencing complex, recognize the 3′-untranslated region (UTR) of target mRNAs, and thereby regulate their expression by translational repression or mRNA degradation.[13] Recently, several papers focused on the differential expression of miRNAs in cardiac pathology, identifying clusters of differentially expressed miRNAs among different human cardiomyopathies.[14,15] Furthermore, the contribution of specific miRNAs in muscle differentiation and cardiac hypertrophy was described.[16,17]

Here, we analyzed miRNA expression during the growth and after cardiomyogenic differentiation of hCMPCs. We explored two identified miRNAs, miR-1 and -499, in more detail for their function in progenitor cells, and we show that miR-1 and -499 repress hCMPC proliferation and enhance *in vitro* differentiation into cardiomyocytes.

MATERIALS AND METHODS

Human cardiomyocyte progenitor cells

Human fetal heart tissue was collected after elective abortion, followed by Langendorff perfusion with Tyrode's solution, collagenase, and protease treatment. Single cells were obtained and hCMPCs were isolated and characterized as described.[8–10] Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. Cells were cultured in M199/EGM (3:1, Gibco) supplemented with 10% FCS (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml epithelial growth factor (EGF), 5 ng/ml insulin like growth factor (IGF-1) and 5 ng/ml hepatocyte growth factor (HGF). To induce differentiation, cells were treated with 5 μM 5′-azacytidine (Sigma) for

72 hours in differentiation medium (Iscove's Modified Dulbecco's Medium/ HamsF12, 1:1, Gibco) supplemented with L-Glutamine (Gibco), 2% horse serum, non-essential amino acids, Insulin-Transferrin-Selenium supplement, and 10-4 M Ascorbic Acid (Sigma), followed by TGF-β stimulation.[8–10] In addition, lentiviral expression of Sox6 and HDAC4 RNAi was used to knock down their expression prior to the start of differentiation. Knock down efficiency was confirmed by flow cytometry as explained below.

Analysis of miRNA expression by microarray

Low-molecular-weight RNA was isolated from proliferating and differentiated hCMPCs by using the mirVana™ RNA Isolation Kit (Ambion). The miRNA expression profile was determined by microarray analysis using the µParaflo™ microfluidic chip (MiHuman_8.2- Based on Sanger miRBase Release 8.2, LC Sciences), according the manufacturer.

Quantitative RT-PCR for miRNA expression

Total RNA was isolated with mirVana™ RNA Isolation Kit or Tripure isolation reagent (Roche Applied Science). In brief, 10 ng purified total RNA was used for reverse transcription (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) followed by Taqman® MicroRNA Assays for quantification of miR-1, 133a, 499, 126, 155, 206, 208, and RNU19 (Applied Biosystems: 4373161, 4373142, 4373224, 4378064, 4373124, 4427975, 4427975, and 4373378, respectively), according to the manufacturer's conditions. Amplification and detection of specific products was performed in a MyIQ single-color real-time PCR system (Bio-Rad) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The threshold cycle (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to RNU19 (ΔCt value). The relative difference in expression levels of each miRNA in proliferating and differentiated hCMPCs (ΔΔCt) was calculated and presented as fold induction ($2^{-\Delta\Delta Ct}$).

In situ hybridization

In situ hybridization to determine hsa-miR-499 localization was performed as previously described.[19] In brief, 10 µm thick sections of human fetal heart tissue were hybridized O/N at 46ºC with 5 nM LNA DIG-labeled probes (Scramble-miR, Exiqon, 99001-01 and hsa-miR-499, Exiqon, 38306-01) in denaturizing hybridization buffer, incubated with an anti-DIG-alkaline phosphatase antibody (1:1500, Roche, 11093274910), an anti-PECAM (1:200, Santa Cruz, sc-1505), and an anti-α-actinin antibody (Sigma) at RT for two hours. After washing, sections were incubated with

fluorescent-labeled secondary antibodies, exposed to Fast-Red Substrate-Chromogen (DAKO, K0597) O/N at RT and mounted in VectaShield (Molecular Probes, Amdsterdam).

Quantitative RT-PCR

Total RNA was isolated with TriPure reagent, cDNA was prepared using the SuperScript First-Strand Synthesis System for RT-PCR (170-8890, Bio-Rad), and amplification was detected in a MyIQ single-color real-time PCR system. Amplifications were performed with iQ™ SYBR® Green Supermix (170-8884, Bio-Rad). Used qRT-PCR conditions are: 5 min at 95°C, followed by 40 cycles of 15s at 95°C, 30s at specific annealing temperature (Sup Table I), and 45s at 72°C, followed by melting curve analysis to confirm single product amplification. Messenger RNA expression levels were normalized to β-actin mRNA expression (ΔCt value), and relative differences were calculated (ΔΔCt value) and presented as fold induction ($2^{-\Delta\Delta Ct}$).

hCMPC transfection

Pre-miR™ miRNA Precursor molecules for miR-1: (5'-UGGAAUGUAAAGAAGUAUGUA-3', PM10660), miR-499: (5'-UUAAGACUUGCAGUGAUGUUUAA-3', PM10496), and a miRNA negative control (AM17121) with or without FAM™ dye-label were obtained from Ambion. Inhibitors for miR-1, miR-499, and negative controls (scr) were obtained from Dharmacon Scientific (Thermo Fisher Scientific Inc., IH-300586-06, IH-300837-06, and IN-001005-01, respectively). hCMPCs were transfected with siPORT™ NeoFX™ Transfection Agent (Ambion) and pre- and anti-miRs (0-100 nM), according to the manufacturer's guidelines.

Transfection efficiency of pre-miR was confirmed by RT-PCR (Taqman miRNA assay) and visually by means of FAM-labeled negative control pre-miR. For fluorescence microscope analysis of FAM-labeled pre-miR expression, cells were washed with PBS and fixated with 4% paraformaldehyde in PBS for 15 min at RT. Nuclei were stained with 0.2 µg/ml Hoechst (Invitrogen) and mounted in VectaShield (Molecular Probes, Amsterdam).

To test whether miR-1 and -499 had an effect on cardiomyocyte differentiation, hCMPCs were stimulated for three consecutive days with 5–aza and followed by miRNA transfection as described above. After overnight transfection, differentiation medium was changed every three days, but without TGF-beta stimulation.

hCMPC proliferation assay

Cells were seeded in a 0.1% gelatin coated 96-well plate and transfected as described above. After four and six days, culture medium was removed and cells were treated

with 0.5 mg/ml of Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich) in RPMI serum free medium (Gibco) and incubated at 37°C for three hours. Subsequently, purple formazan crystals, formed by mitochondrial reductase enzymes, were solubilized in 0.1 ml DMSO and absorbance was measured at 540 nm with a reference filter of 690 nm in a photometric plate reader (Labsystems Multiskan Ascent).

Cell viability assay

Cells were transfected with 100 nM scr-miR, miR-1, and miR-499 and cellular viability was measured by staining for 7-AAD and Annexin V (BD Pharmingen 556421, 559925) after three days, followed by flow cytometric analysis according to manufacturer's protocol.

Western blot

Total protein was extracted using TriPure, and 33 µg, reduced with DTT, was separated by PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked (5% non-fat-dry milk, PBS-0.1% Tween), probed with polyclonal rabbit-anti-human HDAC4 (12171, GeneTex), Sox6 (S7193, Sigma), and α-actinin (sarcomeric, EA-53, A7811, Sigma) and goat anti-rabbit IgG secondary antibodies (P0448, Sigma-Aldrich) in blocking-buffer. Signal was visualized with enhanced chemiluminescence (Amersham) and bands were scanned using a ChemiDoc XRS system (Bio-Rad).

Embryoid body assay

Mouse Embryonic Stem Cells (mESCs), E14-IB10, were cultured in BRL-conditioned medium as described before.[18] mESCs were differentiated by generating embryoid bodies (EBs) via hanging drops. mESCs were transfected with pre-miR-1, -499, and negative control (scr) prior to body formation. EBs were monitored at day seven for percentage of beating EBs, followed by plating them on gelatin coated coverslips allowing visualization of α-actinin fibers via immunohistochemistry. Fifty EBs were collected per condition after four and eight days to check differentiation rate by RT-PCR, as described below.

Luciferase experiments

Primers were generated for the 3'UTR of Sox6 (human and mice 87% homology, all sites conserved), a putative target of miR-499, and extended with restriction sites (see Sup Table I). After RT-PCR, the 3'UTR of Sox6 was inserted into pMIR-REPORT™ miRNA Expression Reporter Vector (#5795, Ambion) by restriction site directed ligation at the 3'UTR of CMV-Luc.

In a 24-wells plate, HEK293 cells were transfected with 200 ng plasmid, containing the CMV-Luc and 3'UTR of Sox6, with and without 30 nM of miR-499, miR-1, or scr-miR, and 200 ng CMV-Bgal for normalization. To block miR-499 activity, an LNA-probe for miR-499 was used at 30 nM. After 48 hours, luciferase and β-galactosidase activity was measured with the Luciferase Assay System and β-galactosidase Enzyme Assay System (Promega), respectively. Cells transfected with the LUC plasmids only were normalized to 1. To confirm specificity of miR-499 and its putative sites, mutational cloning in the seed region of the predicted sites was performed with the QuikChange® Site-Directed Mutagenesis Kit (200518, Stratagene), according to manufacturer's guidelines (see Supplemental Figure IIC for sites). The successful creation of mutations was confirmed by sequencing (data not shown).

RNAi

HEK293 cells were transfected with pLKO.1-puro-shRNA-Sox6 or HDAC4 (Mission library, Sigma) to produce lentiviral transduction particles. hCMPCs were transduced with shRNA-Sox6 or a scrambled (off-target) control for 24 hours, washed and grown in the presence of puromycin (2 ug/ml) for seven days, followed by flow cytometric analysis.

Flow cytometry

Selected cells were trypsinized, fixated in 2% PFA for 15 minutes, followed by permeabilization for 15 minutes in 0.5% NP-40 in PBS, washed with PBS and incubated with Sox6 antibody (1:200, S7193, Sigma) for one hour at 4°C, washed and incubated with anti-FITC secondary antibody (1:600, R&D systems), and measured using a flow cytometer (BD Biosciences). To quantify total cell numbers expressing Sox6, an isotype control was used to set the negative gate. Total number of cells expressing Sox6 / total number of cells was calculated.

Statistical analysis

Data is presented as mean ± SEM of at least three independent experiments and were compared using the two-tailed paired Student's t test or one-way ANOVA, a difference with a $p<0.05$ was considered to be statistically significant.

RESULTS

Expression of miRNAs in proliferating and differentiated hCMPCs

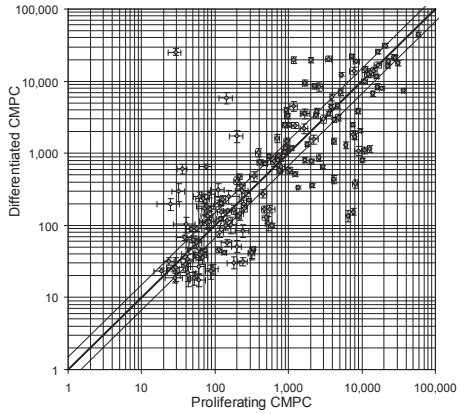
miRNA expression was determined in both proliferating and differentiated hCMPCs

by comprehensive miRNA microarray analysis. From all human targeted miRNAs (total, 453), 188 miRNAs (42%) were detectable in proliferating hCMPCs and 195 (43%) in differentiated hCMPCs. Of these, 19 showed a 4-fold or higher increase (Figure 1A and 1B) and 27 showed a 4-fold or higher decrease (Figure 1A and 1C) in expression on differentiation. Among the highly upregulated miRNAs (Figure 1B), several cardiac and skeletal muscle–specific miRNAs were present, such as miR-1, miR-133a, and miR-133b. In addition to these known muscle-specific miRNAs, other miRNAs were highly regulated as well, including miR-499, a miRNA that is functional but has not yet been studied.

To confirm miRNA expression changes, we performed quantitative TaqMan-based RT-PCR. The expression of miR-1, miR-133a, and miR-499 was significantly increased and miR-126 and miR-155 significantly decreased in differentiated hCMPCs after normalization to RNU19 (Figure 1D–1H). Some discrepancy exists between the observed fold increase in array and quantitative RT-PCR, which is most likely due to the relatively low values in proliferating cells which were used for calculating the ratios. Although miR-206 and -208 were not detected by the microarray because of technical limitations, they are upregulated in differentiated cardiomyocyte progenitor cells (Supplemental Figure I). We selected the strongest regulated miRNAs, miR-1 and miR-499, for functional follow-up studies to investigate their significance for the myogenic differentiation of hCMPCs.

Localization and tissue distribution of miR-499

miR-1 is a muscle-specific miRNA that is known to control myogenic differentiation in the embryonic mouse heart.[20] However, the function of miR-499 is currently not known, and its tissue distribution has not been investigated extensively.[21] This highly conserved miRNA (Supplemental Figure IIA) is located in intronic region 20 of human MYH7B (myosin heavy chain 7B cardiac muscle β) on chromosome 20 (Supplemental Figure IIB). Because we observed high miR-499 levels in differentiated hCMPCs, we investigated and confirmed the endogenous presence of miR-499 in cardiomyocytes *in vivo* by in situ hybridization for miR-499 in fetal and adult human hearts, as demonstrated by co-staining with cardiac α-actinin (Figure 2A, 2C, and 2F); however, miR-499 was not present in endothelial cells (Figure 2E). This myocyte-specific expression was confirmed when analyzing several cell types for miR-499 expression (Figure 2G). Subsequently, analysis of mouse heart, brain, spleen, liver, lung, quadriceps muscle, kidney, and gut tissue showed that the mature miR-499 is abundantly expressed in cardiac tissue and absent in other tissues, including skeletal muscles (Figure 2H). Interestingly, MYH7B mRNA is also restricted to heart muscle (Figure 2I), indicating that both MYH7B and miR-499 are cardiac enriched.



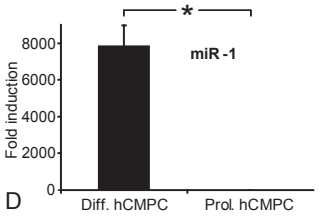
A

B

	miRNA ID	Proliferating CMPC	Differentiated CMPC	log2	fold induction
1	hsa-miR-1	26.1	24058.8	9.91	962.3
2	hsa-miR-499	10.9	1372.2	7.07	133.9
3	hsa-miR-133a	9.7	715.5	6.28	77.6
4	hsa-miR-133b	15.6	727.8	5.72	52.9
5	hsa-miR-335	128.9	5829.0	5.42	42.8
6	hsa-miR-450	32.2	643.7	4.40	21.2
7	hsa-miR-542-3p	12.5	245.1	4.21	18.5
8	hsa-miR-143	1143.5	19731.0	4.04	16.5
9	hsa-miR-217	8.8	122.1	3.79	13.8
10	hsa-miR-204	27.9	330.7	3.68	12.8
11	hsa-miR-203	20.0	181.2	3.54	11.6
12	hsa-miR-210	31.3	295.4	3.35	10.2
13	hsa-miR-145	2015.3	19156.3	3.34	10.2
14	hsa-miR-422b	68.4	630.5	3.21	9.3
15	hsa-miR-218	221.0	1798.7	3.10	8.6
16	hsa-miR-214	3645.1	20597.9	2.55	5.9
17	hsa-miR-152	1709.7	10020.7	2.51	5.7
18	hsa-miR-30d	918.3	4009.8	2.07	4.2
19	hsa-miR-374	59.6	249.2	2.06	4.2

C

	miRNA ID	Proliferating CMPC	Differentiated CMPC	log2	fold repression
1	hsa-miR-146a	7683.9	155.1	-5.59	48.1
2	hsa-miR-155	6457.5	127.6	-5.53	46.2
3	hsa-miR-126	8375.6	380.6	-4.48	22.3
4	hsa-miR-19a	192.5	12.1	-3.99	15.9
5	hsa-miR-31	10019.8	781.9	-3.66	12.7
6	hsa-miR-222	13140.2	1124.8	-3.48	11.2
7	hsa-miR-221	11128.8	1057.9	-3.32	10.0
8	hsa-miR-20b	4227.6	439.9	-3.27	9.6
9	hsa-miR-542-5p	181.9	20.2	-3.13	8.7
10	hsa-miR-625	121.3	13.3	-3.08	8.4
11	hsa-miR-663	9058.1	1057.9	-3.04	8.2
12	hsa-miR-602	303.5	42.3	-2.95	7.7
13	hsa-miR-612	226.6	29.3	-2.93	7.6
14	hsa-miR-323	309.9	44.1	-2.93	7.6
15	hsa-miR-29b	121.7	17.9	-2.84	7.2
16	hsa-miR-299-5p	331.7	47.2	-2.81	7.0
17	hsa-miR-494	2099.8	353.4	-2.60	6.1
18	hsa-miR-212	190.2	33.6	-2.57	5.9
19	hsa-miR-18a	591.4	99.8	-2.57	5.9
20	hsa-miR-638	37354.6	7219.1	-2.33	5.0
21	hsa-miR-329	540.3	110.4	-2.32	5.0
22	hsa-miR-106a	5998.6	1252.7	-2.27	4.8
23	hsa-miR-17-5p	8154.2	1645.0	-2.22	4.6
24	hsa-miR-20a	9392.7	2119.2	-2.21	4.6
25	hsa-miR-19b	2901.6	660.8	-2.14	4.4
26	hsa-miR-505	202.7	50.0	-2.09	4.3
27	hsa-miR-15b	7703.7	1734.1	-2.03	4.1



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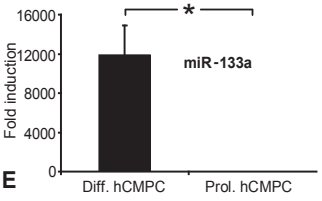

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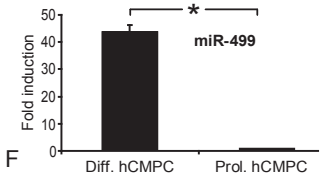

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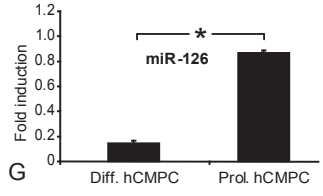

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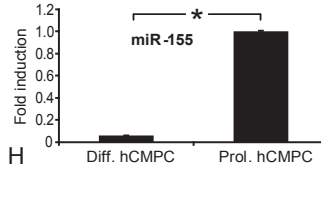

H

Figure 1

miRNA profiling of proliferating hCMPCs and hCMPCs differentiated to cardiomyocytes (A). Variation in detection of each miRNA on the array is plotted in the figure (axes are arbitrary expression units). miRNAs are shown that are highly upregulated (log2 difference) (B) or downregulated (log2 difference) (C) in differentiated hCMPCs (numbers in proliferating and differentiated CMPCs are arbitrary expression units). Quantitative RT-PCR for miR-1 (D), miR-133a (E), miR-499 (F), miR-126 (G), and miR-155 (H) in proliferating hCMPCs (prol. CMPC) and hCMPCs differentiated to cardiomyocytes (diff. CMPC). Data are presented as fold induction +SEM; *p<0.05

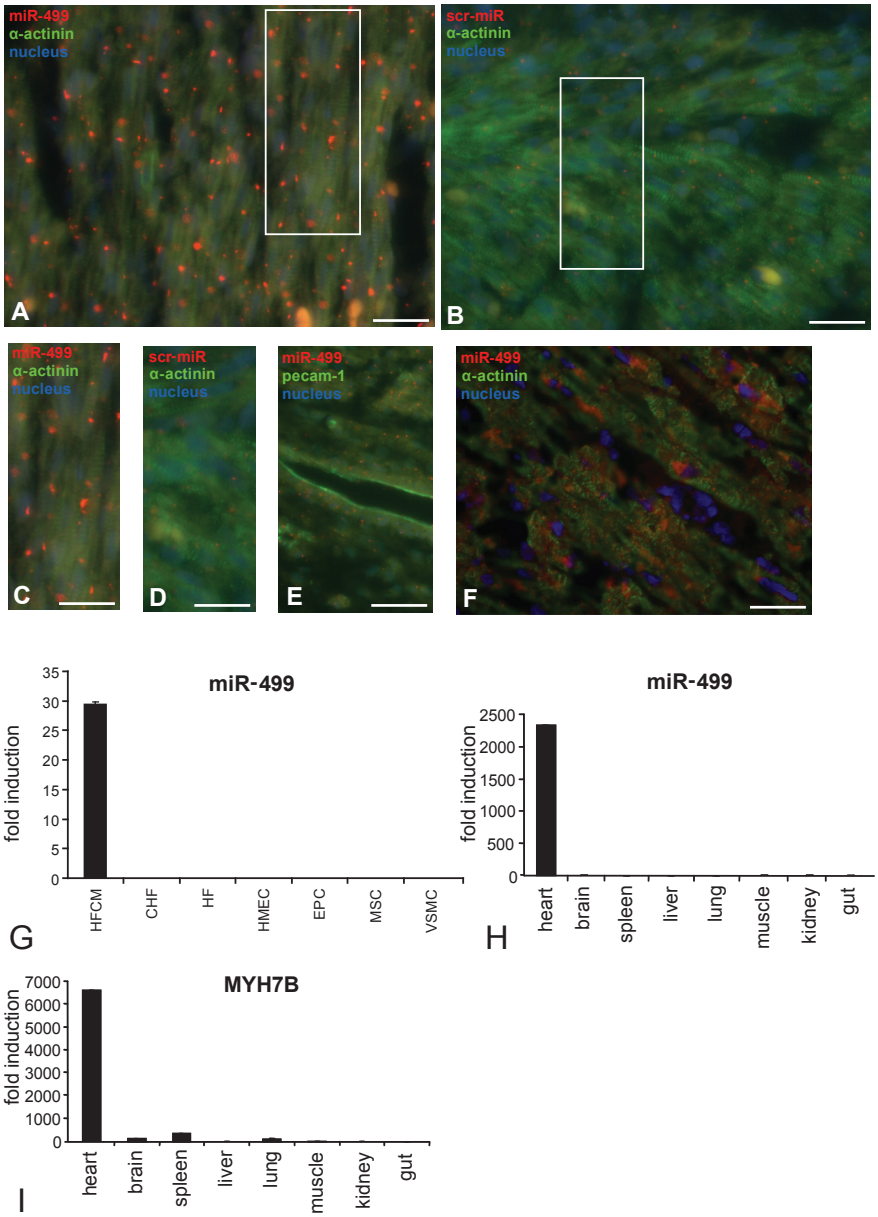

Figure 2.
In situ hybridization for miR-499 (A and C, red dots) and a scramble control probe (B and D) in human fetal heart, double stained for α-actinin (A through D, green) or PECAM-1 (E, green). miR-499 (red) localization in adult human heart, double stained for α-actinin (F, green). Nuclei are stained blue. Bar is 50 μm in A, B, E, and F and 15 μm in C and D. G, Quantitative RT-PCR for miR-499 in human fetal cardiomyocytes (HFCM), cardiac human fibroblasts (CHF), human adventitial fibroblasts (HF), human microvascular endothelial cells (HMEC), endothelial progenitor cells (EPC), mesenchymal stem cells (MSC), and vascular smooth muscle cells (VSMC) (G, all human origin). miR-499 (H) and MYH7B (I) expression in different mouse tissues. Expression is presented as fold induction +SEM and compared with the lowest expression (N=5).

Functional analysis of miR-1 and miR-499 in hCMPCs: cellular proliferation

Because expression of miR-1 and miR-499 increased on differentiation, we used a gain-of-function approach using premiR molecules to study their roles in proliferating hCMPCs. A FAM-labeled negative control premiR was used to optimize transfection procedures (Figure 3A and 3B). A dose-dependent signal was observed on transfection of FAM-premiRs, visible up to 12 days after transfection in proliferating hCMPCs (data not shown). To verify the transfection efficiency, we analyzed mature miR-1 and -499 expression by TaqMan-based RT-PCR. We observed an increase in miR-1 and -499 levels with increasing transfection concentrations, as compared to endogenous expression levels in control hCMPCs (Figure 3C and 3D).

Four days after transfection, the increased miR-1 levels resulted in a significant reduction in cell proliferation up to 25% (100 nM) compared with scr transfected control cells (Figure 3E), as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Transfection of miR-499 in hCMPCs resulted in a significant reduction in cell proliferation as well (15% with 100 nM; Figure 3E). When using similar concentrations of the scramble control miRNA, no significant effect on proliferation was observed (Figure 3E). These effects could still be observed after six days, but a dilution of the miRNA resulted in a less pronounced effect (data not shown). The observed effects were not caused by reduced cell viability (see Supplemental Figure IIIA) or by an increase in cellular apoptosis/necrosis (data not shown). Moreover, by overexpressing a different miRNA expressed in hCMPCs, miR-155, we did not observe an inhibitory effect on proliferation (Supplemental Figure IIIB).

Functional analysis of miR-1 and miR-499 in hCMPCs: differentiation into cardiomyocytes

A prerequisite for differentiation is inhibition of proliferation. Because addition of miR-1 and -499 reduced hCMPC proliferation, and increased levels of these miRNAs are present in differentiated hCMPCs, we studied whether we could enhance cardiomyocyte differentiation by the addition of miR-1 and -499 to our differentiation protocol (only 5-azacytidine [5-aza] stimulation).[8,9,10] Normally, spontaneous beating clusters of differentiated cells are observed after 21 days. However, when adding miRNAs individually, we observed spontaneous beating areas as soon as six days after initiation of differentiation (Online Video). This suggests that the differentiation of our progenitor cells is greatly enhanced by transfection of miR-1 or miR-499. To quantify this, we studied mRNA levels for different cardiomyocyte-specific genes by quantitative RT-PCR after two weeks of differentiation. We observed that the expression of cardiac troponin T, α-cardiac actinin, and Mlc-2v was increased on addition of miR-1 and -499 (Figure 4A through 4H).

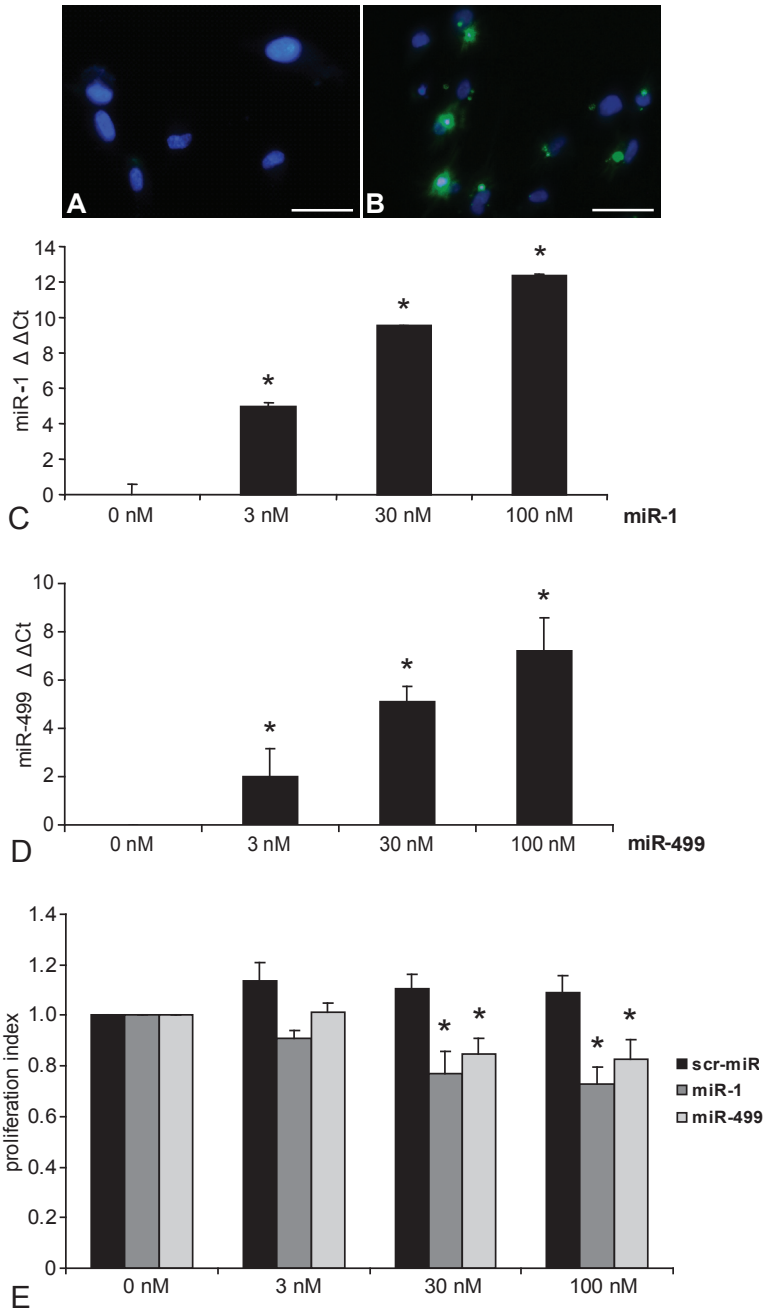

Figure 3
Immunofluorescent detection of untransfected (A) and 100 nM (B) FAM-labeled control miRNA transfected hCMPCs. Nuclei are stained blue. Bar is 25 μm. Transfection with increased concentration of miR-1 (C) or miR-499 (D) resulted in increased detectable expression of mature miR-1 or -499, respectively, as detected by TaqMan RT-PCR. Data are expressed as Ct +SEM. Proliferation of hCMPCs was determined four days after transfection of increased concentrations miR-1, miR-499, and scr-miR (E). *$p<0.01$

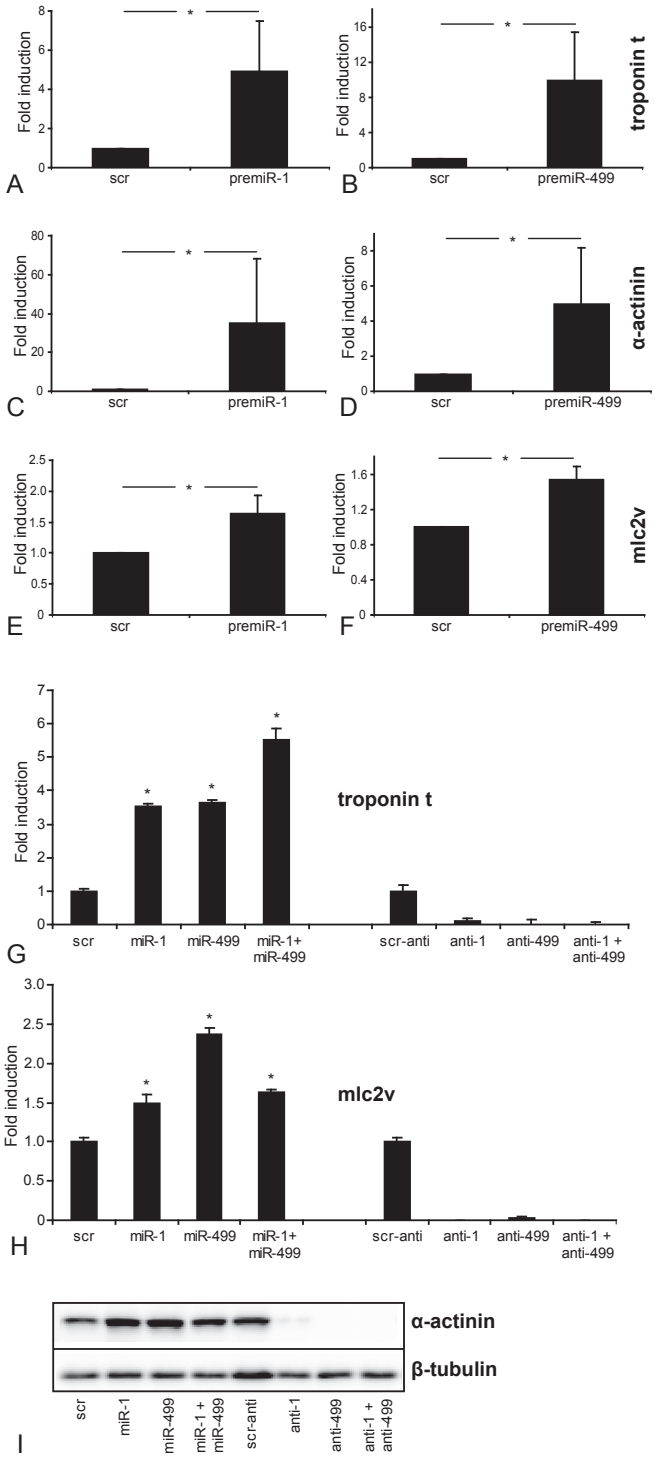

Figure 4
hCMPCs were differentiated into cardiomyocytes by 5-aza stimulation and transfected with 100 nM scr-miR, miR-1 (A, C, and E), or miR-499 (B, D, and F). mRNA expression was determined after two weeks by RT-PCR for troponin T (A and B), cardiac α-actinin (C and D), and Mlc-2v (E and F) and compared with scr-miR-transfected cells. Troponin T mRNA (G), Mlc-2v mRNA (H), and α-actinin protein (I) levels were determined two weeks after transfection of hCMPCs with miR-1 or -499, inhibitors for miR-1 or -499 (anti), a combination of miRs or inhibitors, or their controls (scr and scr-anti). Data are presented as mean fold increase +SEM; N=4. β-Tubulin was used as a loading control.

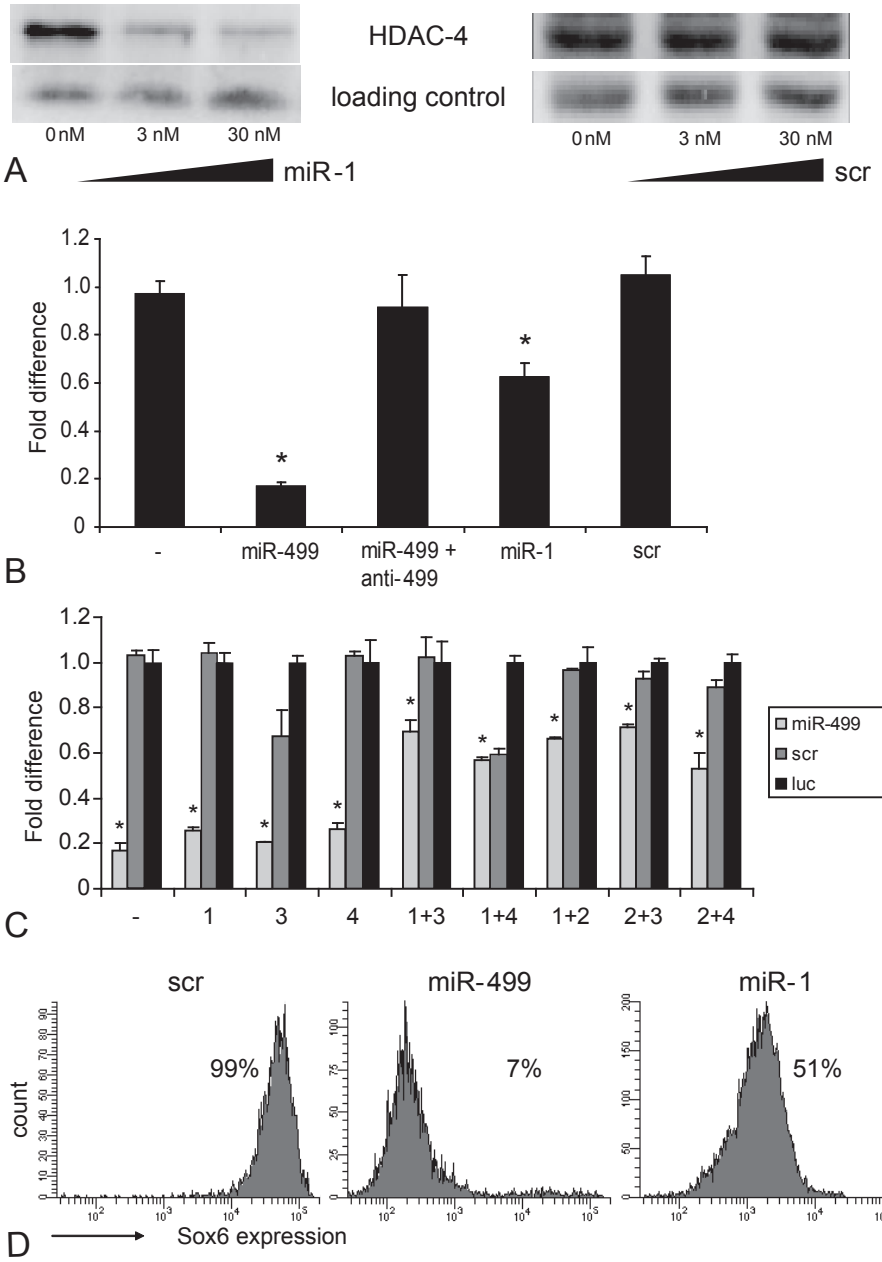

Figure 5

Proliferating hCMPCs were transfected with increased concentrations of miR-1, resulting in decreased concentrations of HDAC4 protein expression (A). The Sox6-3′UTR-luciferase reporter construct was co-transfected with miR-499 (30 nM), a specific inhibitor of miR-499 (anti-499, 50 nM), miR-1, or a scramble miR (30 nM) in HEK293 cells (B). Luciferase reporter assay with mutated miR-499 binding sites (sites 1 to 4) in the Sox6 3′UTR (C).“-“ indicates the wildtype Sox6-3′UTR; 1 to 4 indicate mutated seed regions as in Supplemental Figure IC. Sox6 expression was measured by flow cytometric analysis in hCMPCs three days after transfection of 100 nM scr-miR, miR-499, or miR-1 (D). *p<0.05

To study whether the presence of these miRNAs is a prerequisite for cardiomyogenic differentiation, we used specific inhibitors for miR-1 and miR-499. Compared with the control (scr-anti), cardiomyogenic differentiation was blocked, as determined by troponin T mRNA, Mlc2v mRNA, and cardiac α-actinin protein expression, two weeks after the start of differentiation (Figure 4G through 4I). Combining the two premiRs or antimiRs did not result in an additional general enhancement or inhibition of myogenic differentiation (Figure 4G through 4I).

Interestingly, miR-1 and -499 also enhanced the cardiac differentiation of mouse embryonic stem cells. After treatment with premiR-499 and premiR-1, the percentage of beating embryoid bodies increased by 2.7 and 6.2-fold, respectively, compared to scr-premiR (Supplemental Figure IVA). Enhanced differentiation by introducing miR-499 and -1 was confirmed by increased immunofluorescent α-actinin staining (Supplemental Figure IVB through IVE) and by increased levels of GATA4 mRNA expression after four days (Supplemental Figure IVF) as an early marker, and Mlc-2v at eight days (Supplemental Figure IVG) as a late marker.

miR-1 and -499 targets and potential mechanism

miR-1, which we found to enhance hCMPC differentiation into cardiomyocytes, has previously been reported to promote myogenesis by targeting HDAC4, a transcriptional negative regulator of muscle gene expression.[22] By transfecting miR-1 in hCMPCs, we showed that HDAC4 protein levels were indeed reduced by miR-1 upregulation (Figure 5A).

Because miR-499 function has not been studied before, we used online algorithms (TargetScan and Sanger) to predict potential miR-499 targets that could be involved in myogenic differentiation. Both algorithms predicted four conserved target sites in the transcription factor SOX6 (sex determining region Y–box 6), which is involved in muscle differentiation (Supplemental Figure II).[23] To verify the potential inhibitory effect of miR-499 on Sox6 translation, we cloned the 3′ UTR of Sox6 in a cytomegalovirus-driven luciferase reporter construct. Transient transfection of HEK293 cells with the Sox6-3′UTR-luciferase reporter construct in combination with premiR-499 resulted in a reduction in luciferase activity that could be restored when a specific miR-499 inhibitor was included (Figure 5B). The 3′UTR of Sox6 also contains a predicted target site for miR-1 (Supplemental Figure IIC), and indeed, co-transfection of miR-1 and the Sox6-3′UTR-luciferase reporter construct led to a mild reduction in luciferase activity. When using a scramble miRNA, this effect was not observed (Figure 5B). To confirm the specific binding of miR-499, we mutated two nucleotides in the seed region of the predicted sites. Although single target site mutations (Figure 5C) did not show a reduction in luciferase activity, mutating two sites did result in a

significant reduction in suppression capacity of miR-499 (Figure 5C). In addition, mutating the miR-1 target site reduced the inhibitory effect of miR-1 on the Sox6-3'UTR-luciferase reporter construct (Supplemental Figure V). To demonstrate that Sox6 is a target of miR-1 and -499 in hCMPCs, we transfected miR-499 and miR-1 in hCMPCs and analyzed Sox6 protein levels by flow cytometry, showing that Sox6 protein levels are decreased by miR-1 and -499 (Figure 5D).

Next, we analyzed miR-1 and -499 expression in proliferating, 5-aza–treated, and fully differentiated hCMPCs. On 5-aza treatment, miR-1 levels were mildly induced, whereas miR-499 expression remained similar. The expression of their target genes (HDAC4 and Sox6) was reduced upon 5-aza treatment (Supplemental Figure VI), suggesting that this is a prerequisite for differentiation. To confirm that Sox6 is indeed important for the myogenic differentiation of hCMPCs, Sox6 expression was knocked down via lentiviral-mediated expression of short hairpin RNA against Sox6 (Figure 6A). hCMPCs, deficient for Sox6, stopped proliferating and displayed enhanced expression of muscle markers (Figure 6B and C) after 14 days of differentiation. The induction of differentiation was significantly higher than with standard 5-aza treatment. We were unable to evaluate the role of HDAC4 because cell viability was dramatically affected and myogenic differentiation was not achieved when HDAC4 expression was knocked down (data not shown).

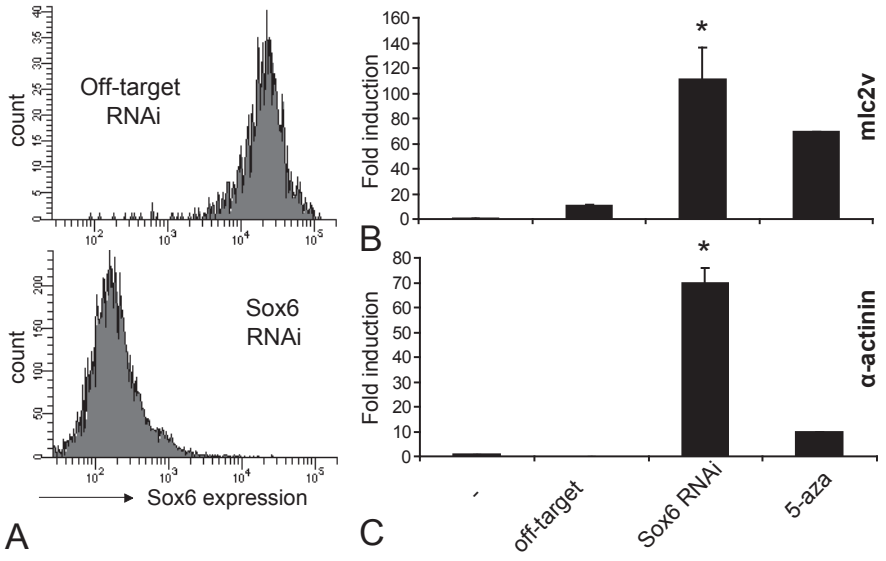

Figure 6
RNA interference (RNAi) mediated knockdown of Sox6 in hCMPCs, as shown by flow cytometric analysis for Sox6 expression (A). Myogenic differentiation was induced as indicated by increased expression of mlc-2v (B) and cardiac α-actinin (C) after 14 days of differentiation.

DISCUSSION

The transcriptional regulation of cardiomyocyte differentiation is highly conserved and requires sequential activation and/or repression of different genetic programs.[24,25] Specific disruption of Dicer, an essential miRNA-processing enzyme, in mouse cardiac progenitor cells resulted in embryonic lethality due to cardiac failure, pointing to an indispensable role of miRNAs in cardiac development and cardiomyocyte differentiation.[20] We observed that hCMPCs express many different miRNAs, some of which highly regulated after differentiation. In this study, we focused on miR-1 and -499[26] and on whether they could be used *in vitro* to induce cardiomyocyte differentiation.

miR-1 is a highly conserved miRNA with a cardiac and skeletal muscle-specific expression pattern. Several essential cardiac transcription factors, such as Mef2, SRF, Nkx2.5, and GATA4, are able to bind to its regulatory region.[17] Several miR-1-targeted genes, such as Hand2,[17] TMSB4X,[17] HDAC4,[22] GJA1,[27] and KCNJ2,[27] play a role in cardiac development or function and have been experimentally confirmed. In miR-1 transgenic mice, the total number of cycling myocardial cells was decreased,[17] whereas in miR-1 knockout mice, an increased proliferation of cardiomyocytes was observed, resulting in severe heart defects.[20] This suggests that miR-1 fine-tunes the balance between cardiomyocyte proliferation and differentiation. Here, we demonstrated that miR-1 also regulates human cardiac-derived CMPC proliferation and differentiation into cardiomyocytes.

In Drosophila, loss of miR-1 is lethal, because it is required for determination or differentiation of cardiac or somatic muscle progenitor cells.[28] Similar observations were reported in embryonic stem cell differentiation, where miR-1 and miR-133 promote mesoderm formation but have opposing functions during further differentiation into cardiac muscle progenitors.[29] Furthermore, introducing miR-1 in C2C12 myoblasts promoted myogenesis by targeting HDAC4.[22] Accordingly, we were able to induce cardiac differentiation via miR-1 in our hCMPCs and mouse embryonic stem cells, with concurrent repression of HDAC4 protein levels. Moreover, we demonstrated that miR-1 function is indispensable for hCMPC cardiogenic differentiation by using a specific miR-1 inhibitor, which completely blocked differentiation. Full knockdown of HDAC4 led to cell death, and therefore myogenic differentiation could not be studied. miR-1 was also capable of partially blocking Sox6 protein expression, suggesting that a single miRNA can bind to more targets to direct cell fate, in this case myogenic differentiation. Taking these data together, miR-1 is a highly conserved miRNA that plays a role in cardiac muscle differentiation, not only in mouse and Drosophila, but also in human-derived cardiac progenitor cells.

Next to miR-1, we have also explored the role of miR-499 in hCMPCs. We observed that miR-499 is expressed in differentiated hCMPCs and, together with its host gene MYH7B, is strongly enriched in cardiac tissue. Another study suggested that miR-499 is expressed in cardiac, but also in slow skeletal muscles.[21] However, this might be caused by different percentages of fast and slow fiber expression in the different skeletal muscles that were used in both studies. miR-499 localization and its cellular expression suggests a role in cardiomyocyte homeostasis and/or maintaining the differentiated state of cardiomyocytes. Indeed, by transfection of miR-499 into hCMPCs and mouse embryonic stem cells, we could enhance their differentiation into cardiomyocytes. We identified Sox6 as a direct target of miR-499. Sox6, which is expressed in the heart and skeletal muscle,[30] contains four miR-499 target sites. Sox6 is a member of the Sox gene family of transcription factors (Sry-related HMG box) that play a key role in embryonic development and cell fate determination, however, its exact role is not clear.[23] Mice homozygous for a Sox6 null mutation show delayed growth, have abnormal muscle architecture, develop cardiomyopathies and die within two weeks after birth.31 In P19CL6 cells, an embryonic carcinoma cell line which is able to differentiate into beating cardiomyocytes, Sox6 is regulated by bone morphogenetic protein and is expressed only when the cells are committed to differentiate into beating cardiomyocytes, suggesting an association with the initiation of the cardiomyogenic program. Furthermore, because the L-type Ca2+ channel, which is critical for cardiomyocyte contraction, is repressed by Sox6,[23] Sox6 needs to be tightly regulated in developing and differentiating cardiomyocytes. In skeletal muscle of Sox6 null mice, an isoform-specific change in muscle gene expression has been observed, indicating that Sox6 is involved in muscle development and the tight balance between different muscle isoforms.[31] As indicated by our results, induction of miR-499 represses the expression of Sox6 in hCMPCs, leading to a reduction in cell proliferation and enhanced myocyte differentiation. Moreover, knockdown of Sox6 induces cardiomyogenic differentiation of hCMPCs, confirming the role of Sox6 in muscle differentiation. In addition, by using miR-499 inhibitors we showed that miR-499 function is required for cardiac differentiation. Thus, our data suggest that when cells are committed to the cardiac lineage and start to express MYH7B, miR-499 is co-expressed, thereby repressing Sox6 to further induce differentiation and modulate or fine-tune fiber expression in developing cardiomyocytes.

In summary, miRNA levels are highly regulated in cultured and differentiated cardiac-derived hCMPCs, with miR-1 and miR-499 expression being significantly higher in differentiated hCMPCs. Our results demonstrate that a single miRNA, miR-1, induces muscle differentiation, possibly via direct repression of HDAC4 and partly by repressing Sox6. Furthermore, miR-499, a miRNA whose function has not yet been explored, is

highly enriched in cardiac tissue, and enhances hCMPC differentiation into cardiomyocytes *in vitro* by targeting Sox6. These results demonstrate that miRNAs are powerful regulators driving hCMPC differentiation and that they can be used to influence cell fate. In addition, hCMPCs can be used as a model to study human *in vitro* developmental processes in order to better understand cardiac development and cardiomyocyte homeostasis.

Acknowledgements

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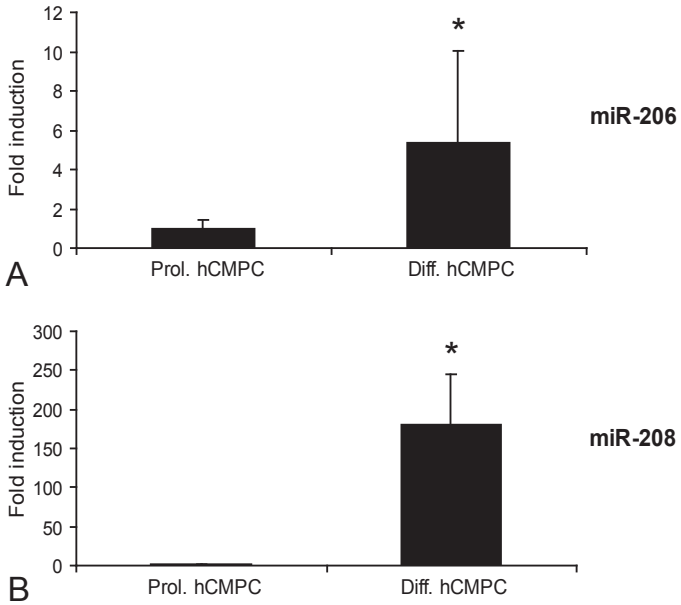
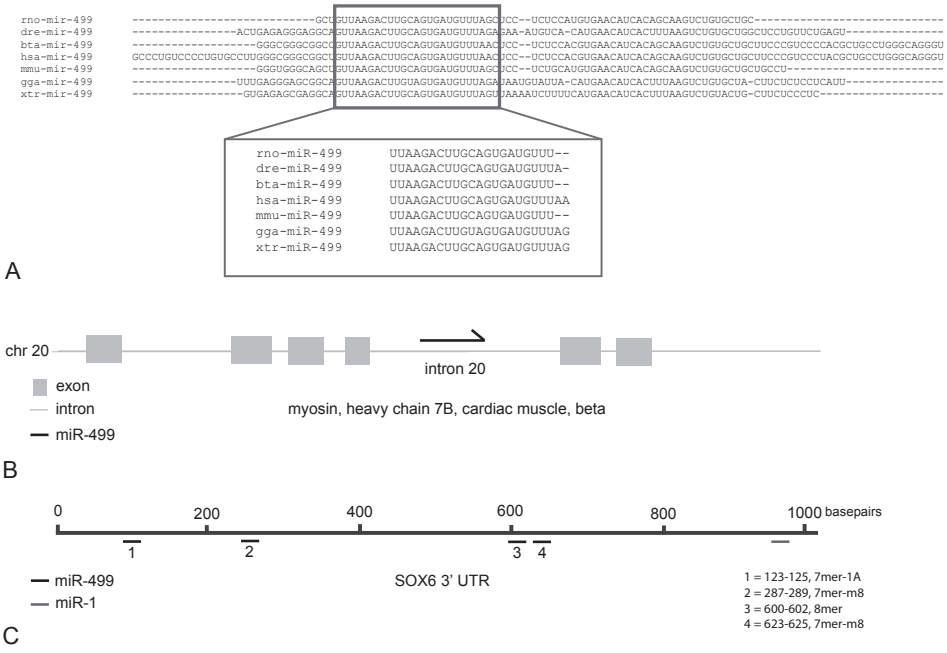
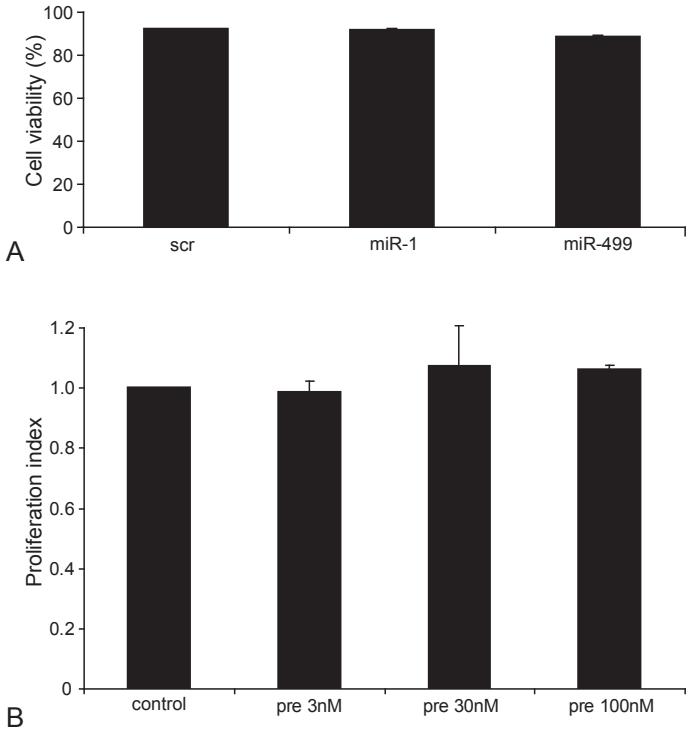


Supplemental Figure I
Quantitative RT-PCR for (A) miR-206 and (B) miR-208 in proliferating hCMPCs (prol. hCMPC) and hCMPCs differentiated to cardiomyocytes (diff. hCMPC). Data is presented as fold induction +/- SEM, * = $p<0.05$.

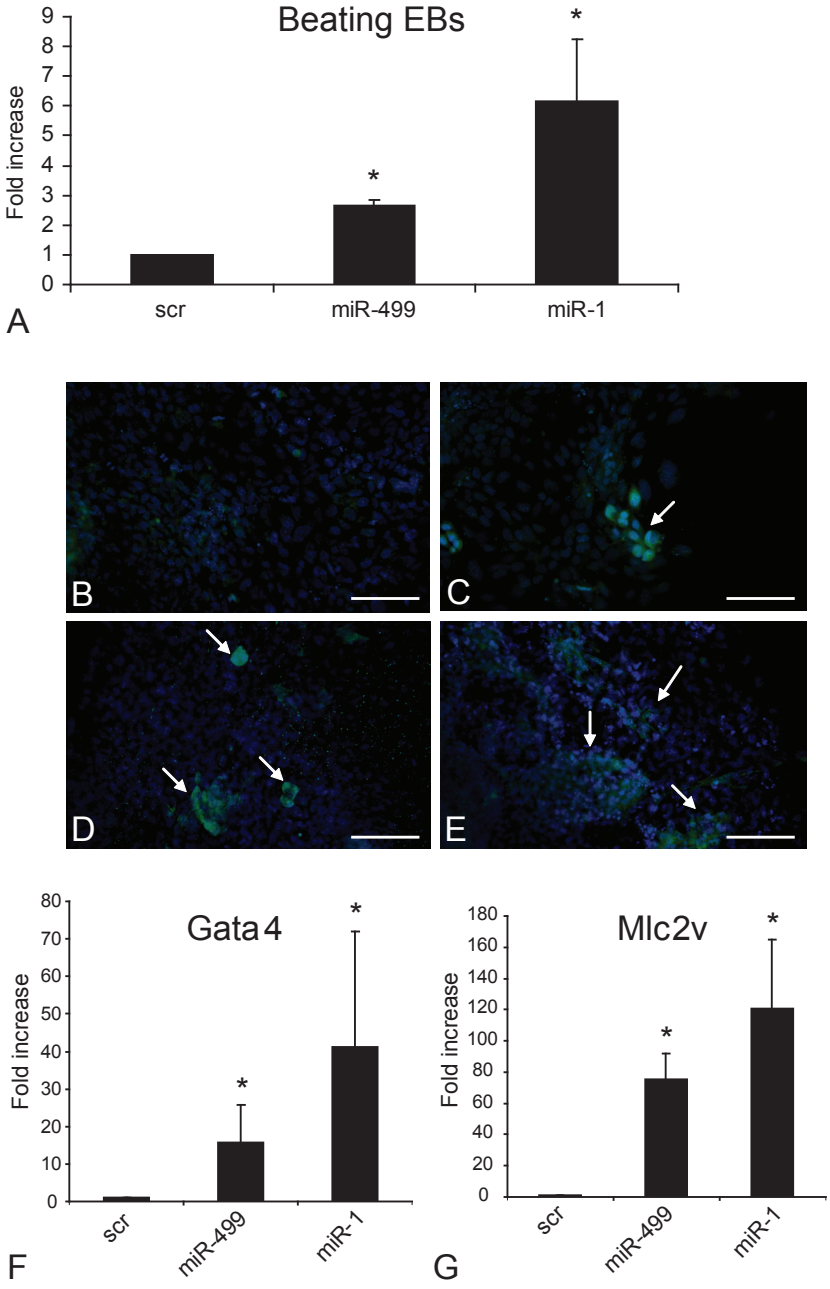


Supplemental Figure II

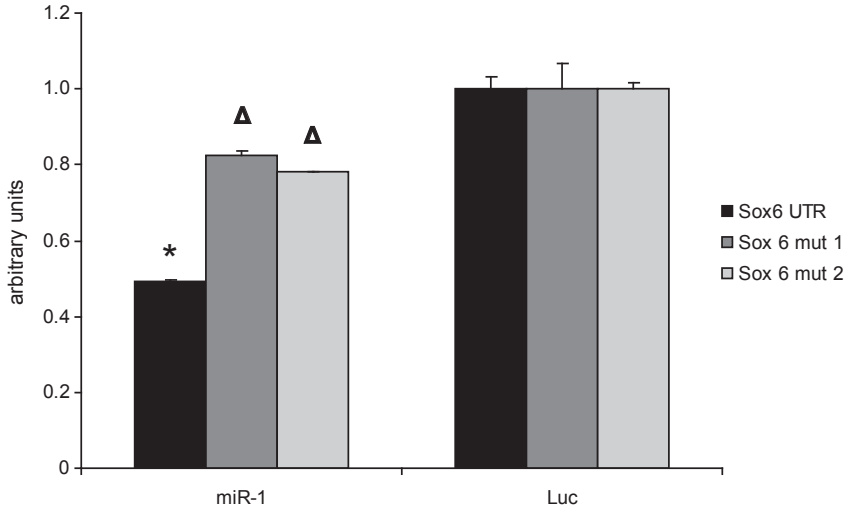
The sequence of miR-499 is well conserved among different species (A), moreover, also its localization within intron 20 of MYH7B (myosin heavy chain, cardiac muscle beta isoform 7B) is conserved among human and mice (B). The untranslated region (UTR) of human and mouse Sox6 has four predicted binding sites for miR-499, one 8-mer and three 7mer-m8. Moreover, one additional miR-1 binding site is present, one 7mer-1A (C).



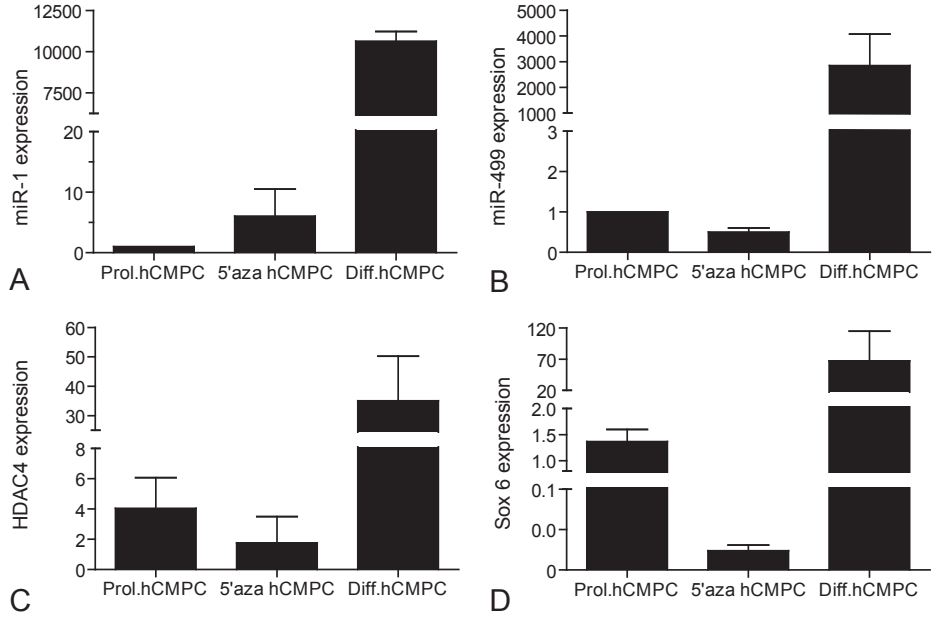
Supplemental Figure III
(A) Transfection of 100 nM scr-miR, miR-1, and miR-499 did not reduce cellular viability as measured by 7-AAD and AnnexinV staining and flow cytometric analysis. (B) Increasing concentrations of pre-miR-155 in hCMPC did not significantly change their proliferation rate.



Supplemental Figure IV
Mouse embryonic stem cells were transfected with 100 nM miRNAs and embryoid bodies were generated via hanging drops. (A) After seven days, the beating aggregates were counted. (B-D) Embryoid bodies were plated after seven days, grown for four days and stained for α-actinin (green, arrows), (B) not, (C) scr-premiR, (D) miR-499, and (E) miR-1 transfected. (Bar = 50 µm, nuclei are stained with Hoechst). GATA4 (F) mRNA expression was measured after four days, and Mlc-2v (G) after eight days of introducing miR-499 and miR-1.



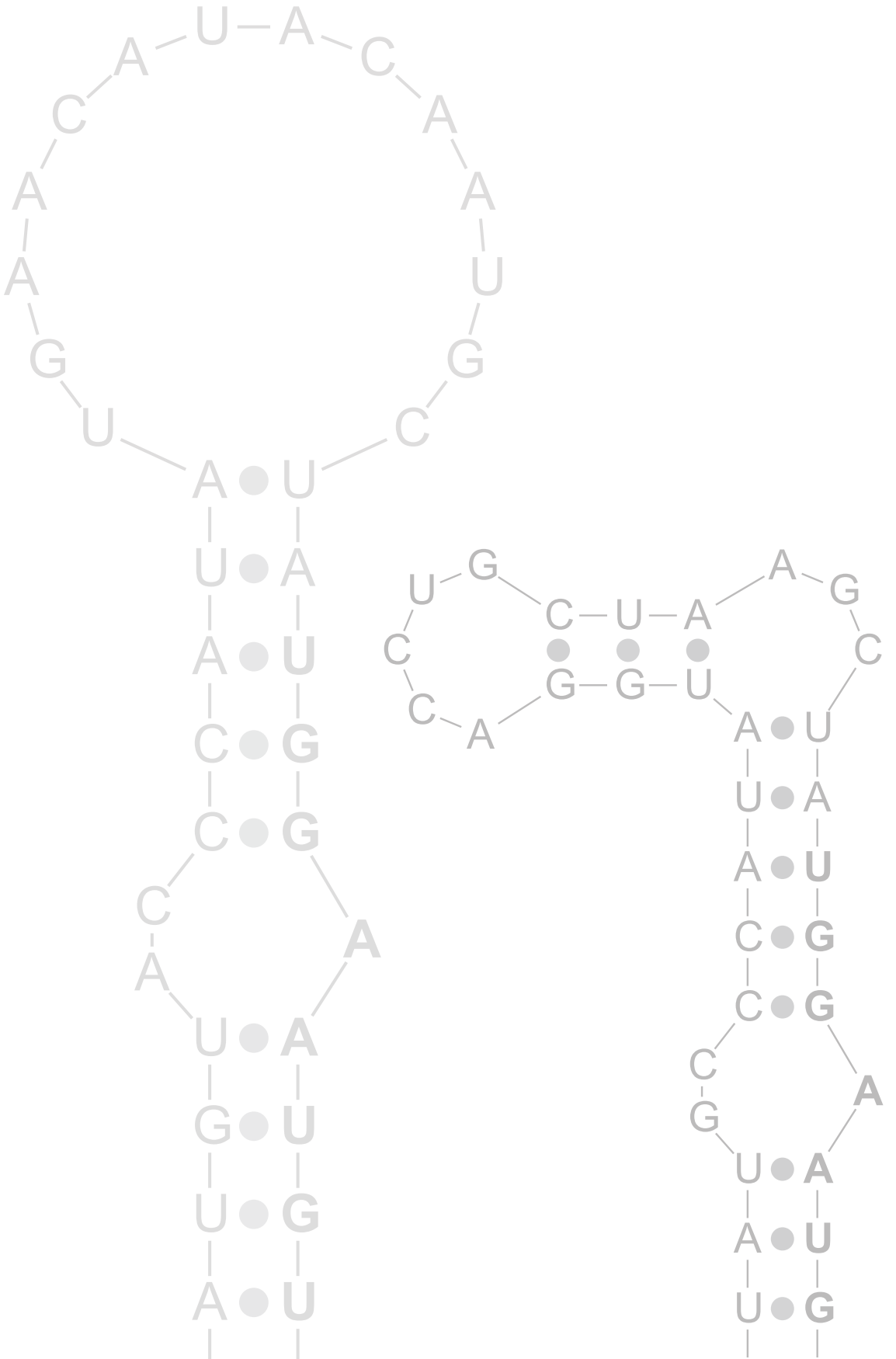
Supplemental Figure V
A CMV-Luciferase plasmid was transfected in HEK293 cells, containing the full 3'UTR of Sox6 or containing different mutations (mut 1: substitution of one nucleotide in seed region, mut 2: deletion of three sites in seed region). Cells were co-transfected with miR-1 (30 nM). (* $p<0.05$ full UTR vs LUC control, Δ $p<0.05$ mut 1 and 2 vs full UTR)



Supplemental Figure VI
miR-1 (A), miR-499 (B), HDAC4 (C) and Sox6 (D) mRNA expression in proliferating, 5-aza-treated and fully differentiated hCMPCs.

Supplemental Table I
Primer sequences that were used to determine mRNA expression and amplify the Sox6 3'-UTR (F=forward primer, R=reverse primer)

Gene	F/R	Sequence	Temp
Troponin T	F	GTGGGAAGAGGCAGACTGAG	62
Troponin T	R	ATAGATGCTCTGCCACAGC	62
β-MyHC	F	TCTTTCCCTGCTGCTCTC	60
β-MyHC	R	GACTGCCATCTCCGAATC	60
α-cardiac actin	F	ACCGACCTTGCTGTGAATC	60
α-cardiac actin	R	AATTGTGCTCCGAAACTAACC	60
β-actin	F	GATCGGCGGCTCCATCCTG	60
β-actin	R	GACTCGTCATACTCCTGCTTGC	60
α-SMA	F	ACTGGGACGACATGGAAAA	62
α-SMA	R	TTTGAGTCATTTTCTCCCGG	62
3'UTR Sox6	F	GGAGCTTTTGTTTGCTGAAT	58
3'UTR Sox6	R	TCCAGCAGGAAAAAGAGGTTA	58
HDAC4	F	CACGAGCACATCAAGCAACAA	55
HDAC4	R	CAGTGGTTCAGATTCCGGTGG	55
Sox6	F	AACCAAAGGCTAAAGGGCCTA	55
Sox6	R	CACCACCATGTTCAAAGGTGT	55



CHAPTER 3

MICRORNA-1 ENHANCES THE ANGIOGENIC DIFFERENTIATION OF HUMAN CARDIOMYOCYTE PROGENITOR CELLS

Under revision

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ABSTRACT

Instigated by the discovery of adult cardiac progenitor cells, cell replacement therapy has become a promising option for myocardial repair in the past decade. We have previously shown that human-derived cardiomyocyte progenitor cells (hCMPCs) can differentiate into cardiomyocyte-, endothelial-, and smooth muscle-like cells *in vitro*, and *in vivo* after transplantation in a mouse model of myocardial infarction, resulting in preservation of cardiac function. However, to allow successful repopulation of the injured myocardium, it is of key importance to restore myocardial perfusion by the formation of new vasculature. Several studies have shown that microRNAs regulate vascular differentiation of different stem/progenitor cells.

Here, we show that miR-1 is upregulated in hCMPCs during angiogenic differentiation. Upregulation of miR-1 enhanced the formation of vascular tubes on Matrigel and within a collagen matrix, and also increased hCMPC motility, as shown by planar and transwell migration assays. By western blot, qRT-PCR and luciferase reporter assays, miR-1 was found to directly target and inhibit the expression of sprouty-related EVH1 domain-containing protein 1 (Spred1), a protein that negatively regulates angiogenesis by controlling growth factor-induced activation of the MAP kinase cascade. Using a systems biology approach, we found that in hCMPCs, miR-1 is proposed to control a network of target genes predominantly involved in angiogenesis-related processes.

Our data shows that by upregulation of miR-1, the angiogenic differentiation of hCMPCs can be enhanced, which may be used as a new therapeutic approach to improve the efficiency of cell-based therapy for cardiac regeneration by enhancing the formation of new vasculature.

INTRODUCTION

Despite the progress that has been made in the prevention and treatment of myocardial infarction (MI), heart failure remains one of the leading causes of morbidity and mortality worldwide. Current treatment options cannot overcome the progressive decline in cardiac function caused by the loss of functional cardiomyocytes, which may result in end-stage heart failure, for which currently the only therapeutic options are heart transplantation or left ventricular assist devices. To regenerate the injured myocardium after myocardial infarction, a considerable amount of new cardiomyocytes are needed. In 2003, the first adult murine cardiac progenitor cells were described[1,2], leading to the discovery of the first adult human cardiac progenitor cells a few years

later[3-6]. Since then, it became increasingly clear that transplantation of these cells can lead to partial regeneration of the injured myocardium. Subsequently, cell replacement therapy and the concept of regenerative medicine became a promising option to stop the development of end-stage heart failure by regenerating both cardiomyocytes and vasculature[7-9]. However, cardiac regeneration is still limited, and to increase regeneration of the injured myocardium it is of key importance to restore myocardial perfusion, enabling free availability of oxygen and nutrients for the myocardium in need. This makes the formation of new vasculature vital to achieve true cardiac regeneration. We have previously shown that human-derived cardiomyocyte progenitor cells (hCMPCs) have the potential to form cardiomyocyte-, endothelial-, and smooth muscle-like cells *in vitro*, and *in vivo* after transplantation in a mouse model of myocardial infarction[5,10]. After transplantation in immunocompromised mice with induced myocardial infarction, hCMPCs were predominantly shown to contribute to the formation of new vasculature, leading to sustained cardiac function[10,11]. However, this effect was mainly due to paracrine effects. Therefore, the formation of new vasculature, and thereby cardiac regeneration, might be greatly enhanced if we can direct the hCMPCs to differentiate into de novo blood vessels.

MicroRNAs (miRNAs) have been described to tightly regulate both angiogenesis and stem/progenitor cell differentiation. We have previously shown that upregulating miR-1 and miR-499 potently enhances the cardiomyogenic differentiation of hCMPCs and mouse embryonic stem cell (ESCs)[12]. This positive effect of miR-1 and/or miR-499 on cardiac differentiation was further demonstrated in human ESCs and the functional relevance was shown *in vivo* after myocardial infarction[13-17]. MiRNAs were also shown to be key regulators of vascular differentiation[18-20]. Clearly, modulation of miRNA expression can be used to regulate the differentiation of adult cardiac progenitor cells and ESCs. Therefore, miRNAs are potential interesting candidates to enhance the angiogenic differentiation of hCMPCs. Compared to the laborious *in vitro* hCMPC myogenic differentiation protocol, *in vitro* angiogenic differentiation is achieved relatively easy by culturing hCMPCs on Matrigel. It is, however, unclear which underlying molecular signaling pathways regulate this process, and if specific miRNAs are important. In this study, we found that miR-1 is upregulated in hCMPCs cultured on Matrigel, indicating a possible role of this miRNA in the angiogenic differentiation. Indeed, we found that miR-1 upregulation enhanced the angiogenic differentiation of hCMPCs, thereby directly targeting sprouty-related EVH1 domain-containing protein 1 (Spred1), a protein that negatively regulates angiogenesis by controlling growth factor-induced activation of the MAP kinase cascade[21,22]. With this study we show that miR-1 overexpression enhances the angiogenic differentiation of hCMPCs, which can potentially be exploited to improve cardiac regeneration.

Cell culture

After obtaining individual informed consent, human fetal hearts were collected after elective abortion at week 17 to 19 of gestation. The investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Medical Ethics committee of the University Medical Center Utrecht. hCMPCs were isolated as described elsewhere[5,23,24]. hCMPCs were cultured on 0.1% gelatin in SP++ medium (22% EGM-2 Lonza, Basel, Switzerland, 66% M199, 10% FBS, 1% penicillin/ streptomycin, and 1% MEM nonessential amino acids, Invitrogen, Carlsbad, CA) and passaged 1:5 when they reached 80% confluence.

Taqman miRNA qRT-PCR

Total RNA was isolated with TriPure Isolation Reagent (Roche Applied Science, Penzberg, Germany) and treated with RNase-free DNase I (Qiagen, Venlo, The Netherlands). miR-1 and miR-126 expression was validated by TaqMan MicroRNA Assay (002222, 002228, Applied Biosystems, Foster City, CA) and normalized to the expression of the small nuclear RNA RNU19 (001003, Applied Biosystems) as previously described[25].

miRNA transfection

hCMPCs were reverse-transfected with 3, 30 or 100 nmol/L Pre-miR™ miRNA Precursor Molecules for miR-1 (PM10617, Ambion, Austin, TX) or Negative Control#1 (AM17110, Ambion), using siPORT™ NeoFX™ Transfection Agent (Ambion). Transfection efficiency was confirmed by transfection of FAM3™ dye-labeled Pre-miR™ Negative Control #1 (AM17121, Ambion) and miR-1 expression was assessed by TaqMan MicroRNA Assay as described. For fluorescence microscope analysis of FAM3-labeled negative control miR transfected cells, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at RT. Nuclei were stained with 0.2 µg/ml Hoechst 33342 dye (Invitrogen) and mounted in Fluoromount-G (SouthernBiotech, Birmingham, AL).

Matrigel assay

hCMPCs were transfected as described. The next day, 15.000 hCMPCs were seeded onto a Matrigel-coated (Merck Millipore, Billerica, MA) 24-well in SP++. After 16 hours, the cells were fixed with 4% PFA and blocked for 30 min using 0.1% saponin and 2% BSA. The cells were incubated with goat anti-PECAM-1 (1:200, sc-1505, Santa Cruz Biotechnology, Santa-Cruz, CA) and mouse anti-α-SMA (1:40, A2547, clone 1A4, Sigma-Aldrich, St. Louis, MO) for 1 h at RT, followed by incubation with Alexa Fluor®

555 donkey anti-goat (1:400, A21432, Invitrogen) and Alexa Fluor® 488 goat anti-mouse (1:400, A11001, Invitrogen). Nuclei were stained using 0.2 µg/ml Hoechst 33342 dye. Cells were embedded in mowiol and images were captured using cellP software (Olympus) on an Olympus BX60 microscope. Tube formation was quantified using AngioQuant software[26].

qRT-PCR

Total RNA was isolated as described. Five hundred ng of total, DNA-free, RNA was transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and quantitative real-time polymerase chain reaction (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad) and specific primers for α-SMA, KDR, β-actin, and Spred1 (Invitrogen) in a MyIQ single-color qRT-PCR system (Bio-Rad) as previously described 25. Primers used are: α-SMA forward: 5`-actgggacgacatggaaaa-3`, reverse: 5`-tttgagtcattttctcccgg-3`, KDR forward: 5`-aaagggtggaggtgactgag-3`, reverse: 5`-cggtagaagcacttgtaggc-3`, β-actin forward: 5`-gatcggcggctccatcctg-3`, reverse: 5`-gactcgtcatactcctgcttgc-3`, Spred1 forward: 5`-agggcttttgatagaggtatccg-3`, reverse: 5`-catttgcttgtaagtcatctgcc-3`.

Spheroid assay

hCMPCs were transfected as described. After 24 h, spheroids consisting of 400 hCMPCs each were generated and embedded in 1 mg/mL collagen (Advanced Biomatrix, San Diego, California) in M199 with SP++ medium on top. *In vitro* sprouting was quantified by measuring the cumulative length of the tubular outgrowth using ImageJ software (NIH).

Scratch wound cell migration assay

hCMPCs were transfected as described. The following day, at 90% confluence, a scratch was made and the medium was renewed with 50% SP++/50% M199. After 8 h, wound closure was measured using ImageJ software.

Transwell cell migration assay

hCMPCs were transfected as described. After 24 h, 60.000 transfected cells were resuspended in 20% SP++/80% M199, and added to the upper chamber of a transwell system (3422, Corning, Tewksbury, MA). 20% SP++/80% M199 was added to the lower chamber and cells were allowed to migrate for 6 h before membranes were fixed with 4% PFA. The non-migrated cells from the upper well were removed with a cotton swab, and migrated cells were stained with Hoechst 33342 dye, followed by quantification using Image J software.

Microarray

Total RNA was isolated from fetal hCMPCs as described and subsequently purified with Nucleospin columns (Machery-Nagel). Five hundred nanogram of total, DNA-free, RNA was transcribed to cDNA as described and subsequent cRNA synthesis was performed with the Illumina® TotalPrep™ RNA amplification kit (Ambion). The Illumina Expression BeadChip (HumanRef-8 v2) microarray assay was performed using a service provider (LC Sciences, Houston, TX). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression).

Luciferase experiments

The Spred1 3′ untranslated region (UTR) was cloned into the pMIR-REPORT Luciferase vector (Ambion). Seed mutations for target site 1, 2, and 3 were generated by QuikChange Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA), and validated by sequencing. To determine the targeting capacity of miR-1, HEK293 cells were co-transfected with 200 ng of the pMIR-REPORT-Spred1-3′ UTR Luciferase vector, or one of the mutated vectors and the pMIR-REPORT β-gal control plasmid to assess transfection efficiency. In addition, 50 nmol/L premiR-1, a combination of premiR-1 and antimiR-1 (AM10617, Ambion), or a ctrl-miR was introduced by using Lipofectamine 2000 (Invitrogen). Luciferase and β-galactosidase activity was assessed after 48 h with the Luciferase Assay System and β-galactosidase Enzyme Assay System (Promega, Fitchburg, WI), respectively.

Western blot

Total protein was isolated with TriPure Isolation Reagent (Roche Applied Science). Equal amounts of protein were separated and then transferred to a PVDF membrane with the Novex® NuPAGE® SDS-PAGE Gel System (Invitrogen). Membranes were blocked (5% non-fat dry milk, PBS-0.1% Tween), and probed with Spred1 (sc-101392, Santa-Cruz Biotechnology) and β-tubulin (#2146, Cell Signaling, Danvers, MA) antibodies, followed by incubation with goat anti-rabbit or anti-mouse IgG secondary antibody, horseradish peroxidase-conjugated (P0448/P0447, Dako, Glostrup, Denmark). The signal was visualized with enhanced chemiluminescence system (Sigma-Aldrich) and detected by using the ChemiDoc XRS system (Bio-Rad).

Ingenuity systems pathway analysis

From each of the six miRNA target prediction databases (Microrna.org v08-2010, TargetScan v6.0, PicTar v03-2007, MicroCosm v5, Diana-microT v3, and ElMMo 11-2011) the 100 top-scoring miR-1 predicted targets were analyzed for expression in

hCMPC and only those expressed in hCMPCs and predicted by 3 or more databases were evaluated with the ingenuity systems pathway analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). The most significant miR-1-predicted target-associated network functions and biological functions were identified.

Statistical analysis

Data are presented as mean+SEM from at least three individual experiments (unless otherwise described) and were compared using a Student's t test (Microsoft Excel). Differences were considered statistically significant when *p*-values were ≤0.05 as indicated by an asterisk.

RESULTS

miR-1 is upregulated during vascular tube formation by hCMPCs

hCMPCs propagate rapidly when cultured on 0.1% gelatin-coated culture flasks, while maintaining an undifferentiated state (Figure 1a) as shown previously[5]. When plated on Matrigel, hCMPCs are capable of reorganizing into cord-like structures, forming a complete vascular network within 16 hours (Figure 1B). As shown earlier, these tubular structures consist of aligned endothelial cell marker positive cells surrounded by cells expressing smooth muscle cell markers[5]. As these markers are absent in hCMPCs, this shows that the cells differentiated towards endothelial and smooth muscle-like cells. To investigate which miRNAs might be involved in the formation of this vascular network by hCMPCs, we analyzed the expression of various miRNAs and found that miR-1 is rapidly expressed when hCMPCs are cultured on Matrigel (Figure 1c).

miR-1 enhances vascular tube formation of hCMPCs

Since miR-1 is upregulated during the angiogenic differentiation of hCMPCs, we investigated its role in this process. Compared to the endothelial-specific miR-126, miR-1 is hardly present in undifferentiated hCMPCs (Supplemental Figure 1), and therefore can easily be upregulated but not inhibited. hCMPCs can be efficiently transfected (up to 99%) as shown by visualization of different concentrations of fluorescent miRNA control (Supplemental Figure 2a), and upregulation of miR-1 could be achieved up to at least 8 days post-transfection in a dose-dependent manner as shown by taqman qRT-PCR (Supplemental Figure 2b).

To study the role of miR-1 during vascular tube formation, 3, 30, or 100 nM of premiR-1 was transfected in hCMPCs which were then plated on Matrigel. Transfection by

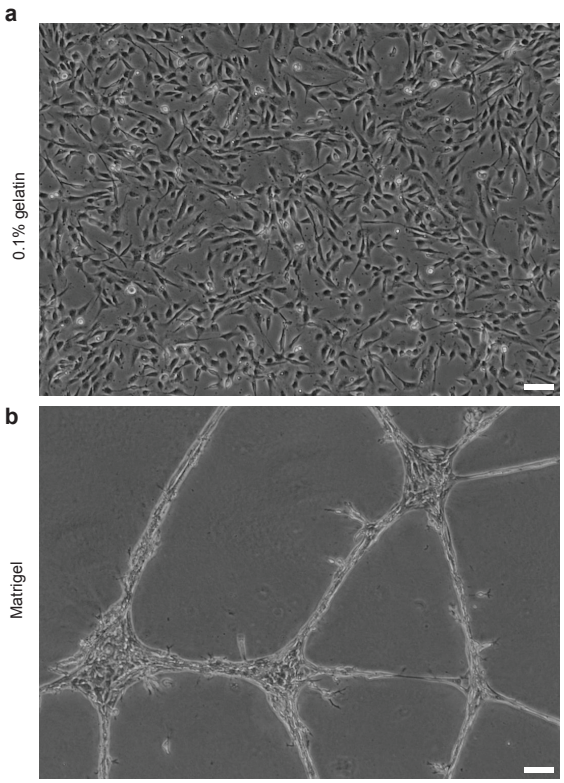

Figure 1 miR-1 is upregulated during vascular tube formation by hCMPCs
(a) Undifferentiated hCMPCs cultured on 0.1% gelatin. (b) hCMPCs, 16 hours after seeding on Matrigel, differentiated and reorganized into cord-like structures. (c) TaqMan qRT-PCR analysis of miR-1 expression in undifferentiated hCMPCs compared to hCMPCs differentiated on Matrigel. Data are represented as fold induction relative to undifferentiated hCMPC.

fluorescent miRNA control showed that the transfected miRNA mimics are retained in the hCMPC during vascular tube formation on Matrigel (Supplemental Figure 3). After 16 h, cells were fixed and stained for αSMA and CD31, revealing a dose-responsive increase in tubular formation in the premiR-1 transfected cells (Figure 2a) and an alignment of CD31+ cells surrounded by spindle-shaped αSMA+ cells (Figure 2b). When performing time-lapse recordings of network formation, we found that miR-1 accelerated the formation of tubular structures and increased the formation of more mature, cord-like structures (data not shown). Accurate unbiased analysis of the vascular organization was accomplished by computational quantification with AngioQuant software, which provides results on tubular length, size, and junctions (Supplemental Figure 4). Quantification of the tubule dimensions showed that miR-1 upregulation increased tubule length up to 2.5 fold and tubule size up to 4.5 fold (Figure 2c). Next to immunofluorescent staining, the expression of KDR/VEGFR-2 and αSMA were analyzed. KDR is expressed on endothelial cells and mediates the cellular response to VEGF, and αSMA is the actin isoform typical of vascular smooth muscle cells and regulates cell motility and contraction. miR-1 upregulation increased

the expression of KDR by almost 20-fold and αSMA was increased over 10-fold compared to ctrl-miR transfected cells (Figure 2d). These data indicate that the differentiation towards both endothelial and smooth muscle-like cells is enhanced by miR-1.

miR-1 enhances hCMPC spheroid sprouting

To further investigate the functional role of miR-1 in regulating hCMPC angiogenic differentiation, we performed a three-dimensional spheroid sprouting assay where we overexpressed miR-1. We found that introducing miR-1 positively affected tubular outgrowth, as shown by an over 2-fold increase in total sprout length after 1 day of culture (Figure 3). Adding the pro-angiogenic growth factor VEGFA to the culture medium increased spheroid sprouting to a similar extend as adding miR-1.

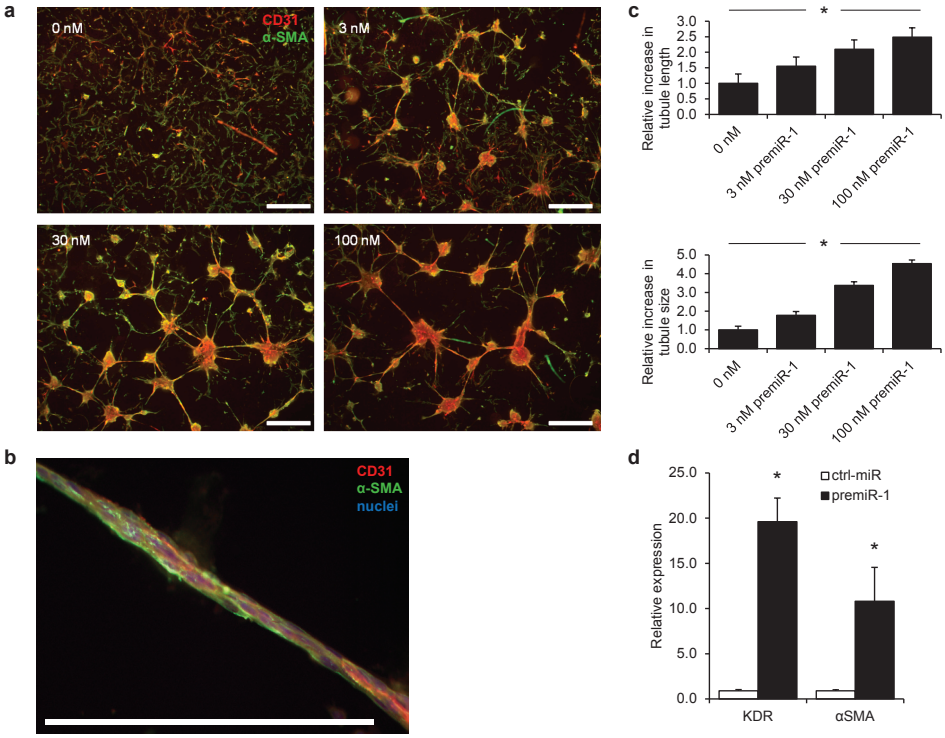

Figure 2 miR-1 enhances vascular tube formation of hCMPCs
(a) Matrigel angiogenesis assay of hCMPCs transfected with 0, 3, 30, or 100 nM premiR-1. Cells were stained for CD31 and αSMA. (b) High magnification of tubular structures formed on Matrigel showing aligned CD31+ cells surrounded by αSMA+ cells. (c) Quantification of tubule length and tubule size. (d) KDR and αSMA expression after miR-1 upregulation relative to ctrl-miR transfected cells.

When adding both VEGFA and miR-1, tubular sprouting was increased over 5-fold. These data indicate a positive role for miR-1 on the angiogenic differentiation needed for the formation of these tubular sprouts.

miR-1 enhances hCMPC migration

Since KDR expression is highly increased upon miR-1 upregulation, and VEGF has been shown to stimulate endothelial cell migration, we studied the effect of miR-1 upregulation on hCMPC migration. Cell migration is one of the key steps necessary to achieve new blood vessel formation, and was studied in two different assays, the scratch or wound closure and transwell assay. PremiR-1 transfection resulted in an induction of wound closure by 46% compared to ctrl-miR transfected cells, 6 h after inducing the scratch (Figure 4a). In the transwell migration assay, miR-1 upregulation resulted in twice as much cells migrating through the porous membrane, as shown by nuclear staining (Figure 4b) These data show that hCMPC migration is regulated by miR-1 abundance.

Spred1 is a target of miR-1

To get insight in the mechanism by which miR-1 enhances the angiogenic differentiation of hCMPCs, we performed a stringent target search. From six different miRNA target prediction databases the 100 top-scoring miR-1 predicted targets were selected for further analysis. Only those predicted by three or more databases were searched in the hCMPC microarray expression data. Only those expressed in hCMPCs were considered

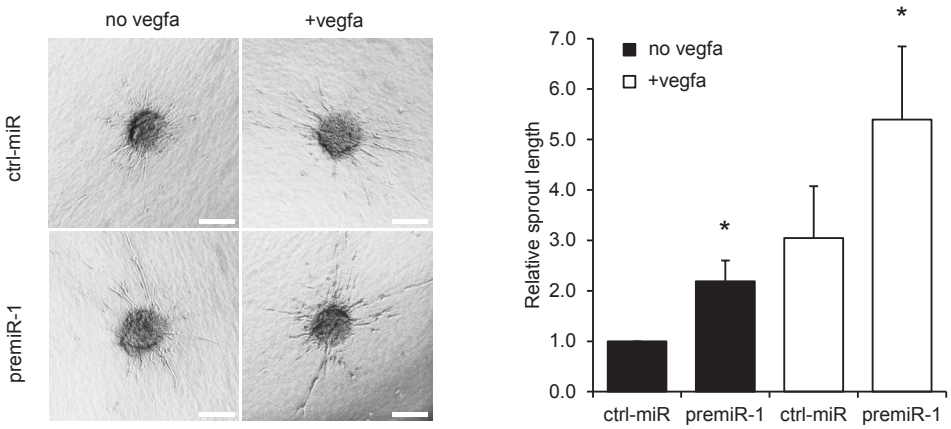

Figure 3 miR-1 enhances hCMPC spheroid sprouting

(a) Spheroid angiogenesis assay of ctrl or premiR-1 transfected hCMPCs, with or without the addition of VEGFA. Quantification of sprout length relative to ctrl-miR is shown below.

for reporter gene analysis and are listed in Table 1. In total, 78 genes were predicted by three or more databases and expressed in hCMPCs. Given the essential role in angiogenesis, and having several conserved miR-1 target sites (Supplemental Figure 5), we identified Spred1 as a potential direct target of miR-1. The function of Spred1 has been explored in detail by several groups, showing that Spred1 negatively regulates angiogenesis by controlling growth factor-induced activation of the MAP kinase cascade[21,22]. To investigate if miR-1 could directly target Spred1, we cloned the Spred1 3' UTR, containing three conserved miR-1 target sites (Figure 5a), into a luciferase

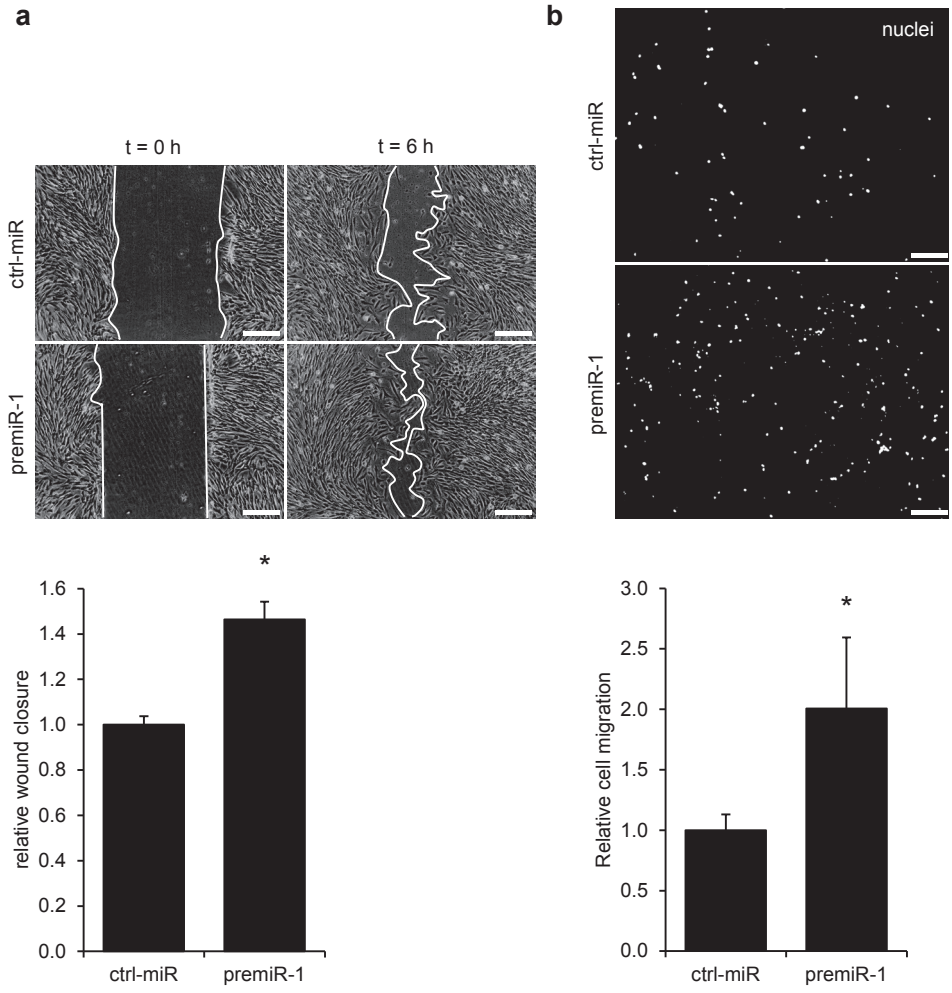

Figure 4 miR-1 enhances hCMPC migration
(a) Scratch wound cell migration assay of ctrl or premiR-1 transfected hCMPCs. Quantification of wound closure relative to ctrl-miR is shown below. (b) Transwell migration assay of ctrl or premiR-1 transfected hCMPCs, with quantification of migrated cells, relative to ctrl.

reporter vector (Figure 5b) and then co-transfected this vector together with premiR-1 into HEK293 cells. We found that Spred1 is indeed directly targeted by miR-1, as we observed that the luciferase activity could be significantly repressed (Figure 5b). Importantly, antimiR-1 co-transfection and mutating the target sequences complementary to the miR-1 seed region (Figure 5a, and Supplemental Figure 6) relieved the repressive effect of miR-1 on luciferase activity, verifying the direct targeting of Spred1 by miR-1 (Figure 5b). The mutation data indicated that target site 1 is the most important site for miR-1 binding. Since miRNA targeting can result in mRNA degradation and/or translational blockage, we analyzed the effect of

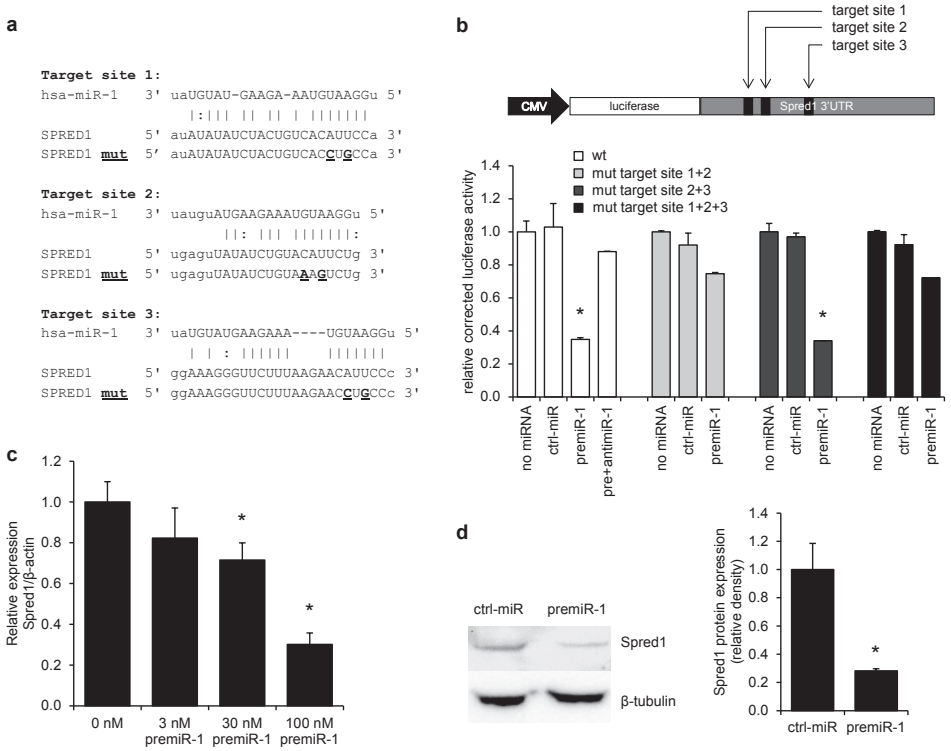

Figure 5 Spred1 is a target of miR-1

(a) Three predicted, conserved miR-1 target sites for Spred1, including sequences with seed-mutations used for validating direct targeting of miR-1. (b) Schematic representation of the Spred1-3'UTR-pmiR luciferase reporter vector, and luciferase reporter gene analysis showing the effect of co-transfecting ctrl-miR, premiR-1, a combination of premiR-1 and antimiR-1, with the Spred1-pmiR-reporter or one of the mutated Spred1-pmiR-reporters. Significance is measured compared to ctrl-miR. (c) qRT-PCR analysis of Spred1 expression after premiR-1 transfection in hCMPCs, normalized to β-actin. (d) The effect of miR-1 upregulation on Spred1 protein levels, as shown by western blot and densitometric analysis. Data is normalized to β-tubulin and relative to ctrl-miR transfected cells.

upregulating miR-1 on Spred1 mRNA and protein levels. Consistent with the conclusion that Spred1 is a direct target of miR-1, we found that miR-1 was capable of dose-dependently decreasing Spred1 mRNA (Figure 5c), as well as reducing the level of Spred1 protein (Figure 5d).

Predicted miR-1 targets are associated with angiogenesis-related processes

To understand the broader role of miR-1 in hCMPCs, we analyzed the selected targets, listed in Table 1, with ingenuity pathway analysis (IPA) software. The most significant molecular, cellular, and developmental categories and two top functions per category are listed in Table 2. IPA analysis showed that miR-1 predicted targets are associated with cellular movement (invasion and migration), gene expression, and cellular growth and proliferation, functions that are commonly associated with vascular development and the process of angiogenesis. Supplemental Figure 7 shows the top network associated with these functions, and includes Spred1. In addition, the physiological and developmental functions associated with the predicted miR-1 targets include tissue development, vasculogenesis, and angiogenesis. These results are in line with the functional effects brought about by miR-1 upregulation.

Table 1 miR-1 targets predicted by three or more databases and expressed in hCMPCs

ADAR	CORO1C	HSPD1	PDCD4	SLC31A1	TMSL3
ANXA2	CREBL2	KLF13	PPIB	SMARCC1	TPM3
ANXA4	CTBP2	KTN1	PPP2R5A	SMARCD1	TRAPPC3
AP3D1	DCP2	LASP1	PRKACB	SNX2	TRIM2
ARCN1	DDX5	MAP3K1	PTBP1	**SPRED1**	UBE2H
ARHGEF18	EFNB2	MATR3	RAB5A	STK39	UBE4A
BDNF	FN1	MEIS1	RAP1B	STXBP4	UNC50
BSCL2	FOSB	MMD	RASA1	SULF1	WDR1
C9orf82	FOXP1	MYLK	RNF38	TAGLN2	XPO6
CCND2	GJA1	NAP1L5	SEC63	TH1L	YWHAQ
CEBPZ	HDAC4	NCOA3	SETBP1	THBS1	YWHAZ
CITED2	HMGCR	NDRG3	SFRP1	TIMP3	ZBTB4
CLTC	HMGN1	NFAT5	SFRS9	TMSB4X	ZFP36L1

Targets are listed in alphabetical order. miRecords, miRWalk, and miRTarBase were used to find experimentally validated targets (underlined).

Table 2 Predicted miR-1 targets are associated with angiogenesis-related processes

Molecular and Cellular Functions		
Category	**p-value**	**#Molecules**
Cellular Movement	1.00E-07 - 8.91E-03	24
Gene Expression	1.55E-07 - 8.91E-03	33
Cellular Growth and Proliferation	4.27E-07 - 8.91E-03	36
Top two functions per category	**p-value**	**#Molecules**
Invasion of cells	1,00E-07	15
Migration of cells	3,82E-06	23
Transcription	1,55E-07	30
Expression of DNA	3,15E-07	29
Proliferation of cells	4,27E-07	31
Growth of cells	4,86E-04	20
Physiological System Development and Function		
Category	**p-value**	**#Molecules**
Tissue Development	1.52E-06 - 8.91E-03	29
Cardiovascular System Development and Function	3.34E-06 - 8.94E-03	17
Organismal Development	3.34E-06 - 8.91E-03	29
Top two functions per category	**p-value**	**#Molecules**
Tissue development	1,52E-06	27
Development of organ	2,07E-04	17
Vasculogenesis	3,34E-06	13
Developmental process of organism	3,44E-06	26
Vasculogenesis	3,34E-06	13
Angiogenesis	1,28E-04	10

Ingenuity Systems pathway analysis of miR-1 targets predicted by three or more databases and expressed in hCMPCs, showing the top three most significant associated molecular and cellular categories and physiological system development categories. For each category, the two top functions are listed. P-values are calculated using the right-tailed Fisher's exact test. # Molecules: the number of molecules related to the specific function.

DISCUSSION

With this study, we have uncovered a role for miR-1 in the angiogenic differentiation of hCMPCs. We found that miR-1 was upregulated when hCMPCs were induced to differentiate into endothelial and smooth muscle-like cells, forming a vascular network *in vitro*. miR-1 is normally absent in hCMPCs, but undifferentiated hCMPCs do

express several vascular development related miRNAs, including the endothelial cell-specific miR-126, underlying the angiogenic potential of hCMPCs. The pro-angiogenic function of miR-126 has already been described in great detail[27,28], and was therefore not explored in this study. By increasing miR-1 levels, we could enhance the angiogenic differentiation as shown by an increase in CD31, KDR and αSMA expressing cells and an enhanced and accelerated formation of tubular structures on Matrigel. The enhanced tube formation corresponds with our finding that miR-1 targets Spred1, thereby sensitizing the progenitor cell to pro-angiogenic stimuli. The increase in αSMA expression agrees with two previous reports showing that miR-1 inhibits smooth muscle cell (SMC) proliferation and promotes SMC differentiation[29,30]. In addition, we have shown that miR-1 upregulation in hCMPCs enhances angiogenic sprouting in a three-dimensional collagen matrix. We found that miR-1 or VEGF augmented tubular sprouting, but the combination of miR-1 and VEGF resulted in an additional improvement, which can be rationalized by Spred1 inhibition, making the cells more sensitive to pro-angiogenic stimuli. Spred1 is also known to inhibit cell motility[31,32], a key process during vascular tube formation. Consistent with the negative role of Spred1 on cell migration, our data shows a positive effect of miR-1 on cell migration. Under normal physiology, miR-1 is considered to have a muscle-specific expression pattern[33,34], however, a recent study has shown its expression in dorsal root ganglion neurons, where miR-1 upregulation positively affected neuronal outgrowth[35]. Intriguingly, inhibition of Spred1 has been shown to increase neuronal outgrowth. Blood vessels and nerve fibers have been shown to share some deep similarities in the mechanisms involved in the differentiation of the cellular players, growth, and navigation, and it is now well established that VEGF also plays a critical role in the nervous system[36]. The positive role of miR-1 on both angiogenic sprouting and neuronal outgrowth may very well add to these similarities.

Among the list of seventy-eight predicted miR-1 targets, which were both expressed in the hCMPC and predicted by three or more databases, thirty-four have already been experimentally validated, underlying the efficiency of this method in finding actual target genes. We validated Spred1 as a direct target of miR-1 by luciferase reporter assay and found that target site 1 was the most important for repression, showing the relevance of mutating each predicted target site to uncover dominant or functional sites. Spred-1 is an intracellular inhibitor of the MAP kinase pathway and diminishes the transmission of intracellular pro-angiogenic signals by different angiogenic growth factors[21]. Thus, when miR-1 is overexpressed in hCMPCs, Spred-1 is silenced, mitigating the repressive influence of Spred1 on the intracellular signaling cascade activated by pro-angiogenic growth factors, thereby sensitizing the hCMPCs to these pro-angiogenic growth factors. Consequently, when these hCMPCs find

themselves in an area where these pro-angiogenic growth factors are present, they can be induced to differentiate and form new vasculature. Importantly, while miR-1 seems to enhance both cardiomyogenic differentiation and angiogenic differentiation of hCMPCs, it is clear that these processes are dependent on the milieu the hCMPC resides in. Where TGF-β is necessary to initiate cardiomyogenic differentiation of hCMPCs, vascular growth factors will induce the angiogenic differentiation. TGF-β is a well-known inducer of cardiomyogenic differentiation and we have previously shown that the differentiation of hCMPCs into cardiomyocytes is enhanced by TGF-β signaling[5]. It has been shown that TGF-β enhances the expression of Spred-1[37]. Therefore, in the presence of cardiomyogenic differentiation factors such as TGF-β, Spred-1 levels are likely to be maintained making hCMPCs less prone to form blood vessels. In addition, it has been shown that miR-1 does not regulate cell fate determination, but rather maturation in lineage-committed cells[38], which is in line with the role of miR-1 in enhancing both the cardiomyogenic and angiogenic differentiation of hCMPCs.

In conclusion, our data show that the angiogenic differentiation of hCMPCs can be enhanced by upregulation of miR-1. Next to improving our understanding of how cardiac progenitor cell differentiation towards vascular structures is regulated, these new insights may help to devise a novel therapeutic approach to improve cardiac regeneration by enhancing the formation of new vasculature.

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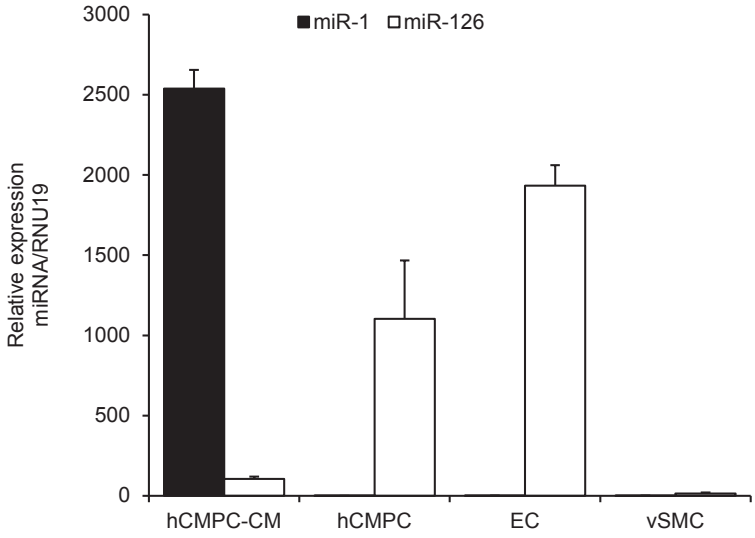
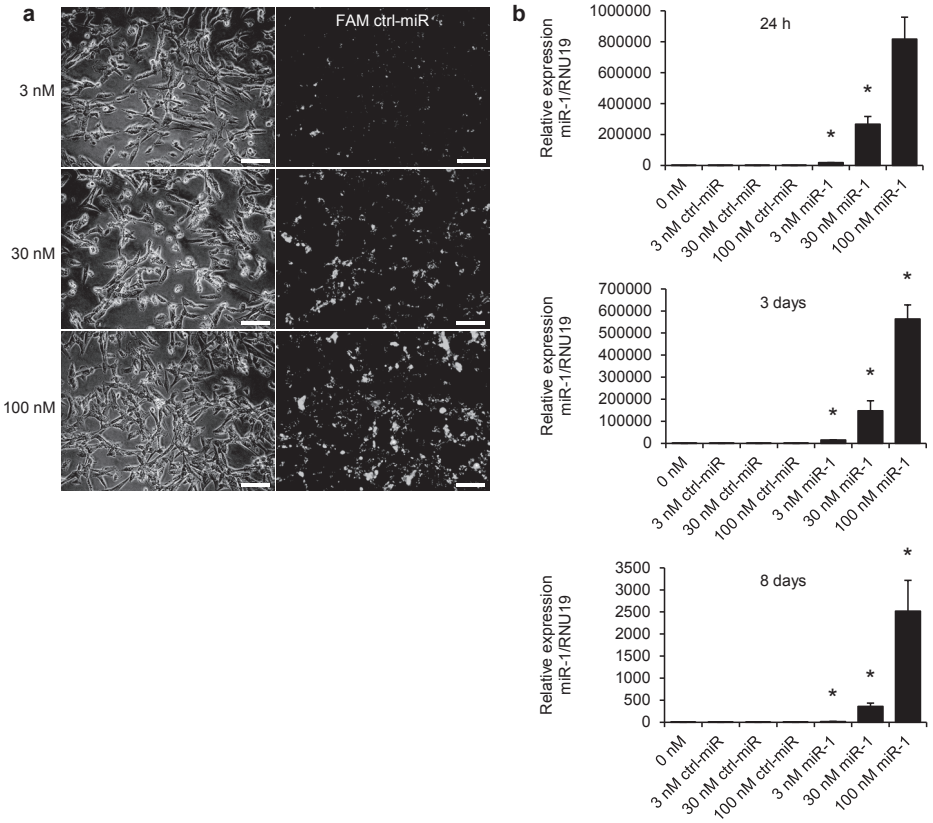
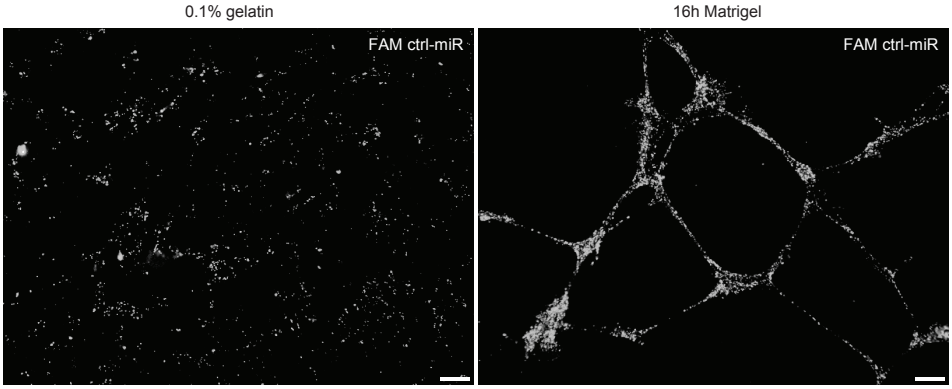


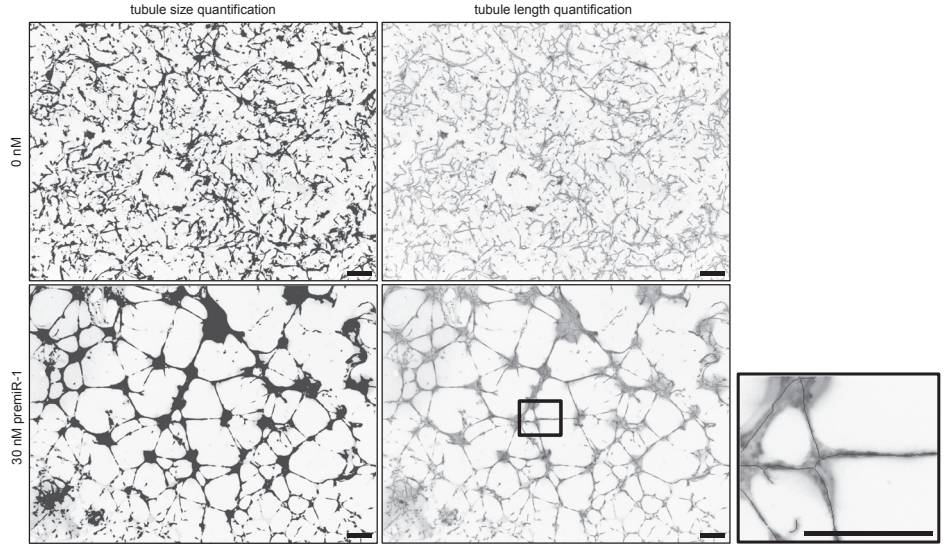
Supplemental Figure 1 miR-1 expression in different cells compared to miR-126
Expression of miR-1 and endothelial-specific miR-126 is shown in hCMPC-derived cardiomyocytes (hCMPC-CM), undifferentiated hCMPCs (hCMPC), human endothelial cells (EC), and human vascular smooth muscle cells (vSMC).



Supplemental Figure 2 Small RNA transfection efficiency
(a) Light and fluorescent microscope pictures of hCMPCs transfected with different concentrations of FAM-labeled ctrl-miR. (b) Taqman qRT-PCR analysis of hCMPCs transfected with 0, 3, 30, or 100 nM of premiR-1, or ctrl-miR, one, thee, or eight days after transfection.



Supplemental Figure 3 Transfection efficiency before and after seeding hCMPCs on Matrigel
FAM-labeled ctrl-miR transfected hCMPCs grown on 0.1% gelatin and 16 h after plating on Matrigel.



Supplemental Figure 4 AngioQuant quantification of vascular tube formation of hCMPCs on Matrigel
Tubule size and length quantification are shown for untransfected and premiR-1 transfected hCMPCs. A zoomed image shows the accuracy of quantification.

Spred1 target site 1:

Homo sapiens:	AUAUAUAUCUACUGUCACAUUCCA
Pan troglodytes:	AUAUA--UCUACUGUCACAUUCCA
Gorilla gorilla:	AUAUAUAUCUACUGUCACAUUCCA
Mus musculus:	*GC*ACAUAUCU*GCCACC*ACAUUCCA

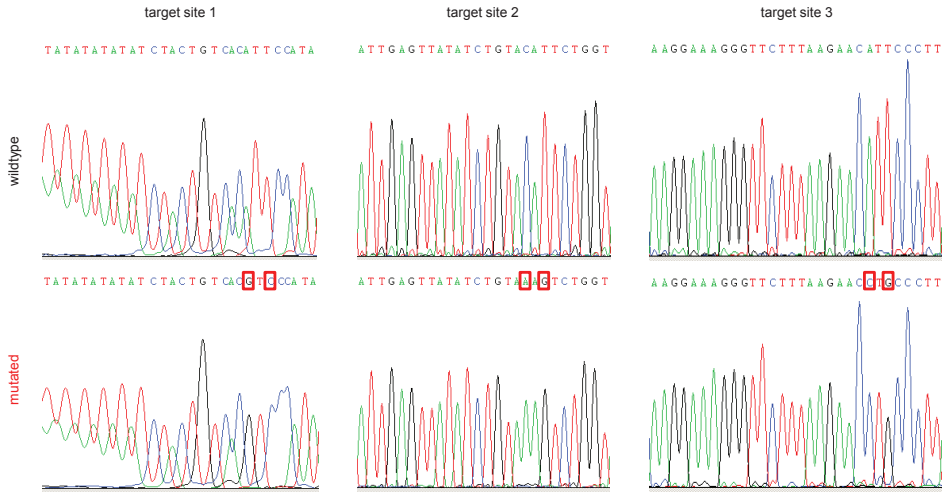
Spred1 target site 2:

Homo sapiens:	UGAGUUAUAUCUGUACAUUCUG
Pan troglodytes:	UGAGUUAUAUCUGUACAUUCUG
Gorilla gorilla:	UGAGUUAUAUCUGUACAUUCUG
Mus musculus:	UGAGU*C*AUAUCUGUACAUUCUG

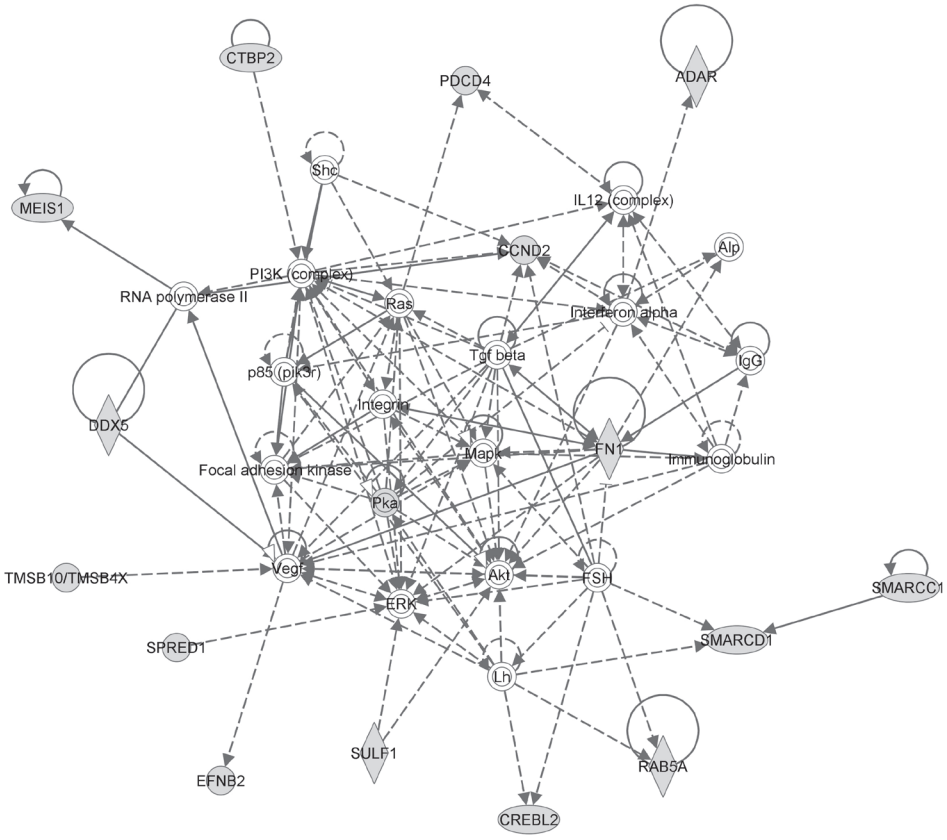
Spred1 target site 3:

Homo sapiens:	GGAAAGGGUUCUUUAAGAACAUUCCC
Pan troglodytes:	GGAAAGGGUUCUUUAAGAACAUUCCC
Gorilla gorilla:	GGAAAGGGUUCUUUAAGAACAUUCCC
Mus musculus:	G*UGA*GGG*A*UU*GUUGC*A*AAA*U*A*C*UCCC

Supplemental Figure 5 Three conserved miR-1 target sites in the Spred1 3′ UTR
Seed sequences are underlined, and nucleotides which are not conserved are shown in italics.



Supplemental Figure 6 Sequence chromatograms of three wild type and mutated miR-1 target sites in the Spred1 3′ UTR pmiR luciferase vector
Mutated nucleotides are encircled in red.



Supplemental Figure 7 IPA top predicted network built from putative miR-1 targets which are expressed in hCMPCs and predicted by at least 3 online target prediction algorithms
In grey: genes from the dataset inputted into IPA. In white: non-dataset genes/molecules, which are incorporated into the network through relationships with other molecules. Solid line: direct interaction. Dashed line: indirect interaction. Oval: transcription regulator. Double circle: complex/group. Diamond: enzyme. Circle: other.


A U
A U
U A
C G
G C
U A
A U
A U
U A
U A
G C
U A
C G
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C C
G C
U A
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CHAPTER 4

MICRORNA-155 PREVENTS NECROTIC CELL DEATH IN HUMAN CARDIOMYOCYTE PROGENITOR CELLS VIA TARGETING RIP1

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ABSTRACT

To improve regeneration of the injured myocardium, cardiomyocyte progenitor cells (CMPCs) have been put forward as a potential cell source for transplantation therapy. Although cell transplantation therapy has shown promising results, many issues need to be addressed before fully appreciating its impact. One of the issues is poor graft-cell survival upon injection, which limits the cells' potential contribution to myocardial repair. In this study, we focused on improving CMPC survival by increasing microRNA-155 (miR-155) levels, to potentially improve engraftment upon transplantation.

Using quantitative PCR, we observed a four-fold increase of miR-155 when CMPCs were exposed to hydrogen-peroxide stimulation. Flow cytometric analysis of cell viability, apoptosis, and necrosis showed that necrosis was the main cause of cell death. Overexpressing miR-155 in CMPCs showed that miR-155 specifically attenuated necrotic cell death by 40±2.3% via targeting receptor interacting protein 1 (RIP1). Inhibiting RIP1, either by pre-incubating the cells with a RIP1 specific inhibitor, Necrostatin-1 or siRNA mediated knockdown, reduced necrosis by 38±2.5% and 33±1.9%, respectively. Interestingly, gene expression analysis by PCR-array showed that increased miR-155 levels did not change cell survival and apoptotic related gene expression.

By targeting RIP1, miR-155 repressed necrotic cell death of CMPCs, independent of activation of the Akt pro-survival pathway. MiR-155 upregulation provides an opportunity to block necrosis, a conventionally considered non-regulated process, and might be a novel approach to potentially improve cell engraftment, and thereby the effect of cell therapy.

INTRODUCTION

Cell replacement therapy offers a novel approach to potentially repair the injured heart. Different cell types have been considered to be transplanted to replace damaged heart tissue. However, to be able to regenerate the heart, these cells should have the capability of differentiating into all cell types required for cardiac repair; functional cardiomyocytes, endothelial cells and smooth muscle cells. In the past decade, it has been shown that the heart is not a terminally differentiated organ, and different progenitor cells could be isolated from the adult myocardium. These progenitor cells were seen as an ideal candidate to regenerate the heart due to their localization. So far, many different types of cardiac progenitor cells or cardiac stem cells have been

isolated from the heart; Sca-1+, c-kit+, Islet-1+, SP (Side Population), and cardiospheres[1-5]. Cardiac progenitor cells are lineage negative and negative for hematopoietic markers like CD34 and CD45.

Previously, we reported the isolation of cardiomyocyte progenitor cells (CMPCs) from the human heart, that are able to proliferate and differentiate into functional cardiomyocytes without requiring co-culture with neonatal myocytes [6,7]. Three months after transplantation of CMPCs in a mouse model of myocardial infarction, we observed less outward remodeling and improved cardiac function as compared to control injections [8]. Although high numbers of injected cells were used in several studies, few implanted cells survived, limiting their potential contribution to myocardial repair. Most of the engrafted cells died in the first 48 hours after transplantation, partially due to the hostile micro-environment of the ischemic myocardium [9-11]. Therefore, there is a lot to gain if we can find a strategy to improve cell survival after implantation.

MicroRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression through translational repression or degradation of target messenger RNAs (mRNAs). MiRNAs are involved in regulating various cellular processes, such as cell proliferation, differentiation and migration. Accumulating evidence has suggested that miRNAs also play an important role in cell survival [12-14]. MicroRNA-155 (miR-155) was demonstrated to be involved in regulating cell death in several cell types [15-17], e.g. macrophages and pancreatic cells. Recently, we have shown that miR-155 is expressed in undifferentiated CMPCs, and demonstrated that miR-155 is not involved in regulating cellular proliferation [18]. Here we show that miR-155 enhances CMPCs survival upon oxidative stress by targeting receptor interacting protein 1 (RIP1), a death domain protein required for the activation of necrosis [19,20]. This study shows that miR-155 inhibits necrosis in CMPCs and suggests that the efficiency of cell-based therapy for cardiac regeneration can potentially be promoted by increasing miR-155 levels in CMPCs.

MATERIALS AND METHODS

CMPC isolation and culture

CMPCs were isolated and propagated as previously described [6]. Briefly, human fetal heart tissue was collected after elective abortion and individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. The heart was cut into small pieces and digested by collagenase, followed by passing through a cell strainer to get

a single cell suspension. CMPCs were further isolated via magnetic cell sorting using an iron-labeled mouse anti-Sca-1 antibody and characterized as described [6,7,21]. CMPC cardiomyogenic differentiation was performed as previously described [6]. For our experiments we used six individual isolated CMPC cell-lines.

Quantitative RT-PCR for miRNA expression

Total DNA-free RNA was isolated with Tripure isolation reagent (Roche Applied Science). 3.3 ng total RNA was used for reverse transcription (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) followed by Taqman® MicroRNA Assays for quantification of miR-155 and RNU19 control transcripts (Applied Biosystems: 4373124, 4373378), according to the manufacturer's conditions. Amplification and detection of specific PCR products was performed using a MyIQ single-color real-time PCR system (Bio-Rad) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The expression level of miR-155 was calculated (ΔΔCt) and presented as fold induction ($2^{-\Delta\Delta Ct}$).

Quantitative RT-PCR

Total DNA-free RNA was isolated with TriPure reagent (Roche). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using Sybr-green mastermix (Bio-Rad) in a MyIQ single-color real-time PCR system (Bio-Rad) as described before [22]. RIP1 primer sequences used are: forward: 5′-AGTCCTGGTTTGCTCCTTCCC-3′; reverse: 5′-GCGTCTCCTTTCCTCCTCTCTG-3′, with 63.9°C annealing temperature. Expression values were normalized to GAPDH (forward: 5′-ACAGTCAGCCGCATCTTC-3′; reverse: 5′-GCCCAATACGACCAAATCC-3′, with 56.1°C annealing temperature). TroponinT (TNNT2) and myosin light chain (MYL2) expression were detected by using a customized PCR array from SABiosciences (CAPH09443). Relative expression values were calculated as ΔΔCt.

Small RNA transfection

Pre-miR™ precursor molecules for miR-155 (PM12601) (pre-miR-155), Anti-miR™ inhibitor for miR-155 (AM12601) (anti-miR-155) and a scrambled miR control (AM17121) (scr-miR) were obtained from Ambion. SiRIP1 duplex (sense strand 5′-GGAGCAAACUGAAUAAUGAUU-3′) and siNS (Non-Significant) duplex (sense strand 5′-CAGAGAGGAGGAAAGGAGAUU-3′) were synthesized by Dharmacon. CMPCs were transfected with 30 nM of miRNAs or 100 nM of siRNA with siPORT™ NeoFX™ Transfection Agent (Ambion), according to the manufacturer's guidelines. Transfection efficiency was confirmed by RT-PCR and western blot.

Luciferase experiments

In a 12-well plate, HEK293 cells were co-transfected with 400 ng luciferase-reporter plasmid, containing the 3' untranslated region (UTR) of Bach1, a validated target of miR-155 [23] (a generous gift from Dr. Erik Flemington), and 30 nM of miR-155, a combination of miR-155 and anti-miR-155, or a scr-miR. 400 ng cytomegalovirus (CMV)-β-galactosidase plasmid was co-transfected as a control for transfection efficiency. After 48 hours, cells were lysed and luciferase and β-galactosidase activities were measured with the Luciferase Assay System and β-galactosidase Enzyme Assay System (Promega), respectively, as described [18].

Cell viability assay

CMPCs were seeded and transfected with 30 nM of miRNAs in a 96-well plate. Twenty-four hours after transfection, CMPCs were challenged with 100 μM H_2O_2 in serum-free medium for 16-18 hours. Subsequently, 10 μl WST-1 reagent (Roche 11644807001) was added to each well. The optical density was measured at different time points by colorimetric multiscan FC (Thermo Scientific) with absorption at 450 nm and reference at 750 nm.

Flow cytometric analysis of apoptosis and necrosis

CMPCs were seeded and transfected with 30 nM of miRNAs in a 12-well plate. Twenty-four hours after transfection, CMPCs were challenged with 50 μM H_2O_2 in serum-free medium for 16-20 hours. All cells were collected and subsequently washed with PBS. To detect necrosis only, cells were incubated with 3 μl propidium iodide (BD Pharmingen, 556463) in 1x binding buffer for 25 min in the dark. For apoptosis and necrosis detection, cells were incubated with 5 μl AnnexinV-PE (AnnV) and 5 μl 7-Amino-actinomycin-D (7-AAD) in 1x binding buffer (BD Pharmingen, 559763) for 25 min in the dark. After incubation, apoptotic, necrotic and live cell populations were detected by flow cytometric analysis.

Western blot

Seventy-two hours after miRNA or siRNA transfection, total protein was extracted. Samples were reduced with 0.5% β-mercaptoethanol, separated by PAGE, and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked (5% non-fat-dry milk, PBS-0.1% Tween), probed with RIP1 antibody (1:500, rabbit polyclonal, sc-7881, Santa Cruz), and β-tubulin antibody (1:2000, rabbit polyclonal, #2146, Cell Signaling), followed by goat anti-rabbit IgG secondary antibody (P0448, Sigma-Aldrich) incubation. Signal was visualized with enhanced chemiluminescence (Amersham) and detected by using the ChemiDoc XRS system (Bio-Rad).

Necrostatin-1 treatment

CMPCs were pre-incubated with 30 μM Necrostatin-1 (Calbiochem 480065, Nec-1) in serum-free medium for 30 min, followed by stimulation with 50 μM H_2O_2. After 16-18 hours, cell viability, apoptosis and necrosis were analyzed by flow cytometry as described above.

Human pro-survival and apoptosis pathway super-arrays

CMPCs were transfected with pre-miR-155 or scr-miR. After 48 hours, total RNA was isolated and cDNA was synthesized as described above. Eighty-four key genes, involved in apoptosis and PI3K-AKT pro-survival pathways (SABiosciences, PAHS-012A and PAHS-058A), respectively, were detected using qRT-PCR, followed by analysis using SABiosciences online RT² Profiler™ PCR Array Data Analysis software.

Statistical analysis

Data are presented as mean ± SEM of at least three independent experiments and were compared using the two-tailed paired Student's t test or one way ANOVA, a difference with a $p<0.05$ was considered to be statistically significant.

RESULTS

Increased expression of miR-155 in CMPCs

Since miR-155 overexpression did not alter cell proliferation [18], we investigated if miR-155 had a role in CMPC survival, as was demonstrated before in other cell types [15-17]. After stimulation with H_2O_2, miR-155 levels increased four-fold (Figure 1A), suggesting a role of miR-155 in the stress response. Next, we modulated miR-155 levels in CMPCs. Transfection of pre-miR-155 resulted in a 280-fold increase in miR-155 levels (Figure 1B) compared to anti-miR-155 or scr-miR transfection. Pre-miR-155 overexpression significantly reduced the activity of a miR-155 luciferase reporter plasmid, containing a 3'-UTR sequence of Bach1, a known target of miR-155 [23], which could be abolished by addition of anti-miR-155 (Figure 1C), showing the effectiveness of the pre and anti-miRs.

MiR-155 overexpression augments cell survival during oxidative stress stimulation

Exposure of miR-155 overexpressing CMPCs to oxidative stress resulted in less cell death (Figure 2A). Both flow cytometric analysis (Figure 2B) and cell counting

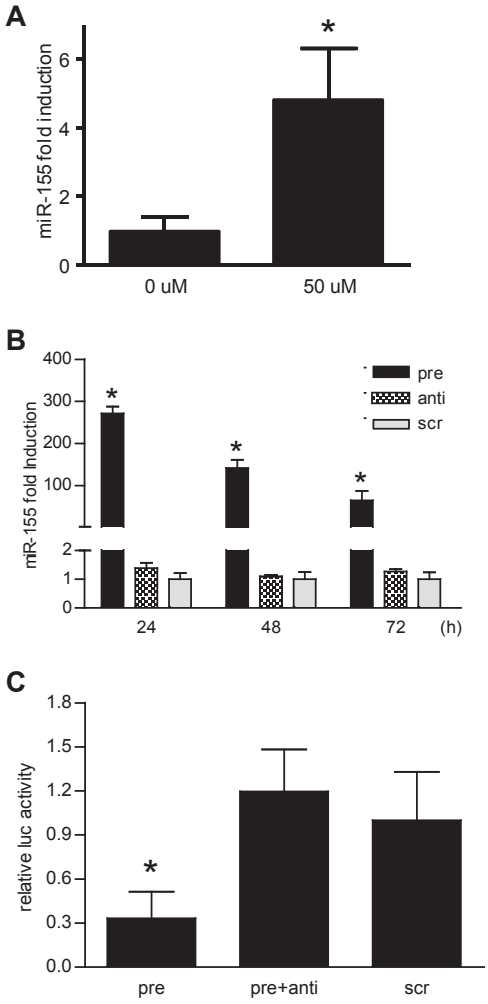

Figure 1
(A) miR-155 expression was increased in CMPCs upon 16h of 50 μM H_2O_2 stimulation, (B) CMPCs were transfected with 30 nM pre-miR-155, anti-miR-155 or scr-miR. MiR-155 expression was detected by Taqman MicroRNA RT-PCR 24 hours, 48 hours or 72 hours after transfection. (C) The functional effectiveness of miRNA molecules was confirmed by luciferase activity assay. CMPCs were co-transfected with luciferase reporter plasmid containing the 3'UTR of a miR-155 target and with different combinations of miRNAs. n=3 and *p<0.05

(Supplementary Figure 1) confirmed this observation and displayed a 42±3.1% improvement of cell survival. A more detailed analysis revealed that the increase in cell number was the result of a 40±2.3% reduction in necrosis, the predominant type of cell death, as compared to scr-miR transfection control and affirmed by a diminished number of PI positive cells (Figure 2D+E). Improved cell survival was also found when performing a cell viability assay (Figure 2C). Interestingly, no significant difference in the number of apoptotic cells was found when comparing CMPCs overexpressing pre-miR-155, anti-miR-155 or scr-miR.

To investigate whether necrosis was a primary result or a secondary event of apoptosis, we performed a time course experiment. In time, apoptosis remained a minor type of cell death, whereas, necrosis increased dramatically after 12 hours of stimulation, being

the predominant type of cell death (Supplementary Figure 2). Additionally, we tested the activities of caspases in CMPCs upon stimulation by a homogeneous caspases assay, but we did not observe the presence of any enzymatic activity (data not shown). Taken together, our observations show that in CMPCs, miR-155 increases cell survival upon oxidative stress by reducing necrotic cell death.

To test whether changing miR-155 levels affect the cardiomyogenic differentiation potential of CMPCs, we transfected CMPCs with pre-miR-155, anti-miR-155 or scr-miR and started differentiation experiments as previously reported [6]. We observed increased levels of Troponin T (TNNT2) and ventricular myosin light chain 2 (MYL2)

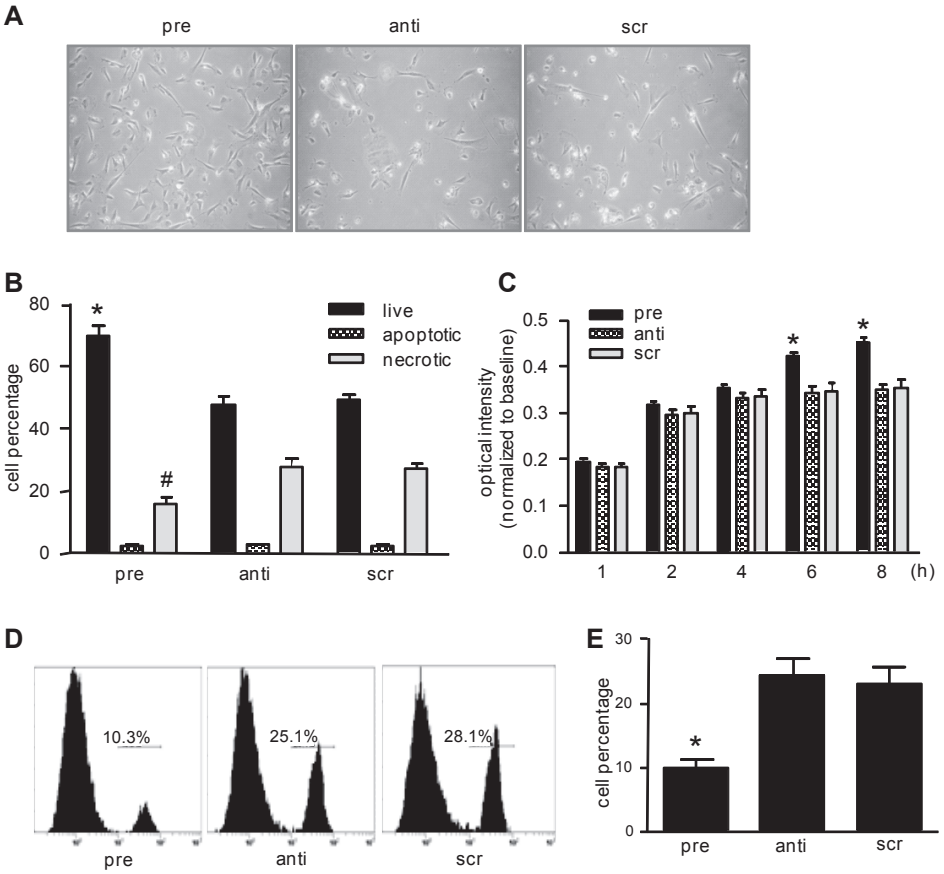

Figure 2

(A) CMPCs were transfected with 30 nM pre-miR-155, anti-miR-155 or scr-miR and challenged by 50 µM H_2O_2 in serum-free medium for 16 hours. (B) Live, apoptotic and necrotic cells were detected by AnnV/7-AAD staining using flow cytometric analysis. (C) A cell viability assay was performed on miRNA transfected CMPCs, followed by exposure to 100 µM H_2O_2. (D) Flow cytometric analysis of propidium iodide (PI) staining shows a reduced necrotic cell population upon miR-155 transfection. (E) Quantification of PI positive cells in D. n=4 and *p<0.05.

one week after the initiation of differentiation, similar as non-transfected CMPCs, indicating that miR-155 does not affect the cardiomyogenic differentiation potential (Supplementary Figure 3).

MiR-155 reduces necrotic cell death via targeting RIP1

Receptor interacting protein 1 (RIP1) is a validated target of miR-155 and functions in the response of macrophages to endotoxin shock [15]. RIP1 is required for death-receptor agonists activated necrosis [20]. Therefore, we tested whether miR-155 inhibited necrosis by repressing RIP1. We first tested if RIP1 is a target of miR-155 in CMPCs

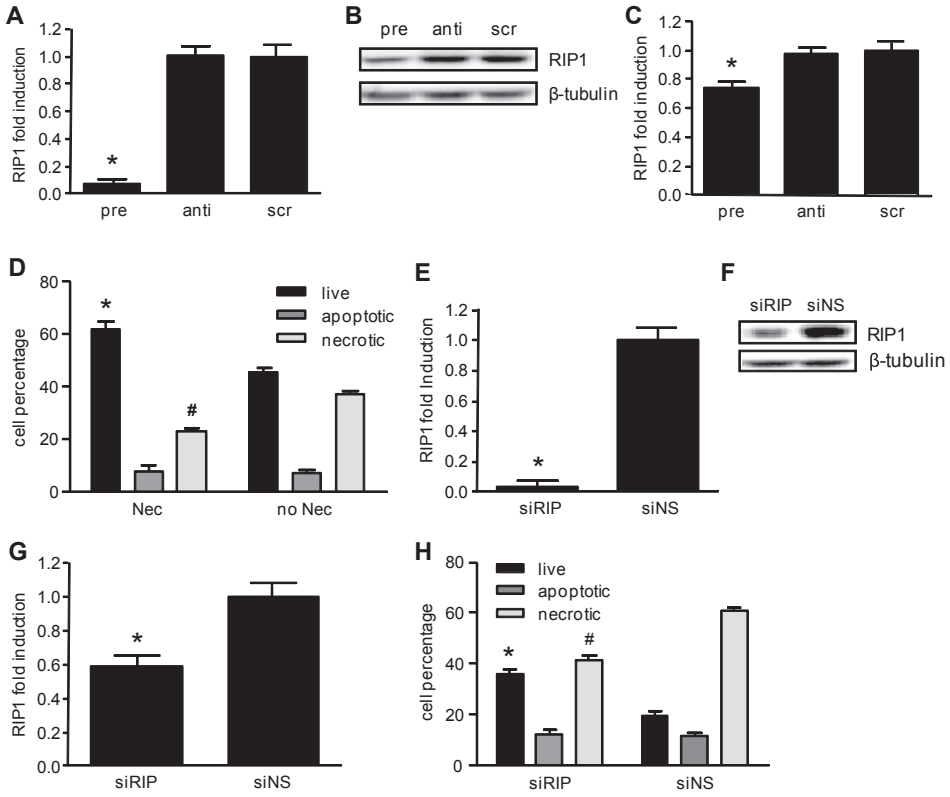

Figure 3

(A) RIP1 mRNA expression in miR-155 transfected CMPCs. (B) RIP1 protein expression detected by western blot and (C) quantification of western blot. (D) CMPCs were pre-incubated with 30 μM Nec-1, inhibitor of RIP1, followed by 50 μM H_2O_2 stimulation. Live, apoptotic and necrotic cells were detected by AnnV/7-AAD staining using flow cytometric analysis. (E) RIP1 mRNA expression after transfection of siRNA against RIP1 (siRIP), or a non-significant control sequence (siNS) in CMPCs as detected by qRT-PCR. (F) RIP1 protein expression detected by western blot and (G) quantification of western blot. (H) Live, apoptotic, and necrotic cell populations were assessed by flow cytometric analysis in siRIP or siNS transfected CMPCs. n=3 and *p<0.05.

and we could indeed show that miR-155 repressed RIP1 expression both at the mRNA and protein level (Figure 3A+B+C). To test the functional relevance of RIP1 in the observed change in necrotic cell death, we added Necrostatin-1 (Nec-1), a specific inhibitor of RIP1 [24,25], to oxidative stress-stimulated CMPCs. In Nec-1 treated CMPCs, cell survival improved and necrosis diminished to a similar extent as miR-155 overexpression (Figure 3D), with no changes in apoptosis. In addition, knockdown of RIP1 expression by siRNA treatment repressed RIP1 mRNA and protein levels (Figure 3E+F+G), thereby preventing necrotic cell death and improving cell survival (Figure 3H). Our results show that miR-155 represses RIP1, a key effector in necrosis, thereby attenuating necrotic cell death induced by oxidative stress.

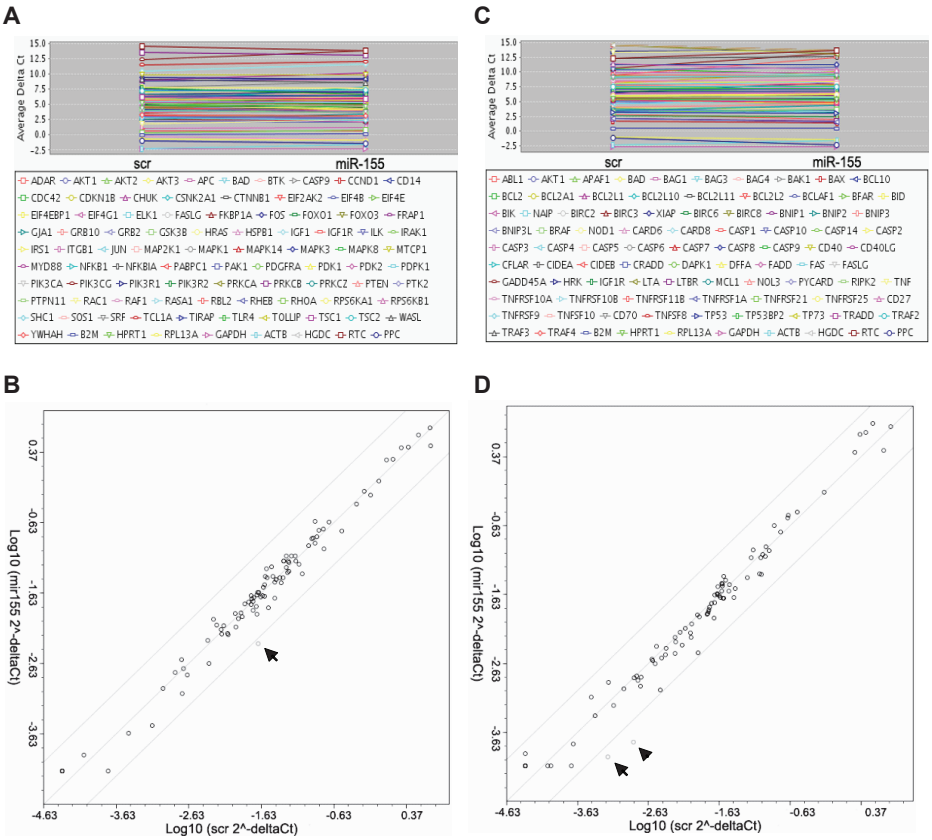

Figure 4
In pre-miR-155 and scr-miR transfected CMPCs, the expression level of 84 genes involved in PI3K-Akt pro-survival (A,B) and apoptosis (C,D) pathways was determined by qRT-PCR and normalized to four housekeeping genes. (A,C) Gene names and average ΔCt are shown, and (B,D) the relative expression was plotted in a scatter plot. (B) NF-κB is indicated by an arrow. (D) BIRC3 is indicated by an arrow, and CASP1 is indicated by an arrowhead.

MiR-155 inhibits necrosis, independent of activation of the Akt pro-survival pathway

Since cell death and survival are tightly regulated processes involving many different pathways, we analyzed whether other cell survival pathways are affected by miR-155 modulation. Using PCR-arrays, we analyzed the expression of 84 key genes involved in the PI3K/Akt pro-survival pathway and apoptosis pathway. We found that increased miR-155 levels did not change the expression patterns of cell survival and apoptotic related genes. In the Akt survival pathway related genes, only a 4-fold downregulation of NF-κB could be observed (Figure 4A+B). Similarly, in the array focused on apoptosis-related genes, only BIRC3 and CASP1 showed a decrease of more than 4-fold (Figure 4C+D). These data confirm that cell survival and apoptosis pathways are not widely affected upon miR-155 overexpression.

DISCUSSION

Cell-based therapy has emerged as a promising, alternative strategy to regenerate or improve function upon myocardial infarction and possibly for chronic heart failure. The feasibility of various cell sources and different cell delivery methods has been explored extensively [21,26-29]. The discovery of resident CPCs that can contribute to both myocardial regeneration and vascularization has provided an ideal candidate for repairing the injured heart. Previously, we reported that upon transplantation, CMPCs reduced the extent of left ventricular remodeling up to three months after myocardial infarction [8], thereby improving cardiac output. Due to the hostile environment of the ischemic heart, however, the survival of newly implanted cells remains problematic[30]. Several strategies of preconditioning or stimulating stem/progenitor cells to increase survival have been studied [31-33]. Although very promising and effective, the effects on stem/progenitor cell survival and cardiac repair in these studies may largely be indirect and a result of paracrine effects that positively influence the recipient tissue, and thereby cell survival. To be able to understand the effect of increasing transplanted cell survival in cardiac repair, new strategies are required that directly improve stem/progenitor survival rates.

MiRNAs are non-coding small RNAs, known to regulate specific cellular processes, including cell survival. Therefore, they provide novel targets to specifically improve cell survival. Several studies have shown the cyto-protective effect of different miRNAs in cardiac tissue [34-36]. In the present study, we found that miR-155 prevents oxidative stress-induced necrosis, the dominant type of cell death in CMPCs. Follow-up experiments demonstrated that apoptosis is a minor contributor to cell death, whereas

necrosis increased dramatically in time. In addition, no caspase activity was observed upon oxidative stress, suggesting that the apoptosis pathway is not activated.
RIP1 emerged as a potential target candidate for miR-155 regulated inhibition of necrosis. RIP1, a death domain protein, is dispensable for the induction of death-receptor-mediated apoptosis [37]. In apoptosis-deficient conditions, however, RIP1 has been found to be required for the activation of necrosis by death-receptor agonists[20]. This process is termed necroptosis by Degterev et al. and they identified RIP1 as a key universal effector molecule, potentially followed by divergent downstream execution steps, depending on the cellular context [24]. RIP1 was described as a target of miR-155 in macrophages in their response to endotoxin shock [15]. In CMPCs, we found that miR-155 reduced RIP1 at the mRNA and protein level and we confirmed a direct effect of RIP1 on necrosis by using a specific inhibitor of RIP1, Nec-1, or via siRNA-mediated knockdown of RIP1, thereby attenuating necrosis without affecting apoptosis. To study whether pro-survival pathways or apoptosis-related genes are also involved, we analyzed over 160 different genes upon miR-155 transfection. We found that the gene expression pattern was not significantly altered, indicating that miR-155 specifically blocks necrosis, independent of pro-survival and apoptosis pathways. This was in line with our finding that miR-155 attenuated necrosis induced by oxidative stress without affecting apoptosis.
Although increased expression of miR-155 could block necrosis, we could not induce necrotic cell death by inhibiting endogenous miR-155. This suggests that the biological role of miR-155 in CMPCs may not be anti-necrotic. However, since pre-miR-155 transfection only transiently increased miR-155 levels and did not affect cell proliferation and cardiomyogenic differentiation potential, preconditioning CMPCs with miR-155 provides a novel opportunity to improve cell survival after transplantation from a therapeutic point of view.
In conclusion, this study demonstrates that oxidative stress-induced cell death in CMPCs is mainly mediated by necrosis. By increasing miR-155 levels, necrosis can be inhibited and cell survival is improved without affecting cardiomyogenic differentiation potential. This is mediated by repressing RIP1. Our data suggests that increasing miR-155 is a potential novel strategy for improving graft-cell survival and suggests the broader possibility of specifically targeting necrosis as a therapeutic strategy.

Acknowledgements
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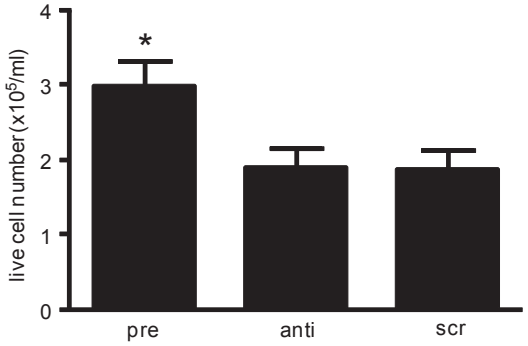
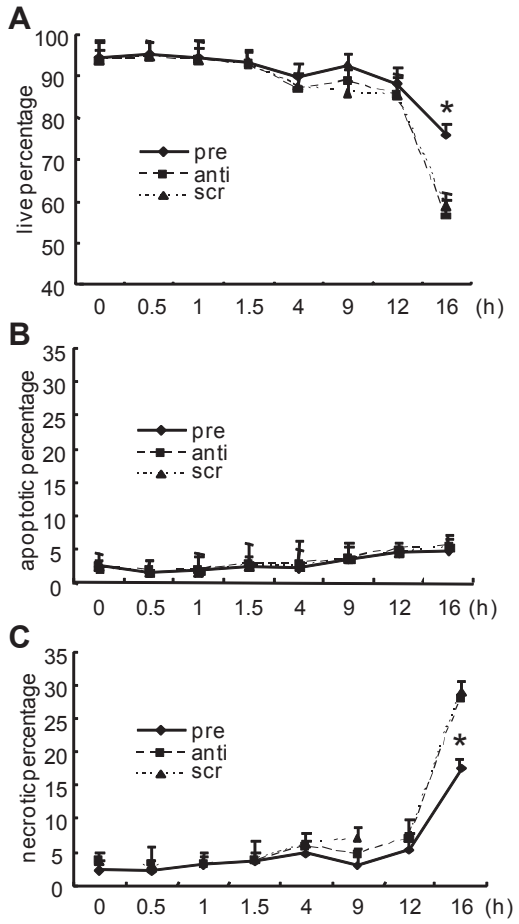
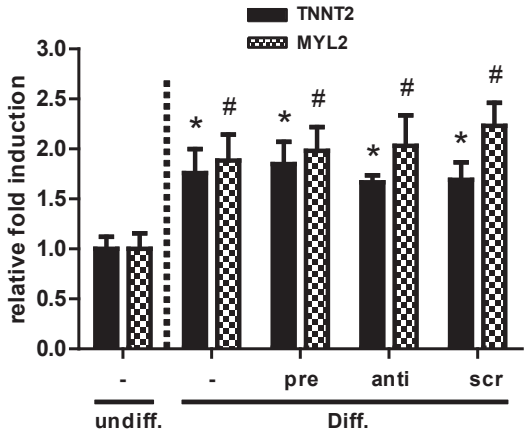


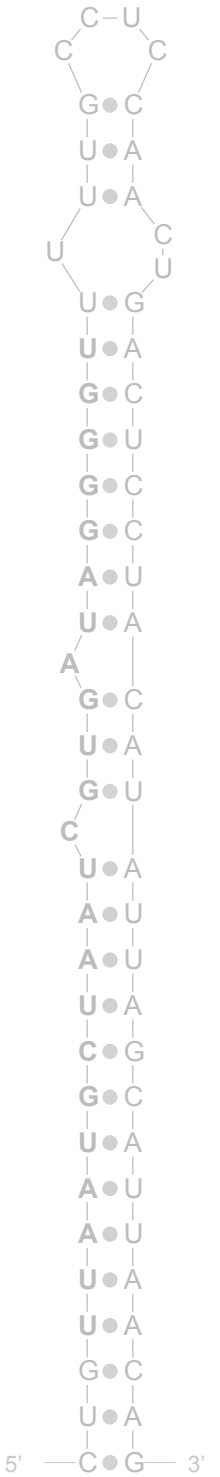
Supplemental Figure 1
Trypan blue cell counting for viable cells upon miRNA transfections followed by 50 μM H_2O_2 stimulation. n=4 and *p<0.05.



Supplemental Figure 2
Time course of live (A), apoptotic (B) and necrotic (C) cells as detected by AnnV/7-AAD staining using flow cytometric analysis in H_2O_2 stimulated, and miRNA transfected cells. n=3 and *p<0.05.



Supplemental Figure 3
CMPCs were transfected with pre-miR-155, anti-miR-155 or scr-miR, followed by stimulation with 5-aza and TGF-beta to induce cardiac differentiation. Troponin T (TNNT2) and ventricular myosin light chain 2 (MYL2) mRNA expression were analyzed before (undiff.) and one week after differentiation (diff.). Non-transfected CMPCs before and after differentiation were used as a negative and positive control, respectively. n=3 and *p<0.05.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CHAPTER 5

MICRORNA-155 INHIBITS CELL MIGRATION OF HUMAN CARDIOMYOCYTE PROGENITOR CELLS VIA TARGETING OF MMP-16

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ABSTRACT

Background

Undesired cell migration after targeted cell transplantation potentially limits beneficial effects for cardiac regeneration. MicroRNAs are known to be involved in several cellular processes, including cell migration. Here, we attempt to reduce human cardiomyocyte progenitor cell (hCMPC) migration via increasing microRNA-155 (miR-155) levels, and investigate the underlying mechanism.

Methods and results

Human cardiomyocyte progenitor cells (hCMPCs) were transfected with pre-miR-155, anti-miR-155 or control-miR, followed by planar and transwell migration assays. These functional assays showed that miR-155 overexpression inhibited planar migration by 38±3.6% and transwell migration by 59±3.7%. Conditioned medium from miR-155 transfected cells was collected and zymography analysis showed a significant decrease in MMP-2 and MMP-9 activities. The 3'UTR of MMP-16, an activator of MMP-2 and -9, was cloned into the pMIR-REPORT vector and luciferase assays were performed. Introduction of miR-155 significantly reduced luciferase activity, which was abolished by co-transfection of anti-miR-155 or by target site mutagenesis. By using MMP-16 siRNA to reduce MMP-16 levels or by using an MMP-16 blocking antibody, we were able to block hCMPC migration as well.

Conclusion

By directly targeting MMP-16, miR-155 inhibits cell migration via a reduction in MMP-2 and -9 activities. Our study suggests that miR-155 may be a potential candidate to improve local retention of hCMPCs after intramyocardial delivery.

INTRODUCTION

Undesired cell migration and low cell retention post intramyocardial cell transplantation potentially limit the therapeutic effect of cell therapy for cardiac regeneration [1]. With the progress made in catheter development for targeted cell injection, the myocardium in need can be targeted more successfully and receive more implanted cells [2-5]. However, cell retention is limited after delivery due to myocardial contraction and catheter misplacement. In addition, migration of transplanted cells out of the myocardium in need can decrease the efficiency of cellular therapy [5].

During recent years, microRNAs (miRNAs) -a class of ~22 nucleotide long non-coding RNAs- have been found to keep tight control of various processes by post-transcriptional gene silencing. Several studies have shown that miRNAs are involved in regulating cell migration [6-10]. These studies suggest the possibility of modulating miRNAs to limit cell migration, which could potentially result in a new strategy to maximize cell retention of transplanted cells within the myocardium after targeted local administration.

Previously, we have shown that miR-155 significantly reduces necrotic cell death in human cardiomyocyte progenitor cells (hCMPC), which suggested the possibility of promoting cell survival upon injection, thereby increasing the efficiency of cell-based therapy [11]. Here we report that miR-155 can also block hCMPC migration by directly targeting matrix metalloproteinase (MMP)-16, an activator of MMP-2 and 9, thereby potentially reducing off-target migration leading to improved targeted cell delivery.

MATERIALS AND METHODS

Cell culture

hCMPCs were isolated and expanded as previously reported [12]. In short, human fetal heart tissue was collected after elective abortion and digested by collagenase to obtain a single cell suspension. hCMPCs were isolated by magnetic cell sorting (MACS, Miltenyi Biotech, 130-090-312) using a mouse anti-Sca-1 antibody (Miltenyi Biotech, 130-091-176) and cultured as described [13]. Three individual cell lines were characterized and used for all experiments (CMPC-1, CMPC-2, and CMPC-3). Standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained.

Small RNA transfection

Pre-miR™ precursor molecules for miR-155 (PM 12601, pre-miR-155), anti-miR™ inhibitor for miR-155 (AM 12601, anti-miR-155) and a negative control miR (AM 17110, ctrl-miR) were obtained from Ambion. Stealth RNAi for MMP-16 (set of 3; low GC, HSS106647, HSS181141 and HSS181142), and Stealth RNAi negative control (Low GC, 12935200) were purchased from Invitrogen. hCMPCs were transfected with 0, 3, 30, or 100 nM of appropriate miRNAs, as reported before [11] or 100 nM of siRNA with Lipofectamine™ Transfection Agent (Invitrogen), according to the manufacturer's guidelines. Transfection efficiency of miRNAs or siRNAs was confirmed by RT-PCR and western blot.

qRT-PCR for miRNA expression

Total RNA was isolated with Tripure reagent™ (Roche Applied Science). 3.3 ng DNAse-free total RNA was used for reverse transcription (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) followed by Taqman® MicroRNA Assays for quantification of miR-155 and RNU19 control transcripts (Applied Biosystems: 4373124, 4373378, respectively). Amplification and detection of specific PCR products was performed in a MyIQ single-color real-time PCR system (Bio-Rad). MiR-155 expression levels were normalized for RNU19 and presented as fold induction ($2^{-\Delta\Delta Ct}$).

MMP-16 mRNA expression was determined using the SuperScript First- Strand Synthesis System (170-8890, Bio-Rad) and qRT-PCR amplification was detected in a MyIQ single-color real-time PCR system using iQ™ SYBR® Green Supermix (170-8884, Bio-Rad) and specific primers (F: GGACAGAAATGGCAGCACAAGC, R: CATCAAAGGCACGGCGAATAGC). PCR product was loaded on a PAGE-GEL to visualize and determine specificity of the amplified products.

Zymography

Cells were transfected with different miRNAs as described above and refreshed with serum free medium the day after transfection. Conditioned medium was collected for 48 h and proteins were separated by SDS-PAGE supplemented with gelatin substrate, followed by incubation with Brij solution (50 mM Tris, 10 mM CaCl2, 0.05% Brij-35 solution) at 37°C O/N and stained with Coomassie blue [14]. The intensities of digested bands were detected by ChemiDoc XRS system (Bio-Rad).

Scratch assay

Cells were transfected with different miRNAs as described above. When cells reached confluency (24-48 h) after transfection, a scratch-wound was made and hCMPC migration was monitored subsequently for 6-8 h. To quantify hCMPC migration, pictures were made immediately after the generation of the scratch and at termination of the experiment. Photoshop and Image J software were used to calculate the area of wound closure.

Transwell assay

Cells were transfected with different small RNAs 48h before transwell assay. 5x10^4 transfected cells were resuspended in basic DMEM culture medium, supplemented with 0.5% FBS, and added to the upper chamber of a trans-well system (8 µm pore size, Corning 3422). Basic culture medium, supplemented with 10% FBS and 10 ng/ml VEGF, was added to the lower chamber. Cells were allowed to migrate for 6-8 h

before membranes were fixed. The non-migrated cells from the upper well were removed by a cotton wrap, and migrated cells were stained with 0.2% crystal violet, followed by quantification using Image J.

Target site cloning

Of the 4.2 kb long MMP-16 3'UTR, the last 1.4 kb, containing the two putative binding sites for miR-155, was cloned into the pMIR-REPORT vector (Ambion, 5795). For this, we used the following primers with restriction sites (Italic) for sub-cloning: mmp16 3'UTR forward with SpeI site 5'- GACTAGTCGGGCCTTGATGTCAAGAAAA-3' and mmp16 3'UTR reverse with SacI site 5'-CGAGCTCGCCCACAGAGAGGGAATGAAA-3'.
The obtained plasmids were isolated, followed by sequencing (Hubrecht Lab, the Netherlands) to confirm sequences of the inserted MMP-16 3'UTR and vector. Mutational cloning in the seed region of predictive sites was performed with QuikChange® Site-Directed Mutagenesis Kit (200518, Stratagene), according to manufacturer's guidelines. To achieve the two mutations in the putative seed regions, we used the following primers (mutated nucleotides are in bold):
mutation site 1 forward:
5'- GGGCTAGAAAATAATCATAGGAGTAAGAAGGAAGGAGTCTATC-3'
mutation site 1 reverse:
5'- GATAGACTCCTTCCTTCTTACTCCTATGATTATTTTCTAGCCC-3'
mutation site 2 forward:
5'- GAGCTAAATTAGACAGGAGTATGTGATACTAGCAAAGACAACTTC-3'
mutation site 2 reverse:
5'- GAAGTTGTCTTTGCTAGTATCACATACTCCTGTCTAATTTAGCTC-3'

Luciferase experiments

In a 24-well plate, HEK293 cells were transfected with 200 ng pMIR-REPORT vector, containing the MMP-16 3'UTR, or seed-mutated vectors, in combination with 100 nM of different miRNAs. A total of 200 ng CMV-β-galactosidase plasmid was co-transfected as an internal transfection control. Cells were lysed 48 h after transfection and luciferase and β-galactosidase activity were measured.

Western blot

Two days after miRNA or siRNA transfection, total protein was extracted using RIPA lysis buffer (50 mM Tris/HCl pH 7.5, 0.1% SDS, 1% Triton-X 100, 0.5-1% sodium deoxycholate, 150 mM NaCl, protease inhibitors (Roche, 04693159001). Samples were loaded on a NuPAGE® Novex® Bis-Tris Gel (Invitrogen, NP0336) with 10x reducing reagent (Invitrogen, NP004). After size separation, proteins were transferred to a PVDF

membrane (Schleicher & Schuell 32-10413096), which was subsequently incubated with MMP-16 antibody (rabbit polyclonal, Abcam 73877, 40 ng/ml) and goat anti-rabbit IgG secondary antibody (DAKO, P0448, 1:2000). Beta-tubulin (rabbit polyclonal, Cell Signaling, 2146, 1:2000) was used as a loading control. Signals were detected after exposure to enhanced chemiluminescence substrate (Amersham) using the ChemiDoc XRS system (Bio-Rad).

Immunohistochemistry

hCMPCs were seeded in chamber slides O/N and fixed with 4% PFA for 10 min at RT. Cells were washed with 0.2% Triton in PBS for 5 min at RT, blocked with 2% PBSA for 60 min, and incubated with rabbit-anti-human MMP-16 antibody (abcam, ab73877) or isotype control (rabbit IgG) in 2% PBSA O/N at 4°C. MMP-16 was visualized by using goat-anti-rabbit Alexa555 for 60 min at RT in the dark and imaged by using a Zeiss LSM 510 Meta confocal microscope.

MMP-16 blocking antibody

hCMPCs were seeded in 24-well plates at 50% confluency in normal culture medium. The next day, a scratch wound was created and the medium was refreshed with 1% FBS culture medium, which contained 20 µ g/ml MMP-16 antibody (Abcam 73877) or IgG isotype control. After 6-8 h, cell recovery was monitored and analyzed using Photoshop software.

Statistics

Data is presented as mean ± SEM of at least three independent experiments and were compared using the two-tailed paired Student's t test. A difference with a $p<0.05$ was considered to be statistically significant.

RESULTS

Introducing miR-155 inhibits cell migration

Since increased miR-155 levels could improve cell survival [11] and thereby potentially increase cell retention, we studied if increasing miR-155 levels might contribute to improved cell retention via other mechanisms like cell migration. To study this, we performed a scratch wound assay and monitored wound closure for 6-8 h. Overexpressing miR-155 was achieved and confirmed by qRT-PCR as previously reported [11]. We observed that increasing levels of miR-155 inhibited cell migration and found that 30 nM pre-miR-155 reduced migration by 38±3.6% compared to ctrl-miR

(Figure 1A, p<0.05). Furthermore, to exclude an effect of hCMPC proliferation, we performed a transwell migration assay. Introducing 30 nM pre-miR-155 decreased migration over a membrane with 59+3.7%, as compared to the ctrl-miR group (Figure 1B, p<0.05). These combined data suggest that miR-155 is effective in blocking hCMPC cell migration.

MiR-155 reduces MMP-2 and -9 activity levels

We have observed before that hCMPCs are able to produce MMP-2 and -9 [15], important proteases that allow matrix turnover and cell migration. We measured secreted MMP-2 and -9 levels from hCMPCs upon transfection of pre-miR-155, anti-miR-155, or ctrl-miR, and found that overexpressing miR-155 decreased active MMP-2 and -9 levels by 68% (Figure 2A+C, p<0.05) and 49% (Figure 2D+E, p<0.05), respectively. Interestingly, pro-MMP-2 levels were not affected (Figure 2A+B), indicating that miR-155 limits cell migration by inhibiting MMP-2 and -9 activation, but not by affecting their expression. This was confirmed by unchanged mRNA levels (Suppl Figure 1). Since miRNAs cannot directly block protease activity and because MMP-2 and -9 are not predicted to be targets of miR-155, we searched for other possible direct targets of miR-155.

MiR-155 directly targets MMP-16

MiR-155 is predicted to target MMP-16 (MT3-MMP, membrane type 3 MMP) (www.microRNA.org), which is a potential activator of MMP-2 and -9 [16,17]. We therefore examined whether miR-155 could directly target MMP-16. First, we confirmed that MMP-16 is expressed in different primary hCMPC cell lines as indicated by gene expression and immunohistochemistry (Figure 3A+B).

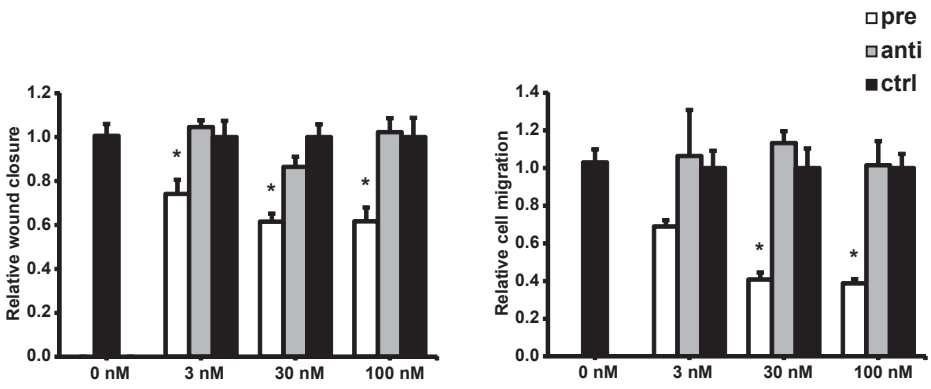

Figure 1

Introducing miR-155 in hCMPCs reduces cell migration in a scratch (A) and transwell assay (B). Cells were transfected with different concentrations (0, 3, 30, 100 nM) of pre-miR-155 (pre), anti-miR-155 (anti), and ctrl-miR (ctrl), and normalized to non-transfected cells. n=3 and *p<0.05

Upon pre-miR-155 transfection, MMP-16 mRNA expression levels did not change in hCMPCs (Figure 4A), however a robust downregulation of MMP-16 protein expression was observed (Figure 4B, $p<0.05$, and Suppl Figure 2). This suggests that MMP-16 is a direct target of miR-155. To validate this, we cloned 1.4 kb of the 3'UTR of MMP16 into a luciferase reporter vector and observed a significant reduction in luciferase

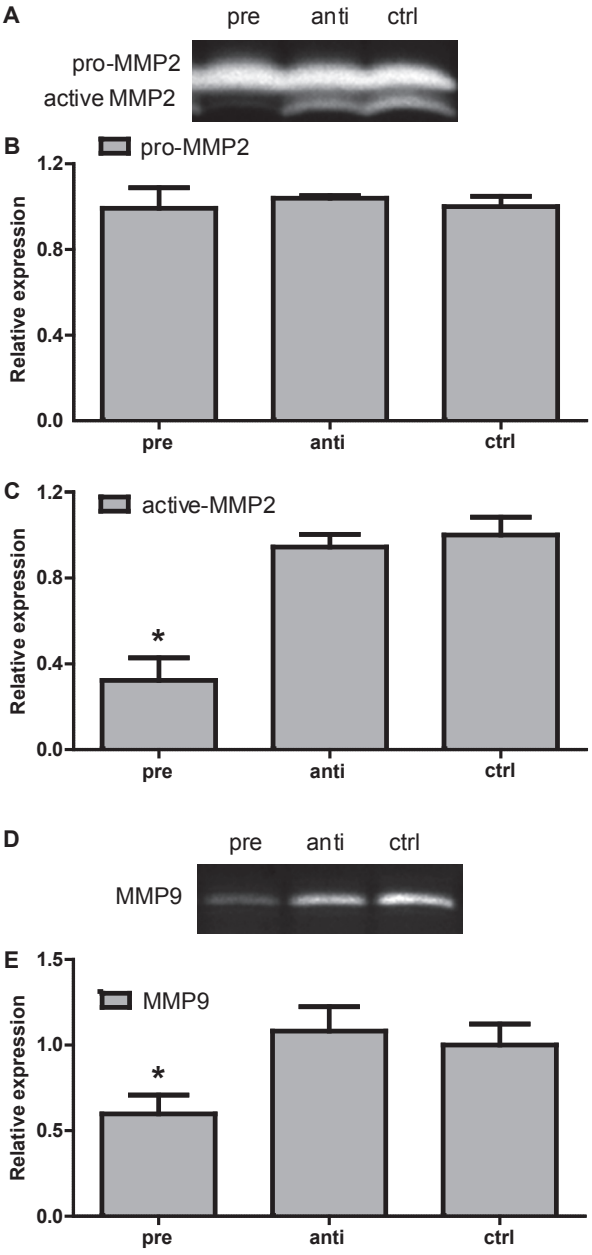

Figure 2
Introducing 30 nM pre-miR-155 in hCMPCs decreased MMP activity levels as detected by zymography. Visualization (A) and quantification of pro- (B) and active-MMP-2 (C) activity. Visualization (D) and quantification (E) of MMP-9 activity levels. n=3 and *$p<0.05$

expression after co-transfecting miR-155 (Figure 4C). This inhibition could be abolished by co-transfection of anti-miR-155, indicating that miR-155 directly decreases MMP-16 production. This was further confirmed by generating mutations in the two target sites in the MMP-16 3'UTR. We found that the decrease in luciferase activity was prevented (Figure 4C), and that target site 1 is probably most powerful in regulating MMP-16 expression.

MMP-16 knockdown inhibits cell migration

To assess the functional relevance of MMP-16 in the observed reduction in MMP activities and cell migration, we used an MMP-16 blocking antibody or knocked down MMP-16 expression by siRNA treatment. As shown, RNAi repressed MMP-16 expression in hCMPCs (Figure 5A+B). MMP-16 knockdown or blocking MMP-16 with blocking antibody both inhibited MMP-2 activity (Figure 5C+D). Scratch assays showed that blocking MMP-16 or knocking down MMP-16 diminished cell migration to a similar extent as miR-155 overexpression (Figure 5E+F). Our combined results show that miR-155 directly targets MMP-16 and thereby consequentially reduces MMP-2 and MMP-9 activity levels, leading to inhibition of cell migration.

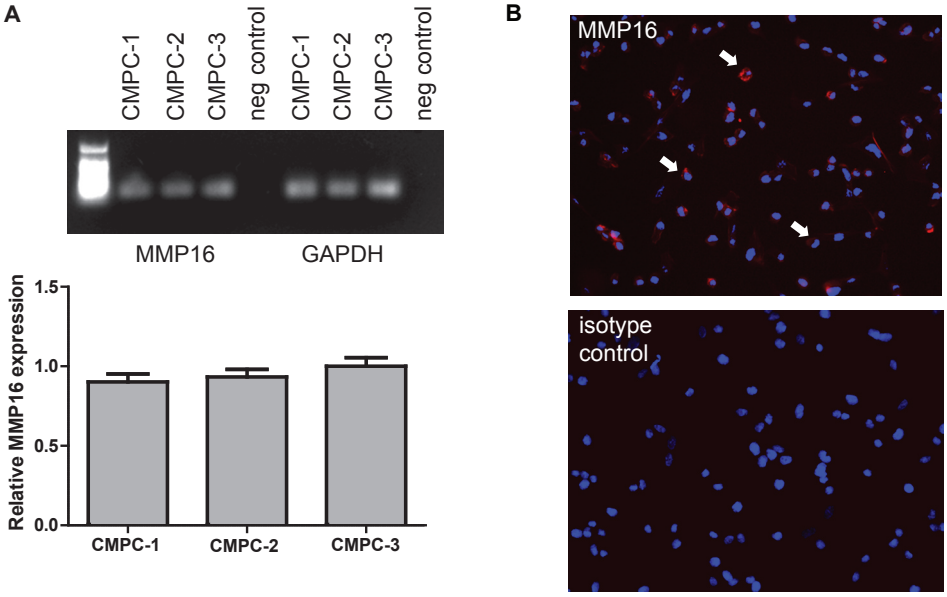

Figure 3
MMP-16 is expressed in hCMPCs as indicated by (A) MMP-16 gene expression in different hCMPC cell lines, and (B) immunofluorescent analysis for MMP-16 in hCMPCs. (MMP-16 expression in red, example positive cells are indicated by arrows)

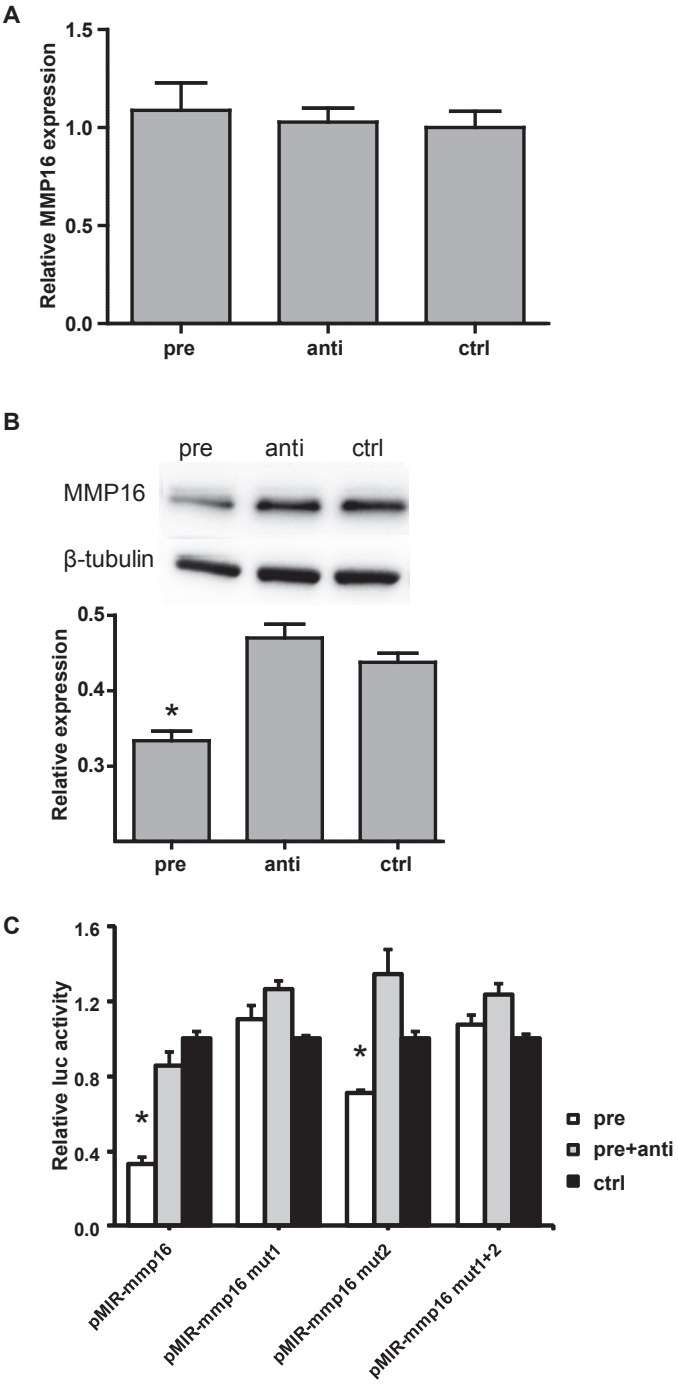

Figure 4

MMP-16 expression in miRNA transfected hCMPCs, on mRNA level (A) and on protein level (B), as demonstrated by western blot, including densitometry. Luciferase activity of pMIR-REPORT vector, containing the 3′UTR of MMP-16 (pMIR MMP-16) or containing the MMP-16 3′UTR with seed-mutated target sites 1, 2, or both (C). n=4 and *p<0.05

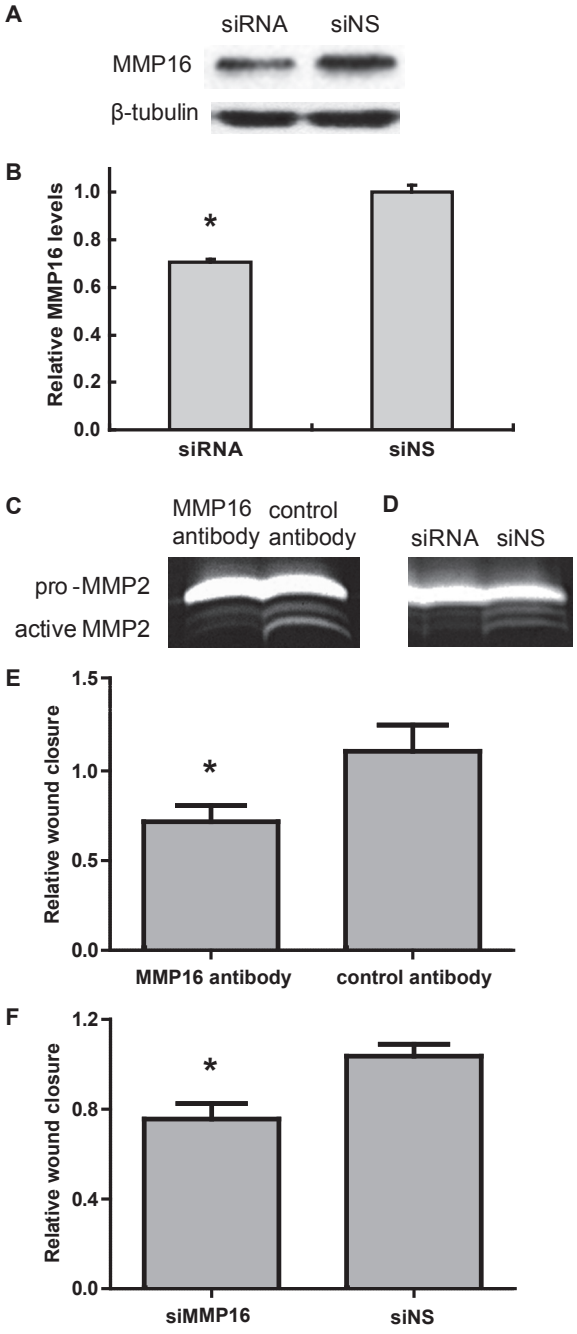

Figure 5

By using siRNAs against MMP-16 (siMMP-16) we could reduce MMP-16 protein levels (A), as quantified in (B). Both a blocking antibody for MMP-16 (C) as the siMMP-16 (D) reduced MMP-2 activity, as visualized by zymography (C+D). Both approaches resulted in a reduction in cell migration in a scratch assay as shown for the MMP-16 blocking antibody (E) or by using siRNA-mediated knockdown of MMP-16 (F). n=3 and *$p<0.05$

DISCUSSION

As indicated previously, high cell survival and limited cell migration could enhance cell retention after cell transplantation [5]. In its turn, short-term cell retention is a prerequisite for long-term cell engraftment in cell transplantation therapy, and thereby enhances cardiac repair. Therefore, much effort has been put into improving cell survival and engraftment after cell transplantation therapy. For example, co-administration of cardiac progenitor cells and IGF-1 has been shown to promote cell engraftment and thereby facilitated cardiac regeneration [18]. In addition, preconditioning MSCs with stromal-derived factor1 alpha (SDF-1) [19] or overexpression of Akt protected MSCs from death after transplantation via a paracrine mechanism [20]. Moreover, ischemic preconditioning augmented miR-210 expression and protected MSC survival by targeting caspase8 associated protein [21]. We have also reported that overexpressing a miRNA, miR-155, inhibited necrotic cell death via targeting RIP1 (receptor interacting protein1), thereby providing a potential novel approach to improve cell engraftment[11].

In addition to increasing cell survival, limiting off-target migration will also potentially lead to better cell retention. Currently, therapeutic cells can be injected accurately into the area of interest with the guidance of e.g. the NOGA system. This system incorporates a cardiac catheter device and a three dimensional electromechanical mapping system, and can thereby define the viable, hibernating and infarcted myocardium [1]. We previously observed that hCMPC transplantation repressed left ventricular remodeling and resulted in better cardiac performance. Upon injection, engrafted cells were not confined to the site of injection, but could also be detected in the remote regions, indicating that cells are able to migrate away from the site where they are needed most; the myocardium in need [22]. This highlights the necessity of limiting post-delivery cell migration in the myocardium.

MiRNAs are known to be involved in various cellular processes, including cell migration [23-25]. Although most studies showing that miRNAs regulate migration are focused on tumor cell invasion [26-28], several studies have now shown that miRNAs play important roles in non-tumor cell migration as well, like miR-9 in human neural progenitor cell migration [9], and miR-21 in pulmonary artery smooth muscle cell migration [24].

In this study, we investigated the potential of modulating hCMPC migration by increasing miR-155 levels. We showed that miR-155 blocked cell migration by directly targeting MMP-16 and subsequently reducing MMP-2 and -9 activities. The role of miR-155 in cellular migration seems cell type and context specific since reports have shown promoting as well as inhibiting roles for miR-155 in cell migration [29,30]. We

found that miR-155 directly downregulated MMP-16, an activator of MMP-2 and -9, and that inhibition of MMP-16 by a blocking antibody or via siRNA-mediated knockdown reduced hCMPC migration to a similar extent as miR-155 overexpression. MMP-16 is a membrane anchored MMP that is able to activate other MMPs, growth factors, and receptors, and thereby facilitates a local cellular mechanism for migration. Our results confirm the functional relevance of MMP-16 in the downregulation of hCMPC cell migration. This is in concordance with a previous report showing that decreasing MMP-16 levels led to a reduction in cell migration [31].

Even though increased expression of miR-155 could modulate cell migration, we did not observe a block in migration when endogenous levels of miR-155 in hCMPCs were blocked. This suggests that the biological endogenous role of miR-155 in hCMPCs may not be migratory. However, since pre-miR-155 transfection only transiently increases miR-155 levels, preconditioning of hCMPCs with miR-155 provides a novel opportunity to improve cell survival and reduce migration after transplantation from a therapeutic point of view.

In conclusion, increasing miR-155 levels can inhibit hCMPC migration via direct targeting of MMP-16 *in vitro*. In combination with the cytoprotective effect [11], miR-155 upregulation might provide a novel and promising approach to augment the therapeutic effect of hCMPC transplantation. Additional proof of improved cell retention by miR-155 after local cell delivery in the infarcted heart is needed for further evaluation of its potential to improve cell transplantation therapy.

Acknowledgements

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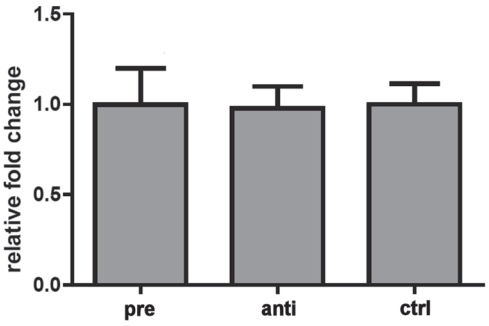
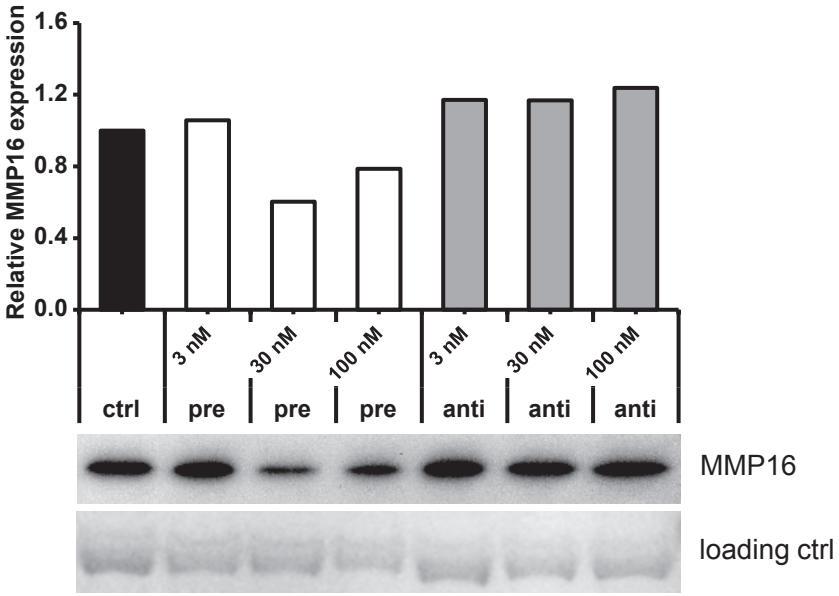


Supplemental Figure 1
MMP-2 mRNA expression in pre, anti and ctrl-miR transfected cells. n=3.



Supplemental Figure 2
Western blot of MMP-16 expression after transfection of different concentrations of pre-miR-155, or anti-miR-155 (3, 30, and 100 nM) as compared to control transfected hCMPCs.

CHAPTER 6

THE POTENTIAL OF MODULATING SMALL RNA ACTIVITY IN VIVO

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ABSTRACT

Small RNAs have shown to be ubiquitous, useful, post-transcriptional gene silencers in a diverse array of living organisms. As a result of homologous sequence interactions, these small RNAs repress gene expression. Through a process called RNA interference (RNAi), double strand RNA molecules are processed by an enzyme called Dicer, which cleaves RNA duplexes into 21-23 base pair oligomers. Depending on their end-point functions, these oligomers are named differently, the two most common being small interfering RNAs (siRNAs) and microRNAs (miRNAs). These small RNAs are the effector molecules for inducing RNAi, leading to post-transcriptional gene silencing by guiding the RNAi-induced silencing complex (RISC) to the target mRNA. By exploiting these small RNAs, it is possible to regulate the expression of genes related to human disease. The knockdown of such target genes can be achieved by transfecting cells with synthetically engineered small RNAs or small RNA expressing vectors. Within recent years, studies have also shown the important role of miRNAs in different diseases. By using several chemically engineered anti-miRNA oligonucleotides, disease related miRNAs can be specifically and effectively silenced. Since RNAi has developed into an everyday method for *in vitro* knockdown of any target gene of interest, the next step is to further explore its potential *in vivo* and the unique opportunities it holds for the development of novel therapeutic strategies. This review explores the various applications of small RNA technology in *in vivo* studies, and its potential for silencing genes associated with various human diseases. We describe the latest development in small RNA technology for both gene knockdown, and the inhibition of translational silencing in animal studies. A variety of small RNA formulations and modifications will be reviewed for their improvement on stability and half-life, their safety and off-target effects, and their efficiency and specificity of gene silencing.

INTRODUCTION

Many pathological conditions depend on abnormal gene expression levels. This could include the aberrant expression of endogenous or mutant genes, or the expression of foreign genes in an infected organism. With the introduction of nucleic acid-based inhibitors or antisense agents, a novel view on how to fight disease was established. In addition to strategies based on the inhibition of target proteins, the possibility of specific downregulation of pathologic genes emerged as an appealing strategy for treating human disease. Targeting the molecular level of disease by modifying gene expression with several types of antisense agents has advanced rapidly over the past

20 years, especially with the discovery of certain small RNA molecules with remarkable properties. The rapid advancement was primarily initiated by the sequencing of the human genome and the accompanied rapidly growing knowledge of the molecular causes of disease. After successful application *in vitro* and in small eukaryotic organisms like Caenorhabditis elegans, several of the antisense gene-silencers were prepared for *in vivo* studies in mammals.

The use of antisense agents started with antisense oligonucleotides (ASOs), short stretches of single-stranded RNA or DNA with sequence complementary to their target messenger RNA (mRNA). The idea that these ASOs could be used as specific inhibitors of gene expression was introduced in 1978 [1, 2]. The silencing mechanism of ASOs showed to vary depending upon the charged characteristics of the ASOs backbone [3]. Although much research was put into ASOs, interest eventually declined when the development of predicted therapeutic possibilities proved to be very time-consuming. Fortunately, gene-targeting strategies were given a boost with the discovery of RNA with catalytic activity, the so-called ribozymes (from *rib*onucleic acid *enzyme*) in 1982 [4], which changed the perception of RNA as a simple bridge between DNA and protein. Since RNA can serve as a catalyst and as a carrier of genetic information, it holds both properties needed for life. This provided the basis for the "RNA world hypothesis", which proposes that our current DNA-, RNA- and protein-based world has evolved from an earlier exclusively RNA-based world, and started an exciting age of exploration of the functional RNA world. A unique property of the ribozyme is that it is able to break covalent bonds in RNA molecules with sequence specificity when guided by a unique substrate sequence [5] or when covalently joined to a specific antisense component [6]. This new knowledge further expanded the use of nucleic acid-based inhibitors of gene expression. Subsequently, both ASO and ribozyme strategies were further improved regarding stability, delivery, and efficiency of gene targeting, although issues still exist.

Following ASOs and ribozymes, a novel gene-targeting mechanism was discovered in 1998 in the nematode C. elegans [7]. This small RNA-based, naturally occurring, sequence-specific, posttranscriptional gene silencing phenomenon was termed RNA interference (RNAi). RNAi is triggered by the presence or introduction of double-stranded RNA molecules (dsRNA). Through an intracellular multistep process, specific small RNAs, called siRNAs, elicit powerful, targeted degradation of complementary RNA sequences [8]. It soon became clear that RNAi is evolutionary conserved as it also exists, although somewhat more complex, in vertebrates, including human. Because of its easy-to-use method *in vitro* and high specificity, RNAi showed to be a particularly powerful tool for targeted inhibition of gene expression of any selected target gene. As a result, our understanding of gene function improved rapidly and RNAi is now a

well-established tool in biomedical research where it is being explored in high-throughput analysis, *in vitro* and *in vivo* functional studies, and for the development of gene-specific therapeutics. The success of RNAi was acknowledged by the Nobel Prize committee and the 1998 discovery of RNAi by Andrew Z. Fire and Craig C. Mello was awarded the 2006 Nobel Prize for physiology and medicine. Although being the most promising gene silencing tool so far, efficient delivery and side-effect issues have held back the *in vivo* applicability of this technique as well.

BASICS OF RNAi

Prior to the discovery of RNAi in 1998, the phenomena of RNAi had been observed eight years earlier in transgenic plants where it was termed co-suppression [9]. This study demonstrated that, in an attempt to promote violet pigmentation in petunias, the introduction of dsRNAs for the pigmentation gene, resulted in complete and/or partly white flowers [9]. RNAi was demonstrated experimentally in C. elegans by Fire et al. [7], who showed that the injection of specific dsRNAs resulted in marked inhibition of gene expression, complementary to the dsRNA. Injection of dsRNA resulted in great efficiency of gene silencing, whereas sense or antisense RNA strands alone did not result in a significant reduction of targeted mRNA. A few years later, the mechanism of RNAi was experimentally demonstrated in a wide range of eukaryotic organisms including flies [10, 11], zebrafish [12], and finally in mammalian cells, including human [13]. The effector molecules of the RNAi mechanism were revealed by Zamore et al., who showed that the dsRNA was rapidly cleaved into small dsRNA strands with a length of 21 to 23 nucleotides (nt) called siRNAs [8]. The pivotal role of siRNAs in initiating RNAi was confirmed by the introduction of chemically synthesized siRNAs, which by themselves were sufficient for the induction of gene silencing [13]. Through biochemical analysis of the siRNAs, two distinctive features were found. The siRNA molecules possessed 2 to 3 nt overhangs at the 3′ end and a monophosphate group on the 5′-terminal nucleotide, which indicated that siRNAs were the cleavage product of an endoribonuclease of the RNase III family [14]. This quickly led to the identification of Dicer as the enzyme required for cleaving dsRNA into siRNAs [15].

It is now clear that RNAi is an intracellular multistep process which initially begins with the cleavage of dsRNAs or short hairpin RNAs (shRNAs) [16] into siRNAs by Dicer (Figure 1). Dicer consists of two RNase III domains, a dsRNA binding domain, an N-terminal helicase domain and the RNA binding domain Piwi Argonaute Zwille (PAZ) [15, 17]. After cleavage, single stranded siRNAs are incorporated into the RNA induced silencing complex (RISC), constituted of at least Dicer, Transactivation

Response Binding Protein (TRBP), and one Argonaute protein (Ago2 in human) [10]. The siRNAs are bound to Ago2, the catalytic protein component of the RISC, which is partially responsible for the selection of the siRNA guide strand on the basis of the 5′ end stability in Drosophila [18, 19], and for the destruction of the siRNA passenger strand [20]. The RISC is activated upon ATP-dependent unwinding of the double-stranded siRNA into the single-stranded siRNA guide strand by RNA helicase activity [20]. Next, the activated RISC is brought in proximity to its target mRNA [21, 22], mediated through the hybridization of the antisense siRNA guide strand to its perfect complementary mRNA target site, which is then cleaved by the RISC nuclease Ago2 and further degraded as it has lost its protective ends [23, 24].

Anti-viral defense is one of the biological functions ascribed to RNAi, since RNAi has been shown to take part in a nucleic-acid-based immune system, protecting human cells from viral infection by degrading viral transcripts [25, 26]. Next to its important role as a regulator of gene expression through miRNAs, which will be discussed next, RNAi is also thought to be important in preventing transposon jumping [27].

Finally, RNAi is thought to contribute to genomic imprinting [28], to silencing of translationally aborted or overproduced mRNAs [29], or to tissue-specific gene expression by modulating DNA conformation [30], since RNAi is also capable of inducing heterochromatin formation [31] and DNA methylation [32].

MicroRNAs

Since RNAi could be induced by foreign dsRNA, and was therefore shown to be endogenous in several eukaryotic organisms, it was hypothesized that the mammalian cellular genome might encode some sort of RNAi inducing RNA. This was confirmed with the discovery of miRNAs, small RNA molecules that negatively regulate endogenous gene expression [33]. An important difference between siRNAs and miRNAs in mammalian cells is that the latter is endogenously present, whereas siRNAs are exogenously derived from e.g. viruses. The action of a miRNA had already been observed in 1993 [34], when the mechanism of RNAi was still unknown. MiRNAs are described as a class of short (~22 nucleotides), endogenously present, non-coding RNA molecules that negatively regulate gene expression by partially complementary base pairing to mRNA, inducing translational repression through mRNA destabilization and degradation [35-37]. In mammals, the cellular biochemical pathway is very similar to that of siRNA [29, 38]. Initially, a miRNA gene is transcribed by RNA polymerase II into variable length (100 to 1000's nt) primary transcripts called pri-miRNAs. These are then processed by the microprocessor complex, which in human consists of at least the RNase III protein Drosha and a dsRNA binding protein DGCR8 [39-41]. This complex binds the pri-miRNA and specifically cleaves at the base of the hairpin loops, releasing

the 60 to 70 nt hairpin-shaped precursor miRNA (pre-miRNA) [39-41]. The pre-miRNAs are exported to the cytoplasm by exportin 5 [42, 43], and are further processed by Dicer into 22 nt long single stranded RNAs (mature miRNAs) and incorporated into the RISC, as described above (Figure 1). In contrast to siRNAs, which primary mode of action is target cleavage through perfect complementarity, miRNAs are partially complementary to their targets. MiRNAs bind predominantly to the 3′UTR of their target genes and only require a "seed" match of 7 to 8 base pairs between the 5′ region of the miRNA and the 3′UTR of the target mRNA [36, 37]. Most miRNA targets are translationally repressed, however, mRNA cleavage can also occur [44]. Due to the partial complementarity, one miRNA could potentially regulate several distinct mRNA targets, thereby regulating a whole set of genes. Furthermore, target prediction algorithms have been generated, predicting that one specific gene could be targeted by numerous miRNAs.

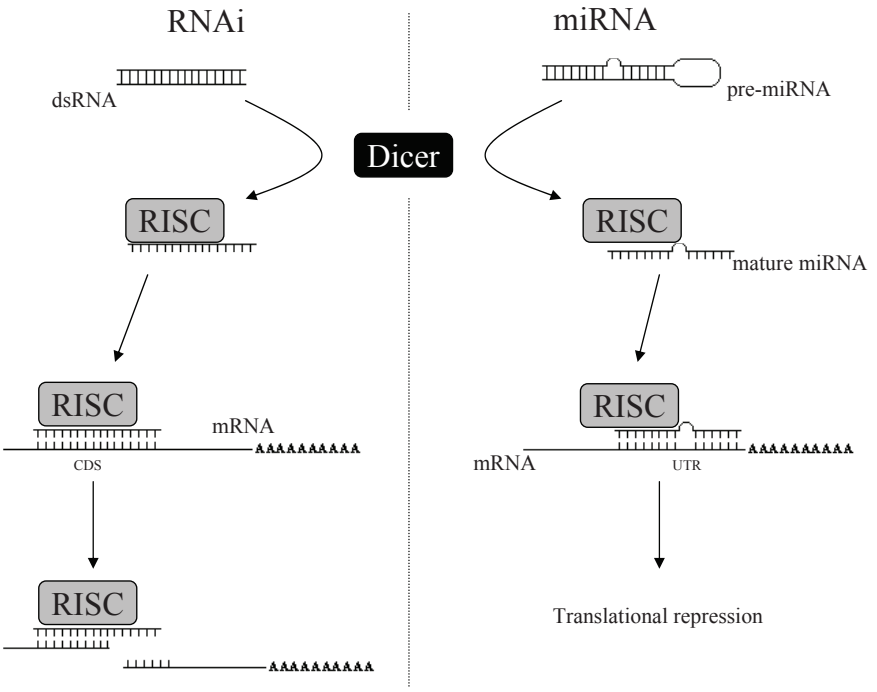

Figure 1
Mechanism of RNAi and miRNA induced gene silencing. Both dsRNA and pre-miRNA molecules are cleaved into a single strand, loaded into the RISC complex, thereby leading to cleavage of the targeted mRNA via perfect binding (RNAi) or translational silencing of the gene via imperfect complementarity (miRNA). (Dicer = endoribonuclease of the RNase III family, RISC = RNA-induced silencing complex, CDS = coding sequence, UTR = untranslated region, mRNA = messenger RNA).

So far, more than 550 miRNA genes have been identified in humans alone and many more have been predicted to exist [45-47]. The importance of miRNAs as biological regulators is recognized by predictions that miRNAs target over one third of all human genes and are often highly conserved across a wide range of species [30, 48, 49]. Moreover, many miRNAs are expressed in a tissue-specific manner which goes as far as organ-specificity or even expression restricted to single tissue layers within one organ [50, 51]. Because of their important roles in biological processes, abnormal expression or mutations in miRNAs or their target sites can affect cellular processes, even resulting in pathological changes, as shown for different forms of cancer [52]. Therefore, not only the silencing of coding genes is an appealing strategy for treating human disease, but also the silencing of disease-related miRNAs. Because miRNAs function through binding to their complementary mRNA sequences, two groups investigated whether oligonucleotides that were complementary to the miRNA would act as inhibitors of miRNA function [53, 54]. This allows miRNA loss-of-function studies *in vivo*, which lead to a better understanding of the precise molecular and biological functions of miRNAs, which are currently largely unknown for mammals. Understanding miRNA function will eventually lead to the development of new therapeutic applications.

IN VIVO DIFFICULTIES FOR SMALL RNA MODULATION

As described above, small RNAs provide two ways of modulation, namely, knocking down gene expression, and the inhibition of translational silencing. Both can be exploited to study specific gene function *in vivo*, create loss-of-function animal models of human disease, or develop small RNA-based therapeutics for a variety of human diseases. For the successful application of small RNA therapeutics *in vivo*, it is essential to stably deliver these small RNAs to specific target tissues, with prolonged activity to inhibit gene function for a sufficient amount of time. However, small single stranded RNA molecules have a highly charged hydrophilic backbone, which makes them particularly vulnerable to enzyme degradation and complicates the diffusion through the cell membrane. In addition, efficient delivery is hampered by non-specific uptake by cells and fast elimination by kidney filtration due to the small molecular mass. In general, the lifetime of small RNAs *in vivo* is insufficient for most human diseases. To achieve a therapeutic effect, sustained delivery is crucial. Vector based systems might provide a solution to this problem as they permit stable expression, but do require specialized delivery methods. Improving the efficient intracellular delivery of ASOs and siRNAs to target sites within the body is still a real challenge [55, 56]. Next to small RNA stability and delivery, the most important

factor in gene-silencing experiments is the efficacy of the small RNA to target the mRNA or miRNA of choice. The targeting efficacy determines the time required to reduce protein or miRNA expression below the threshold level, critical for normal protein or miRNA function.

MODULATING SMALL RNA ACTIVITY *IN VIVO*: GENE SILENCING THROUGH RNAi

To date, RNAi is the most promising strategy for the specific downregulation of pathologic genes. However, to achieve efficient gene-silencing, siRNAs need to be carefully designed. The efficacy and efficiency of gene-silencing can be strongly influenced by the composition and thermodynamic stability of siRNA duplexes [57]. Currently, several guidelines on designing siRNA and shRNA have been published[57-61]. In addition, one can now make use of several online siRNA/shRNA design tools from both academic institutions and commercial companies. However, although predictions are improving, the gene-silencing efficiency of a number of selected candidate siRNAs still needs to be experimentally validated, because RNA-binding proteins and/or intramolecular folding of the target mRNA may hinder antisense binding [62-65]. Therefore, selection of the target sequence is of great importance as well.

Yet, even the most carefully designed siRNA may still have significant sequence specificity problems [66, 67], since a match of only 7 nt is enough to induce miRNA-based gene-silencing [37]. In this way, siRNAs which are introduced into the cell, could exhibit miRNA function, inducing translational repression of one or more targeted genes. In addition, introducing siRNA or shRNA might disrupt the endogenous miRNA pathway through si/shRNA competition with pre-miRNA for exportin-5 or other parts of the processing machinery. This was shown in a study on the effect of high doses of shRNA in the livers of mice, where a significant number of mice died of dose-dependent liver injury, associated with the down-regulation of liver miRNAs [68]. Interestingly, it was shown that both siRNAs and shRNAs can compete against each other and with endogenous miRNAs for transport and for incorporation into the RISC *in vitro*, though the same siRNA sequences did not show competition when expressed from a miRNA backbone [69]. In contrast, a recent *in vivo* study showed effective target-gene silencing by systemic administration of synthetic siRNA without any demonstrable effect on miRNA levels or activity [70]. In general, when the goal is to silence a specific gene by means of siRNA, possible siRNA competition with the endogenous miRNA pathway should be taken into account.

Next to off-target effects due to sequence specificity, siRNAs are also able to provoke immune related side effects by inducing a type I interferon response through Protein Kinase R (PKR) [71, 72], and by activating the innate immune system via toll-like receptors (TLRs) [73-75], both RNA-sensing immunoreceptors. Fortunately, these immune responses can largely be avoided by delivering minimal amounts of siRNA, which are of appropriate length and depleted from certain TLR-associated RNA sequence motifs[76-78]. Altogether, the siRNA/target combination must function with great efficiency, so that only a minimal amount of siRNA is needed to effectively and specifically induce a translational block, minimizing non-specific and off-target effects which are often dose dependent. Unfortunately, most off-target and non-specific effects occurring *in vivo* haven't been documented in great detail. It is evident that, to fully exploit the *in vivo* potential of small RNAs, we need innovative delivery systems and optimal modes of administration, which minimize off-target and non-specific effects.

Vector-based delivery

The *in vivo* delivery of siRNA molecules can be categorized into two general approaches: 1) the transient delivery of siRNA to the target tissue and 2) the inducible delivery of siRNA through shRNA-expressing vectors [79, 80]. Since mammalian cells lack the RNAi amplification mechanism that can occur in C. elegans, gene silencing is dependent on the effective number of siRNA copies delivered into the cells [81]. The use of shRNA-expressing vectors has the advantage that the RNAi effect can be more stable and sustained for a longer period of time [80]. In addition, inducible regulation has the advantage of keeping expression levels within physiological boundaries, whereas transient delivery of a single high dose or multiple doses of siRNAs might result in non-physiological responses. Additionally, vector-based RNAi allows the co-expression of reporter genes and the incorporation of regulatory elements to the promoter region of the expression vector. Successful shRNA delivery and gene silencing *in vivo* has been achieved by using adeno-associated viral (AAV) vectors[82-85] and lentiviral vectors [85-87]. Although the latter is associated with insertional mutagenesis and oncogenic transformation [88, 89]. Recombinant AAV vectors do not cause an inflammatory response, require a helper virus, and they integrate site specifically into the AAVS1 region of chromosome 19, which makes them more safe for *in vivo* use and gene therapy [90, 91]. Still, oncogenic mutagenesis cannot be excluded entirely, since approximately 10 percent of stably AAV transduced genomes have been reported to integrate into host chromosomes *in vivo* [92]. While the use of plasmid shRNA-expressing vectors provides a more safe approach, the successful application of this method is challenged by low transfection efficiencies and

immunogenic side-effects [93]. Overall, strategies based on vector mediated small RNA delivery may possibly go together with serious side effects, which will hamper their *in vivo* use [94].

Unmodified small RNA delivery

Non-viral carrier systems allow a more safe delivery of catalytically active siRNAs. However when not using viral vectors, unmodified siRNAs are generally harder to deliver into the cell. Nevertheless, numerous *in vivo* studies have shown the systemic or local delivery of unmodified siRNAs. A major disadvantage of systemic delivery is the requirement of very high amounts of unmodified siRNA, which is accompanied by an increase in non-specific effects, like concentration-dependent immune responses. Moreover, the standard method used for systemic delivery of unmodified siRNA; hydrodynamic transfection (high-pressure high-volume injection) [79], has been shown to produce membrane defects and disturb the cell interior in mice [95]. Additionally, hydrodynamic delivery primarily targets highly vascularized organs, such as the liver, kidneys, and spleen. On top, the hydrodynamic transfection procedure is highly unsuitable for human clinical use. Local delivery of unmodified siRNA surmounts the use of very high doses since systemic (renal and hepatic) elimination and nonspecific delivery to other tissues is reduced. However, organ-wide gene-silencing through local administration is only successful in a very limited number of organs like liver, eye, lung and brain [80, 96-98], and subcutaneous tissue or tumors [99]. Local delivery in other tissues requires the use of more invasive methods. Overall, systemic delivery is the favorable route for administrating small RNAs, though, especially to become effective in human, this requires the protection of the small RNA against systemic degradation, and special agents for targeting and entering specific cells and tissues.

Small RNA modifications and formulations for *in vivo* delivery

Alternative strategies for systemic delivery of small RNAs consist of backbone modifications, peptide-conjugations, pre-complexation with protecting and uptake-enhancing polymers and incorporation into lipids. All these siRNA modifications and formulations enhance systemic small RNA stability. In addition, polymer pre-complexation, lipid incorporation, and peptide-conjugations protect siRNAs against systemic elimination, enhance cellular uptake, and provide opportunities to target any specific organ, tissue or even cell type with smaller amounts of siRNA. Already many different small RNA modifications and formulations to improve stability and delivery have been employed by several groups.

Chemical modifications

Chemical modifications, including 2′-OH ribose residue substitutions, and phosphodiester backbone modifications have been shown to increase systemic siRNA stability. However, inside the cell, unmodified siRNAs show to be as resistant to degradation as modified siRNAs [100]. The RISC might be responsible for the protection of the siRNA guide strand from intracellular nucleases, which suggests that antimiR oligonucleotides, which will be discussed later, do not experience protection as they do not function through RNAi. Backbone modifications are primarily applied to the siRNAs passenger strand, because this strand plays no direct role in target silencing. Chemical modifications that block phosphorylation of the 5′-end of the guide strand impair RNAi, since the 5′-end phosphate of the siRNAs guide strand is required for Ago2 binding [101]. One major advantage of chemically modifying the siRNA passenger strand is that cells will not incorporate this strand into the RISC, preventing the non-target complementary strand to induce unwanted off-target effects. Partial substitution of the phosphodiester backbone with thioate linkages (Figure 2) at the end of one of the siRNA strands increases siRNA stability [102, 103] and biodistribution [104]. However, phosphorothioate backbones were shown to be cytotoxic and loss of silencing activity could occur [101-103, 105]. 2′-OH ribose modifications like 2′-fluoro (2′-F) (Figure 2) have shown diverging results; substitution of all pyrimidines with 2′-F increased plasma half-life to 1 day, compared to 1 minute for unmodified siRNAs, thereby retaining target silencing activity 100, whereas 2′-F substitutions for all the uridines decreased target silencing 106. Interestingly, the increase in plasma stability did not lead to an *in vivo* extension or improvement of target gene silencing, indicating that *in vivo*, 2′-F modified siRNAs are no more potent than unmodified siRNAs 100. Increased *in vivo* gene silencing has been achieved by chemically modifying all 2′-OH residues on both strands of the siRNA duplex, with 2′-F substitutions on all pyrimidine positions, deoxyribose and 2′-O-methyl (2′-O-Me) (Figure 2) substitutions in all purine positions on the sense and antisense, respectively [107, 108]. Additionally, in contrast to unmodified siRNAs, chemically modified siRNAs did not activate the immune response [108]. This was later confirmed by showing that immune activation by siRNAs can be completely abrogated by selective incorporation of 2′-O-Me, uridine or guanosine nucleosides into one strand of the siRNA duplex [109], by introduction of as little as three 2′-O-Me substitutions into the sense strand [110], or by 2′-O-Me modification of siRNA sense-strand uridine or uridine/adenosine residues [111].

One very promising backbone modification for siRNA is the so-called locked nucleic acid (LNA) (Figure 2). LNA nucleotides contain a methylene bridge between the 2′ and 4′ carbons of the ribose ring, which has been shown to greatly improve bio- and thermal stability of siRNAs without adversely affecting their silencing efficiency [102, 112].

The LNA content and positioning are important for efficient gene inhibition, and reducing off-target effects. This was mediated by increased sequence specificity, lowering RISC incorporation of the siRNA passenger strand and by reducing the ability of improperly loaded passenger strands to cleave the target RNA [112]. Moreover, minimal 3′ end LNA modification effectively stabilizes the siRNA and reduces off-target gene regulation compared with unmodified siRNA, *in vivo* [113].

In addition to backbone modifications, which primarily increase systemic small RNA stability, siRNA delivery formulations like lipid and polymer siRNA have shown to increase stability and enhance cellular uptake due to their positive charge. This facilitates complex formation with the small RNA, and allows electrostatic interaction with the negatively charged cell membrane. Moreover, complex formation and incorporation into liposomes prevents elimination by kidney filtration, allows the

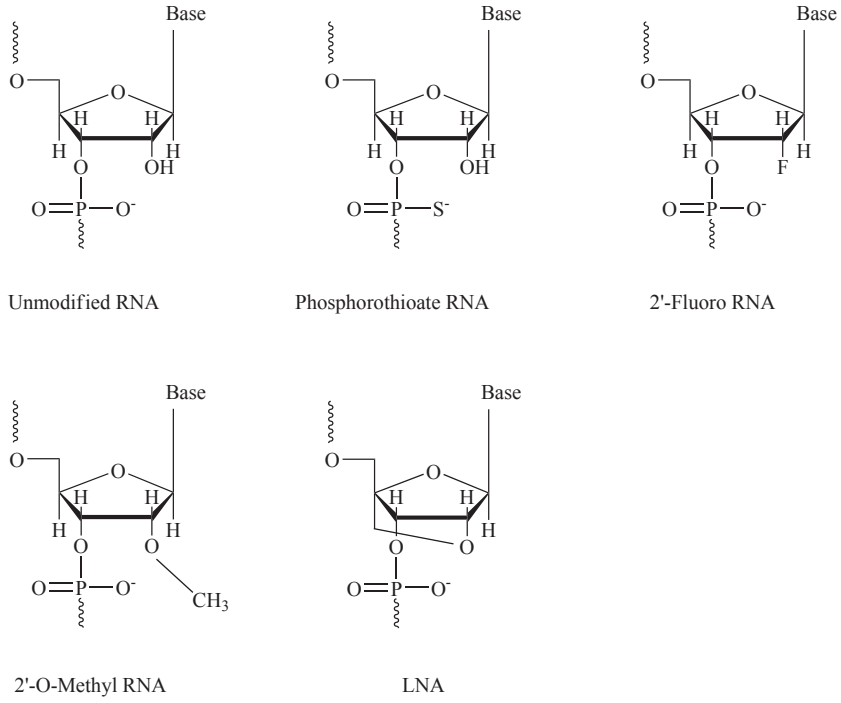

Figure 2
Chemical structures of unmodified and chemically modified RNA used in the different studies. A phosphodiester backbone modification (Phosphorothioate), 2′-OH ribose residue substitutions (2′-Fluoro, 2′-O-Methyl) and a ribose moiety modification (LNA) are depicted. (LNA = locked nucleic acid).

addition of surface molecules, and enables tissue specific targeting. The cellular uptake of these complexes occurs through vesicular mechanisms. For successful delivery, the release of the small RNAs from the endosome into the cytosol is essential. However, how these delivery systems facilitate endosomal release is not yet understood entirely.

Lipid carriers

Since their widespread use in *in vitro* studies, cationic liposomes (Figure 3a) have been one of the first adopted methods for the *in vivo* delivery of small RNAs. They can be seen as non-viral envelopes that mediate cellular uptake, and protect the small RNAs against nuclease degradation and renal excretion. Several groups have used Roche's cationic lipid DOTAP for successful delivery of siRNAs *in vivo* [114], resulting in a 70% and 37% reduction of functional expression of TNF-αand vasopressin receptor V2, respectively [115, 116]. Several other types of cationic liposomal/siRNA formulations have been successfully used for systemic delivery, including LIC-101 liposomes/siRNAs [117], NeoPhectin-AT cardiolipin/siRNAs [118], and AtuFECT01 cationic liposomes/siRNAs [119]. This last study also demonstrated the advantage of using poly ethylene glycol (PEG)-ylated liposomes (Figure 3a). PEGylation sterically stabilizes the nanoparticle, and can reduce immunogenicity and non-specific interactions. However, multiple administrations of PEGylated liposomes have been shown to induce an anti-PEG immune response [120, 121]. The neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) has been used successfully, though at very high concentrations, making it very expensive for human clinical use. Injections of 150 mg/kg body weight of neutral DOPC liposome/siRNAs targeting the oncoprotein EphA2 twice a week (for 4 weeks) resulted in a 10-fold and 30-fold higher tumor accumulation than that of DOTAP/siRNAs and naked siRNAs, respectively [122]. Stable nucleic acid lipid particles (SNALPs) (Figure 3a) have also mediated effective siRNA gene targeting; SNALPs increased systemic half-life from 2 minutes to approximately 6.5 hours [108], and a small single dose of 2.5 mg/kg body weight reduced target gene expression by more than 90% in non-human primates [123].

Lipid carriers can be modified with cell type-specific ligands for tissue or cell specific delivery of small RNAs, thereby minimizing off-target effects. Lactosylated and galactosylated cationic liposomes have been used for hepatic parenchymal cell specific delivery of siRNAs, with significant gene knockdown and no toxicity [124, 125]. However, even with cell-specific delivery, certain problems still remain for liposomal systems, since cationic liposomes can significantly induce the immune response [126-128]. Consequently, modifications of naturally occurring lipids like cardiolipin, a component of the inner mitochondrial membrane, are being developed to minimize liposomal

toxicity. Another point that should be addressed, is that cationic lipids alone were shown to alter gene expression of treated cells when analyzed by microarray-based gene expression profiling [129].

Nanoparticles/cationic polymers

In vivo studies have shown some success in polymer and nanoparticle delivery of siRNAs. Positively charged macromolecules used for *in vivo* delivery of small RNAs include atelocollagen, chitosan, and polyethylenimine (PEI). Atelocollagen is a highly purified pepsin-treated type I collagen which increases cellular uptake, is resistant to nucleases, has prolonged release of oligonucleotides, and displays low immunogenicity and toxicity *in vivo* [130]. *In vivo* delivery of enhancer of zeste homolog 2, phosphoinositide 3'-hydroxykinase p110-alpha-subunit and fibroblast growth factor siRNAs complexed to atelocollagen (Figure 3b) have shown efficient inhibition of tumor growth [131, 132]. Moreover, the complexes remained intact for at least 3 days and did not activate the immune response [132]. Other non-cancer related *in vivo* studies showed that siRNA/atelocollagen complexes were effectively delivered into the brain [133], and detectable in graft vein wall after at least 7 days [134].

Chitosan is a linear polysaccharide, produced by deacetylation of chitin, and has been used in a number of studies to coat nanoparticles for *in vivo* siRNA delivery (Figure 3c). The use of siRNA/chitosan at very small amounts of 0.15 and 1.5 mg/kg body weight administered intravenously every 3 days in mice resulted in tumor growth inhibition of over 90% and no toxicity 135. Effective *in vivo* RNAi was also achieved through nasal [136], and intratumoral [137] administration of siRNA/chitosan formulations. It is however crucial to mention that next to its anti-bacterial activity, chitosan can cause anti-tumor activity via activation of the immune system [138].

Several *in vivo* studies have used polyethylenimines (PEIs) as polymeric delivery systems for small RNAs (Figure 3d). PEIs are synthetic polymers of various shapes and sizes, which allow noncovalent complexation with nucleic acids. Next to protection against nucleolytic degradation, PEI increases cellular uptake through endocytosis, and enhances cytosolic release. Intraperitoneal injections of low molecular weight PEI-complexed, but not of naked siRNAs targeting the HER-2 receptor led to significant reduction in tumor growth in a mouse tumor model [139]. Intraperitoneal and subcutaneous injections of PEI-complexed siRNAs targeting BCR/ABL1 leukemia fusion protein also led to significant inhibition of tumor growth, without a measurable induction of siRNA-mediated immunostimulation [140]. Tissue specific uptake of siRNA/PEI complexes can be enhanced by adding peptide-conjugations. This was shown *in vivo* in a mouse tumor model, demonstrating tumor specific complex uptake, target specific downregulation, and a 90% reduction in tumor growth rate upon intravenous

injection of siRNAs complexed with PEI. The PEI was PEGylated with an RGD peptide ligand, attached to the distal end of PEG (Figure 3d), targeting tumor-specific integrins, thereby providing tissue specific delivery [141]. Targeted nanoparticles have also been used for systemic delivery of siRNAs in non-human primates and mice, for this a cyclodextrin-containing polycation with a transferrin protein-targeting ligand (Figure 3e) for delivery to transferrin receptor-expressing cells was used [142, 143]. Several studies report high toxicity for PEIs, however toxicity is related to the exact composition (length, charge density [144], and primary amine groups [145]) of the used PEI. Therefore, for the successful *in vivo* application of small RNA/PEIs, it is crucial to analyze PEI structure-toxicity. Also, next to chitosan, cationic polymers like PEI have been known to have intrinsic anti-tumor effects [138], which have to be taken into account when developing PEIs for the delivery of small RNAs.

Conjugations

Alternatively to siRNA pre-complexation and incorporation into liposomes, certain siRNA conjugations have also shown to increase small RNA stability and enhance cellular uptake. More importantly, tissue specific delivery can be facilitated. This has been realized through chemical conjugation of cholesterol to the 3'-end of the siRNA sense strand via a pyrrolidine linker (Figure 3f). Chol-siRNAs showed improved *in vivo* pharmacokinetic properties as compared to unconjugated siRNAs, presumably because of enhanced binding to human serum albumin. The *in vivo* elimination half-life was prolonged to approximately 95 minutes, compared to 6 minutes for unmodified siRNA, after an intravenous injection of 50 mg/kg body weight into rats and resulted in an approximate 60% knockdown of the target mRNA in the liver. More importantly, cholesterol attachment improved efficacy and specificity in liver and jejunum tissue uptake [146]. A major advantage of cholesterol conjugated siRNA is that the modification is minor and does not significantly alter the chemical and biological properties of the siRNA formulation, as is seen for siRNA-lipid and -polymer complexes [147]. Although this approach has high potential for *in vivo* rodent studies, one potential problem remains when extrapolating the rodent data to human clinic; the high dosage required for a desired effect would be very expensive.

A very promising method for cell type specific delivery of small RNAs is antibody mediated delivery. In 2005 it was shown that systemic delivery of a siRNA-protamine-antibody conjugate (Figure 3g) improved efficacy and specificity in tissue uptake in mice with subcutaneously injected gp160 expressing tumor cells, and caused a significant anti-tumor effect [148]. The fragment antibody, which is linked to protamine, targets the HIV-1 envelope protein gp160. The efficiency of this study is proven by the use of much lower amounts of siRNA to achieve significant target downregulation,

with 2 to 2.5 mg/kg body weight. Another advantage of this technique is the flexibility, ease-of-use and preparation, since no specialized chemistry is involved. Given the large availability of humanized monoclonal antibodies, this method can be easily adapted to target nearly any given cell type. However, reaching certain cell types still remains a challenge.

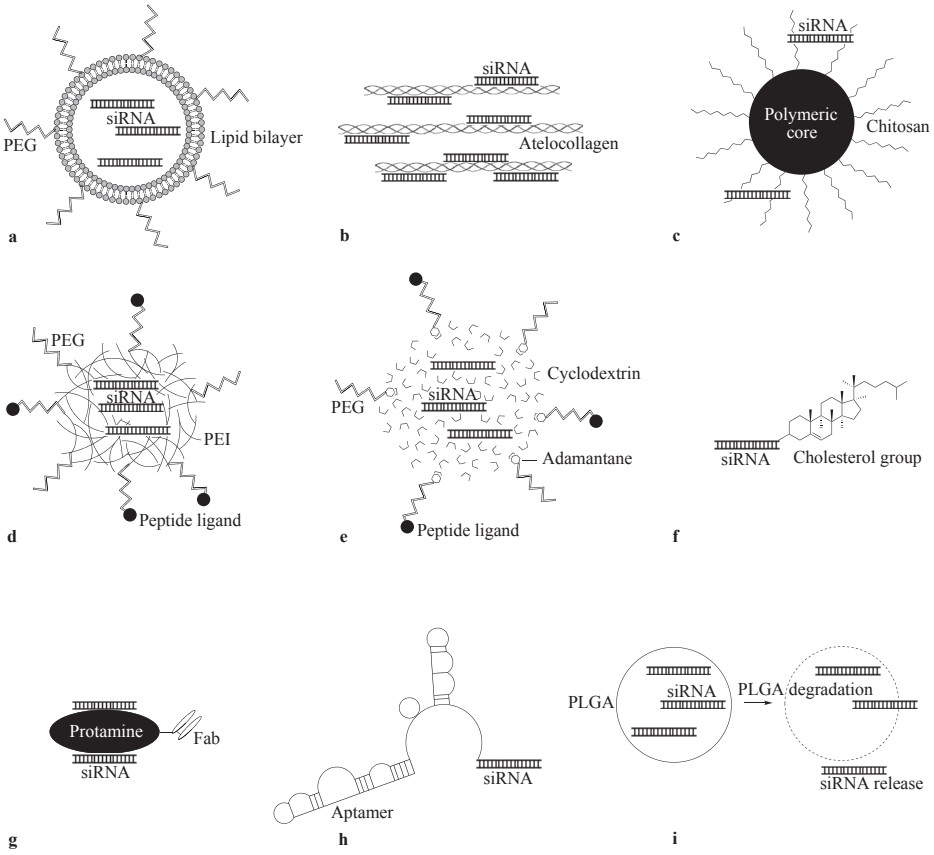

Figure 3
Schematic representation of the different in vivo siRNA delivery formulations. a) Liposomal delivery system with a PEGylated lipid bilayer entrapping siRNAs. b) Atelocollagen, which is positively charged, forms complexes with the negatively charged siRNAs. c) Chitosan coated nanoparticles enable noncovalent binding of siRNAs to the positively charged chitosan. d) Polymeric nanoparticle composed of PEI noncovalently complexed with siRNAs. PEI is PEGylated and a peptide ligand is coupled to PEG, allowing cell type specific delivery. e) Cyclodextrin-containing polycation nanoparticle with PEG linked to the cyclodextrins through adamantane. A protein-targeting ligand on the distal end of PEG enables cell type specific delivery. f) Chemical conjugation of cholesterol to the 3'end of the siRNA sense strand. g) Antibody Fab fragment-protamine fusion protein; a targeted delivery system for siRNAs. h) Aptamer-siRNA chimaeras are capable of cell type-specific binding. Dashes in the structure represent base pairs. i) Delivery with PLGA biodegradable microspheres provides sustained release of siRNA molecules through degradation of the polymeric microspheres. (PEG = poly ethylene glycol, PEI = polyethylenimine, PLGA = poly(lactic-co-glycolic) acid).

An alternative method for cell type-specific binding and delivery of small RNAs is the use of aptamer-siRNA chimaeras (Figure 3h). Intratumoral injections of siRNA-aptamers in mice resulted in a marked reduction in tumor size only in tumors that expressed the aptamer binding ligand. Moreover, siRNA aptamers were non-toxic, and the effect was siRNA specific [149].

One drawback of all these small RNA formulations is the lack of long-term sustained release as in vector-mediated delivery. Although small RNA stability has increased enormously, with cells being exposed to the effect of the small RNA molecule for longer periods, the amount of small RNA gradually decreases. One method using poly(lactic-co-glycolic) acid (PLGA) biodegradable microspheres has shown to provide sustained release of siRNA molecules (Figure 3i) at the site of administration in mice even after 7 days [150]. Recently, the use of PEI as a carrier was added to this delivery system, and siRNA release was shown to last for over one month in a pH 7.4 buffered phosphate solution [151].

MODULATING SMALL RNA ACTIVITY *IN VIVO*: MICRORNA INHIBITION

Next to the *in vivo* gene silencing through siRNAs, small endogenous RNAs, like miRNAs, can be targeted *in vivo* to inhibit translational repression. As described earlier, many pathological conditions depend on abnormal gene expression levels, including miRNA genes. Therefore, silencing of endogenous disease-associated miRNAs may have therapeutic value. However, the delivery of miRNA inhibitors faces the same problems as siRNA delivery. Nevertheless, the *in vivo* inhibition of miRNA function can be achieved by the use of several techniques, which all act through steric blocking rather than RNAi. The first applied technique was a chemically modified (2'-O-Me-modified nucleotides, phosphorothioate linkage), cholesterol-conjugated (through a hydroxyprolinol linkage) single-stranded RNA analogue, complementary to the miRNA, termed 'antagomir'. Antagomirs were administered on three consecutive days at doses of 80 mg/kg body weight, leading to the targeted miRNA being undetectable for as long as 23 days after injection, whereas the unmodified single-stranded RNA had no effect on miRNA levels. In addition, antagomirs achieved broad bio-distribution and efficiently silenced miRNAs in most tissues *in vivo* without apparent toxicities. Moreover, antagomir silencing was highly sequence specific, even discriminating between miRNAs derived from the same primary transcript [152]. It was later demonstrated that antagomirs are able to discriminate between single nucleotide mismatches of the targeted miRNA and require >19-nt in length and a significant number of phosphorothioates for highest

efficiency [153]. A study on cardiac hypertrophy showed 70% lower levels of targeted miR-133 in antagomir-treated mice compared to controls after a single infusion of 80 mg/kg body weight, causing marked and sustained cardiac hypertrophy [154]. Altogether, antagomirs can effectively and specifically silence miRNAs *in vivo*, which makes them highly suitable to study gene regulation *in vivo*. Moreover, antagomirs provide a straightforward and fast method for the generation of mice lacking specific miRNAs and could potentially become a therapeutic strategy for human diseases.

Besides antagomirs, unconjugated forms of single-stranded RNA analogues have been used for the *in vivo* silencing of miRNAs as well. 2′-O-methoxyethyl-phosphorothioate-modified ASOs, targeting miR-122, an abundant liver-specific miRNA implicated in cholesterol and fatty acid metabolism as well as hepatitis C viral replication, were injected twice weekly for over 4 weeks at doses ranging from 12.5 to 75 mg/kg body weight, resulting in a specific 3-fold to over 10-fold reduction of miR-122 activity in the liver with low toxicity. This miR-122 reduction resulted in reduced plasma cholesterol levels, increased hepatic fatty-acid oxidation, and a decrease in hepatic fatty-acid and cholesterol synthesis rates [155]. 2′-O-Me modified ASOs, targeting miR-21, were delivered locally into a balloon injured carotid artery without showing toxicity. MiR-21 expression was significantly and specifically decreased, as no inhibitory effect was found on other miRNAs, resulting in inhibited neoitima formation [156]. MiR-1-targeting ASOs, containing 2′-O-Me modifications at every base and a 3′C3-containing amino linker, were pre-treated with lipofectamine 2000 and injected into the infarcted myocardium at quantities of 80 μg total ASO, resulting in significantly suppressed arrhythmias [157].

A recently described strategy for the *in vivo* inhibition of miRNAs is the use of LNA-antimiRs [158]. Here, the use of an unconjugated 16-nt mixed LNA/DNA fully phosphorothiolated oligonucleotides with two methylated cytosines complementary to the 5′region of miR-122 was explored. Single intravenous injections of LNA-antimiR for three consecutive days, at doses ranging from 2.5 to 25 mg/kg per day, led to specific and dose-dependent miRNA-122 antagonism in mice without observed hepatotoxicity. Moreover, single intravenous doses of LNA-antimiR for three consecutive days at 25 mg/kg per day resulted in over 85% reduction of miR-122 at 24 h, followed by a gradual increase in mature miR-122 with complete normalization at 3 weeks, implying that the inhibition of miR-122 by LNA-antimiR is reversible. More recently, the same group successfully demonstrated the use of their miR-122 targeting LNA-antimiRs in non-human primates [159]. LNA-antimiRs show to be a promising tool for studying the biological role of miRNAs and for identifying their targets. Additionally, the LNA modification, which has been discussed before, highly increases nuclease resistance, decreases off-target effects and does not show toxicity.

This makes it a promising modification for the *in vivo* delivery of siRNAs and ASOs, possibly leading to novel therapeutic strategies for disease-associated genes and miRNAs.

CONCLUSION

Clearly, the *in vivo* modulation of gene expression by using small RNAs has great potential, but successful *in vivo* modulation falls or stands with the efficiency of small RNA delivery into the target tissue, together with the efficiency and selectivity of long-term target silencing. Several of the siRNA and antimiR oligonucleotide modifications and formulations described in this review are efficiently delivered into their target tissue and effectively knockdown their targets. Nevertheless, non target-specific knockdown, sequence specificity problems, immune responses, and other off-target effects like siRNA competition with the miRNA pathway are less well understood and are still hurdles to tackle. For the *in vivo* and especially clinical applicability of small RNA formulations, minimally invasive delivery methods would be preferable. However, local more invasive administration will allow the use of low doses, thereby also minimizing systemic off-target effects and reducing costs. Nonetheless, the majority of diseases require treatment through intravenous or intraperitoneal injection, making systemic administration the more widely applicable strategy for the clinic, thereby requiring small RNA modifications and formulations that increase protection against serum nucleases and kidney elimination, target specific tissues and overcome biological barriers without inducing toxic and non-specific effects.

Table 1 Delivery systems for in vivo delivery of small RNAs and their effects

Formulation	Effect	Example references
Liposomes	Complex formation or incorporation protects against nucleolytic degradation and renal clearance Enhances cellular uptake through electrostatic interaction with the negatively charged cell membrane Facilitates cytoplasmic delivery by destabilizing the endosomal membrane	Sioud, M. and Sorensen, D. R. (2003) Sorensen, D. R., et al. (2003) Hassan, A., et al. (2005) Yano, J., et al. (2004) Pal, A., et al. (2005) Landen, C. N., Jr., et al. (2005)
PEGylation	Steric stabilization Increases the hydrodynamic size which protects against renal clearance Improves solubility which enhances biodistribution	Santel, A., et al. (2006)
SNALP	Full encapsulation protects against nucleolytic degradation and renal clearance	Morrissey, D.V., et al. (2005) Zimmermann, T. S., et al. (2006)
(Ga)lactosylated liposomes	Facilitates tissue/cell specific delivery in addition to liposomal effects	Watanabe, T., et al. (2007)
		Sato, A., et al. (2007)
Atelocollagen, chitosan	Complex formation protects against nucleolytic degradation and renal clearance Enhances cellular uptake through electrostatic interaction with the cell membrane Prolonged release of small RNAs from complex	Ochiya, T., et al. (2001) Minakuchi, Y., et al. (2004) Takeshita, F., et al. (2005) Matoba, T., et al. (2007) Banno, H., et al. (2006) Pille, J.Y., et al. (2006) Howard, K. A., et al. (2006) de Martimprey, H., et al. (2008)
PEI	Complex formation protects against nucleolytic degradation and renal clearance Enhances cellular uptake through electrostatic interaction with the cell membrane Enhances cytosolic release	Urban-Klein, B., et al. (2005) Grzelinski, M., et al. (2006)
RGD-targeted PEG-PEI	Facilitates tissue/cell specific delivery in addition to PEI effects	Schiffelers, R. M., et al. (2004)
Transferrin receptor-targeted cyclodextrin nanoparticles	Incorporation protects against nucleolytic degradation and renal clearance Enhances cellular uptake Tissue/cell specific delivery	Hu-Lieskovan, S., et al. (2005) Heidel, J. D., et al. (2007)
Cholesterol	Binding to albumin and lipoprotein particles protects against renal clearance Indirectly enhances tissue/cell specific uptake by binding lipoprotein receptors	Soutschek, J., et al. (2004)
Protamine-antibody	Complex formation protects against nucleolytic degradation and renal clearance Tissue/cell specific delivery	Song, E., et al. (2005)
Aptamer	Tissue/cell specific delivery	McNamara, J. O. 2nd, et al. (2006)
PLGA microspheres	Complex formation protects against nucleolytic degradation and renal clearance Sustained release of small RNAs from complex	Khan, A., et al. (2004)

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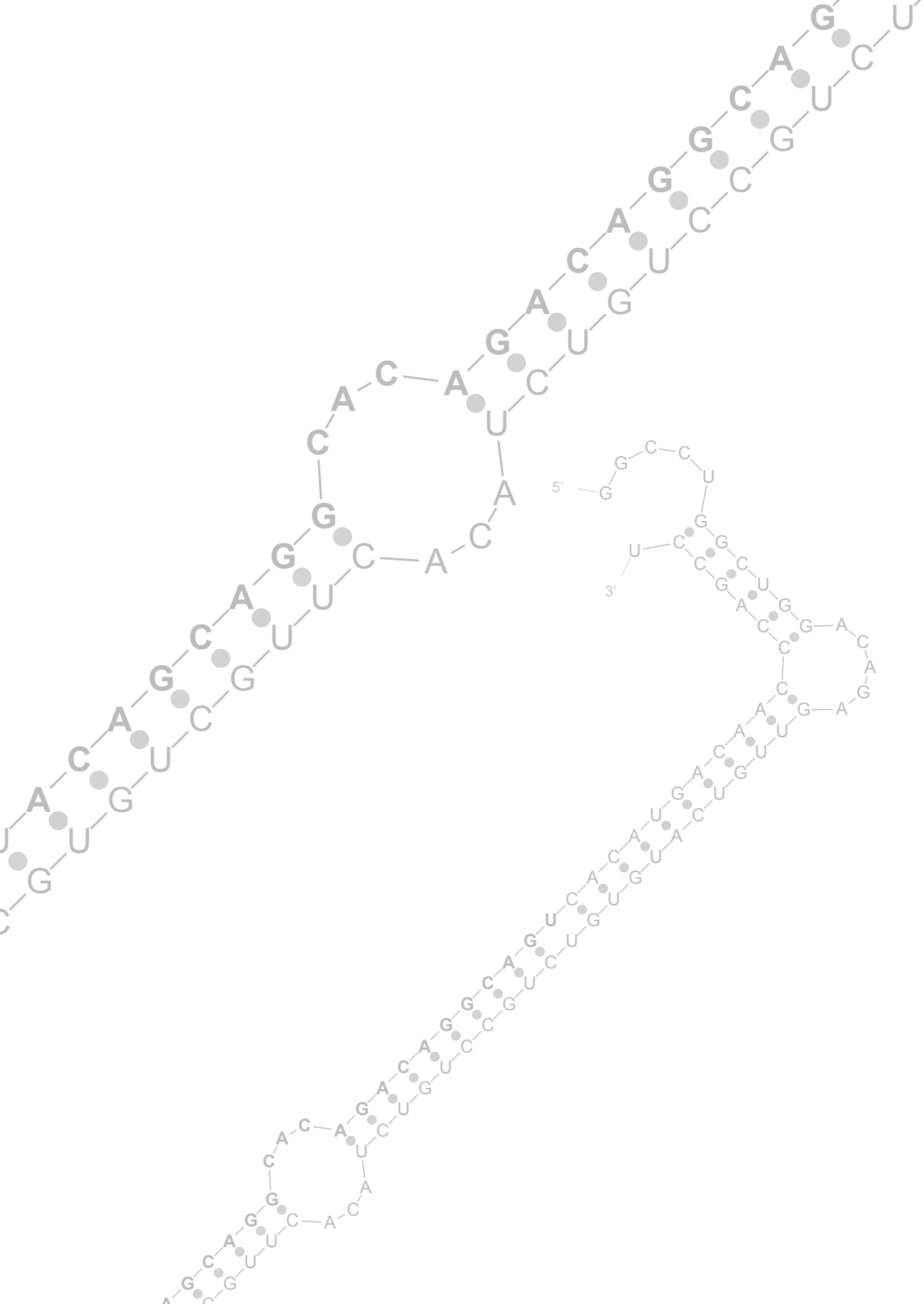
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CHAPTER 7

MICRORNA-214 INHIBITS ANGIOGENESIS BY TARGETING QUAKING AND REDUCING ANGIOGENIC GROWTH FACTOR RELEASE

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ABSTRACT

Aims

Angiogenesis is a critical component of many pathological conditions in adult tissues and essential for embryonic development. MicroRNAs are indispensable for normal vascular development, but their exact role in regulating angiogenesis remains unresolved. Previously, we have observed that miR-214 is differentially expressed in compensatory arteriogenesis. Here, we investigated the potential role of miR-214 in the process of angiogenesis.

Methods and results

miR-214 is expressed in all major vascular cell types, and modulation of miR-214 levels in endothelial cells significantly affected tubular sprouting. *In vivo* silencing of miR-214 enhanced the formation of a perfused vascular network in implanted matrigel plugs and retinal developmental angiogenesis in mice. miR-214 directly targets Quaking, a protein critical for vascular development. Quaking knockdown reduced pro-angiogenic growth factor expression and inhibited endothelial cell sprouting similar to miR-214 overexpression. In accordance, silencing of miR-214 increased the secretion of pro-angiogenic growth factors, including vascular endothelial growth factor, and enhanced the pro-angiogenic action of the endothelial cell-derived conditioned medium, whereas miR-214 overexpression had the opposite effect.

Conclusions

Here, we report a novel role for miR-214 in regulating angiogenesis and identify Quaking as a direct target of miR-214. The anti-angiogenic effect of miR-214 is mediated through the downregulation of Quaking and pro-angiogenic growth factor expression. This study presents miR-214 as a potential important target for pro- or anti-angiogenic therapies.

INTRODUCTION

In angiogenesis, new blood vessels develop from existing endothelium in response to angiogenic stimuli. These blood vessels are crucial for organ growth during embryonic development and tissue regeneration in the adult. In many diseases the body loses control over angiogenesis, leading to an imbalance in the growth of blood vessels. This imbalance results in either excessive angiogenesis, occurring in diseases such as cancer, diabetic blindness, and age-related macular degeneration, or in

insufficient angiogenesis, occurring in diseases such as coronary artery disease and stroke.[1] Angiogenic growth factors of the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) families stimulate essential signaling pathways in vascular development and control both physiological and pathological angiogenesis in the adult. Different components of these pathways have been put forward as targets for anti-angiogenic therapies for cancer, and pro-angiogenic therapies for vascular occlusive diseases.[2, 3] Unfortunately, these cytokine-based approaches did not lead to the expected results.

Although the proteins and pathways controlling angiogenesis and vascular development are well studied, the modulation of angiogenic signaling cascades by microRNAs (miRNAs) is not well understood. MiRNAs are short, non-coding RNAs that regulate gene expression through translational repression or degradation of target messenger RNAs (mRNAs), resulting in the fine-tuning of gene expression.4 MiRNAs are increasingly seen as master regulators of many processes, including angiogenesis and vascular development, because of their ability to target numerous mRNAs, in particular those with similar functions or within related pathways.

A powerful indication for the indispensable role of miRNAs in angiogenesis was shown by EC- or vascular smooth muscle cell (VSMC)-specific deletion of Dicer, an enzyme essential for miRNA biogenesis, resulting in defective blood vessel development and EC function.[5-7] To date, the exact regulation of angiogenesis by miRNAs has not been resolved, though a handful of miRNAs were shown to stimulate (let-7f, miR-27b, 130a, 210) or inhibit (miR-17, 18a, 19a, 20a, 92a, 100, 221, 222, 320) angiogenesis, regulate vascular development (miR-126), or tumor angiogenesis (miR-15, 16, 17~92 cluster, 296, 378).[8, 9] Especially the role of specific miRNAs in non-neoplastic angiogenesis in the adult organism remains largely unexplored.

Previously, we have observed that miR-214 is differentially expressed in compensatory arteriogenesis.[10] In the present study, we demonstrate, through modulation of miR-214 levels, that miR-214 is a potent regulator of angiogenesis both *in vitro* and *in vivo*. We found that miR-214 directly targets Quaking (QKI), a protein critical for vascular development and remodeling, and regulates the secretion of several angiogenic growth factors like VEGF, bFGF, and PDGF.

MATERIALS AND METHODS

Animals

All experiments were carried out in accordance with *the Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health, as well as the

Directive 2010/63/EU of the European Parliament, after securing approval of the committee of animal care of the University Hospital Freiburg and the animal welfare committee of the Leiden University Medical Center. Name and numbers: "miR-214 dependent regulation of neovascularization", 35/9185.81 G-10/02, and DEC10212.

Ingenuity systems Pathway Analysis (IPA)

From six different online miRNA target prediction databases; Microrna.org v08-2010, TargetScan v5.1, PicTar v03-2007, MicroCosm v5, Diana-microT v3, and ElMMo 05-2009 only the top scoring (max. 500) miR-214 targets were evaluated with the Ingenuity Systems pathway analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). IPA uses a database of molecular interactions described in literature to elucidate functional relationships among transcripts. The top five most significant miR-214 predicted target associated network functions and molecular and cellular biological functions were identified. A network with a score of i.e. 33 indicates a 10^{-33} chance of this network occurring randomly. P-values are calculated using the right-tailed Fisher's exact test, which is a measure of the likelihood that the predicted miR-214 target genes from the analyzed dataset participate in the corresponding function.

Cell culture

Mouse embryonic endothelial cells (MEECs) were cultivated on 0.1% gelatin in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs) (C2517A, Lonza) were grown on 0.1% gelatin in endothelial cell growth medium-2 (EGM-2) (Lonza) supplemented with 10% FBS. Human microvascular endothelial cells (HMECs) (Centers for Disease Control and Prevention)[11] were grown on fibronectin in MCDB 131 supplemented with EGF, hydrocortisone, L-glutamine, and 10% FBS. Human embryonic kidney 293 cells (HEK 293) were cultured in DMEM (Invitrogen) supplemented with 10% FBS. Endothelial colony-forming cells (ECFCs), and circulating angiogenic cells (CACs) were cultured in cell-specific medium.

Quantitative real time-PCR for miRNA expression

Total RNA was isolated with TriPure Isolation Reagent (Roche Applied Science) and treated with RNase-free DNase I (Qiagen). MiR-214 and miR-199a-3p expression was validated by TaqMan MicroRNA Assay (Applied Biosystems) as previously described.[12] In brief, 10 ng total DNA-free RNA was used for miRNA-specific (assay ID 002306 or 002304, Applied Biosystems) reverse transcription (Taqman® MicroRNA Reverse Transcription Kit, Applied Biosystems). Amplification and detection of specific

mature miRNAs was performed by specific TaqMan hybridization probes and TaqMan® Universal Master Mix II, no UNG (Applied Biosystems) in a MyIQ single-color quantitative real time polymerase chain reaction (qRT-PCR) system (Bio-Rad, Hercules, CA) at 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The cycle threshold (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to the expression of the small nuclear RNA RNU19 (assay ID 001003, Applied Biosystems) (ΔCt value). The relative difference in expression levels was calculated (ΔΔCt) and presented as fold induction ($2^{-\Delta\Delta Ct}$).

Small RNA transfection

MEECs, HUVECs, HMECs or CACs were reverse-transfected with 30 or 100 nmol/L Pre-miR™ miRNA Precursor Molecules for miR-214 (PM12921, Ambion), custom designed[13] antimiR-214 (Thermo Scientific), or Negative Control #1 (AM17110, Ambion) using siPORT™ NeoFX™ Transfection Agent (Ambion) according to the manufacturer's instructions. Transfection efficiency was confirmed by transfection of FAM™ dye-labeled Pre-miR™ Negative Control #1 (Ambion, AM17121) and miR-214 up or down-regulation was assessed by TaqMan MicroRNA Assay as described. For fluorescence microscope analysis of FAM-labeled negative control miR transfected cells, cells were fixated with 4% paraformaldehyde in PBS for 15 min at RT. Nuclei were stained with 0.2 µg/ml Hoechst 33342 dye (Invitrogen) and mounted in Fluoromount-G (SouthernBiotech).

Spheroid angiogenesis assay

MEECs were transfected with 100 nmol/L pre-miR-214, anti-miR-214 or negative control miR (ctrl-miR) as described. After 24 h, spheroids consisting of 750 cells each were generated in 0.1% methylcellulose in DMEM and embedded in 1 mg/mL collagen (Advanced Biomatrix) containing cell-specific medium. *In vitro* sprouting was quantified at day three by measuring the cumulative length of the tubular outgrowth and the number of branch points using ImageJ software (NIH), analyzing a minimum of 30 spheroids per group.

Matrigel tube formation assay

HUVECs were transfected with pre-miR-214, anti-miR-214, or ctrl-miR as described. The next day, HUVECs were counted and 7000 cells were cultured for eight hours in a µ-slide for angiogenesis (Ibidi) coated with 10 µL ECMatrix™ (Millipore) in 50 µL EGM-2 containing 10% FBS. HMECs were transfected with pre-miR-214, anti-miR-214, or ctrl-miR as described. After five hours, transfection medium was replaced

by medium containing 2% FBS and without additional growth factors. Conditioned medium (CM) was collected after 72 h. Ten thousand untransfected HMECs were cultured on 10 µL ECMatrix™ in a µ-slide for angiogenesis in 50 µL conditioned medium. Tube formation was quantified with AngioQuant after 16 hours.

Scratch wound cell migration assay

HUVECs were transfected with pre-miR-214, anti-miR-214, or ctrl-miR as described. The following day, HUVECs were counted and plated in 24-well plates. At 90% confluency, a scratch was made with a 100 µl pipet tip and medium was replaced with EGM-2 after washing with PBS. Wound closure was measured after 11 hours. CACs were transfected with ctrl-miR or premiR-214 as described. CAC conditioned medium was collected after 48 hours and used in a scratch wound assay with untransfected HUVECs. Pictures were made after 7 hours and wound closure was measured using ImageJ software (NIH).

Cell proliferation and viability assay

MEECs or HMECs were transfected with pre-miR-214, anti-miR-214, or ctrl-miR as described and seeded in 96-well plates. In addition, HMECs were grown in ctrl-miR, pre-miR-214 or anti-miR-214 transfected HMEC conditioned medium. After 3 or 10 days, cell proliferation reagent WST-1 (Roche Applied Science) was added to the medium (1:10) and incubated at 37°C for six hours. Subsequently, wells were shaken thoroughly and absorbance was measured at 440 nm with a reference filter of 690 nm in a photometric plate reader (Labsystems Multiskan Ascent).

Antagomir treatment

Antagomirs were designed as previously described and custom synthesized (VBC Biotech).[14] Antagomir sequences used were: antagomir-214 (5`-acugccugucugugccugcugu-3`), and antagomir-mismatch-ctrl (5`-acucccugccuuuccuuaugu-3`). AntagomiR-214 consists of the inverse complementary mature miR-214 sequence. The mismatch control antagomir (ctrl-antagomiR) contains six mismatches (underlined). Antagomirs are 3`cholesterol-modificated, 2`O-methylated, and contain PTO-linkages at the first two and last four nucleotides. Twelve male C57BL/6J mice (12 weeks old, Charles River Lab) per treatment group underwent intravenous (tail vein) injections of 8 mg/kg body weight antagomir before matrigel injection and 1 and 2 days later. The efficiency of miRNA knockdown was confirmed by TaqMan MicroRNA Assay, as described above, in two mice per treatment group three days after initial injection and in six mice per group after two weeks.

Matrigel plug angiogenesis assay

The *in vivo* angiogenesis assay was performed as previously described.[10] We subcutaneously injected 500 µL growth factor reduced (to minimize the angiogenic response from the host), phenol red free, Matrigel™ Basement Membrane Matrix (BD Biosciences) into twelve male C57BL/6J mice (12 weeks old, Charles River Lab.) along the abdominal midline. Tail vein injections of antagomirs were performed as described. After 14 days, mice were sacrificed by cervical dislocation and matrigel plugs were carefully dissected and processed for paraffin sectioning.

Immunohistology

From the matrigel plugs, 5 µm paraffin tissue sections were obtained from three non-consecutive regions with 1 mm intervals. One section for each region was stained with hematoxylin and eosin (H&E) and cell infiltration and capillary formation were quantified in three random areas by ImageJ software (NIH) and averaged per antagomir group. Additionally, αSMA-positive cells were stained using anti-actin-α-SM-FITC (1:400, F3777, Sigma-Aldrich) and CD31 positive cells with anti-CD31 (1:1500, SC-1506R, sc: 200 µg/mL, Santa-Cruz Biotechnology), biotinylated goat anti rabbit IgG (1:200, BA-1000, sc: 1.5 mg/mL, Vector Laboratories) and streptavidin Alexa Fluor® 555 (1:1000, S21381, sc: 2 mg/mL, Invitrogen), counterstained with Hoechst 33342 dye (Invitrogen) and embedded in Fluoromount-G (SouthernBiotech). Images were captured using cellP software (Olympus) on an Olympus BX60 microscope and processed with Adobe Photoshop CS4 v11. The number of αSMA and CD31 positive cells were counted in three random areas and averaged per treatment group.

Mouse retinal developmental angiogenesis

Postnatal day two C57BL/6J mice were fixated and stabilized by hand for intracardial injection. Pups were closely monitored for signs of airway obstruction and distress. 8 µg/g body weight of antagomiR-214 or ctrl-antagomiR was injected intracardially in the left ventricle of three mice per group. In C57BL/6J mice, the superficial vascular plexus is formed during the first postnatal week by radial outgrowth of vessels from the optic nerve towards the retinal edges. After seven days, mice were sacrificed by decapitation, the eyes were formalin-fixed overnight, and retinas were carefully dissected for histological analysis. Retinas were permeabilized for 30 minutes in PBS/1% Triton X-100 and visualization of the vasculature was performed after preincubation in PBS/1% BSA by overnight incubation with biotinylated isolectin B4 (1:80, B-1205, Vector Laboratories) in PBS/1% Triton X-100 at 4°C. Retinas were washed in PBS, and incubated for two hours with streptavidine DyLight-488 (1:500, 21832, sc: 1 mg/mL, Thermo Scientific) in PBS/1% BSA prior to flat mounting with

Fluoromount-G (SouthernBiotech). Quantification of capillary density was performed on 15 to 19 random fields at the vascular migration front from three ctrl-antagomiR and three antagomiR-214 treated mice using ImageJ software (NIH).

Quantitative real time-PCR

Total RNA was isolated with TriPure Isolation Reagent (Roche Applied Science) and treated with RNase-free DNase I (Qiagen). 500 ng total DNA-free RNA was transcribed to copy DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad) and qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and specific primers (Invitrogen, see below) in a MyIQ single-color real-time polymerase chain reaction system (Bio-Rad). Used PCR procedure is: 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at specific annealing temperature, and 45 s at 72°C, followed by melting curve analysis to confirm single product amplification. The expression levels were normalized to beta-actin or GAPDH, calculated as (ΔCt), relative differences were calculated (ΔΔCt) and presented as fold induction ($2^{-\Delta\Delta Ct}$). All measurements were performed in duplo. Primers used (and annealing temperatures) are: QKI (50°C) forward: 5`-gagagcagttgaagaagtgaag-3`, reverse: 5`-gggctggtgatttaatgttgg-3`, VEGFA (51°C) forward: 5`-ccaccacaccatcaccatc-3`, reverse: 5`-gcgaatccaattccaagagg-3`, beta-actin (60°C) forward: 5`-gatcggcggctccatcctg-3`, reverse: 5`-gactcgtcatactcctgcttgc-3`, GAPDH (56°C) forward: 5`-acagtcagccgcatcttc-3`, reverse: 5`-gcccaatacgaccaaatcc-3`, p0 (50°C) forward: 5`-ggacccgagaagacctcctt-3`, reverse: 5`-gcacatcactcagaatttcaatgg-3`, bFGF (59°C) forward: 5`-aactacaactccaagcagaag-3`, reverse: 5`-cacacttagaagccagcag-3`, and PDGF (55°C) forward: 5`-aggatgccttggagacaagt-3`, reverse: 5`-caggaatggcttcctcaatac-3`.

Luciferase experiments

The conserved miR-214 binding sequences in the QKI 3'untranslated region (UTR), extended with SpeI and HindIII restriction sites were cloned into the pMIR-REPORT Luciferase vector (Ambion) according to the manufacturer's protocol. Seed mutations for target site 1, 2, or both were generated by QuikChange Site-Directed Mutagenesis (Agilent Technologies). The cloned QKI sequences were confirmed by sequencing. To determine suppression efficiency of miR-214, HEK 293 cells were grown in 24-well plates to 50-60% confluence and co-transfected with 200 ng pMIR-REPORT-QKI-3UTR Luciferase vector, or one of the mutated vectors and a pMIR-REPORT β-gal Control Plasmid to assess transfection efficiency. In addition, 50 nmol/L pre-miR-214, a combination of pre-miR-214 and anti-miR-214, or a ctrl-miR was introduced by using Lipofectamine 2000 (Invitrogen). After 48 h, cells were lysed and the activity of Luciferase and β-galactosidase was assessed with the Luciferase Assay System and

β-galactosidase Enzyme Assay System (Promega), respectively, according to manufacturer's instructions.

siRNA experiments

MEECs were seeded in 6-well plates two days before transfection with either 20 nmol/L QKI siRNA (sense: GGACUUACAGCUAAACAACtt, antisense: GUUGUUUAGCUGUAAGUCCtc, Invitrogen), scramble siRNA (Invitrogen), or 100 nmol/L custom designed anti-miR-214 (Thermo Scientific) together with 20 nmol/L QK siRNA using 5 µl Lipofectamine™ RNAiMAX (Invitrogen) per well. The MEEC spheroid angiogenesis assay was performed as described. Total RNA was isolated after 72 hours.

Western blot

Total protein was isolated with TriPure Isolation Reagent (Roche Applied Science) 72 hours after miRNA transfection. For western blot, 20 µg of total protein or equal amounts of conditioned medium was reduced with 0.5% beta-mercaptoethanol, separated by PAGE, and transferred to a nitrocellulose membrane (Sigma-Aldrich). Membranes were blocked with 5% non-fat dry milk, PBS-0.1% Tween, probed with either VEGF (1:500, rabbit polyclonal, sc-152, sc: 200 µg/mL, Santa Cruz), or TSP1 (1:250, mouse monoclonal, MS-421-P0, Thermo Scientific) antibody, and GAPDH antibody (1:1000, rabbit monoclonal, 14C10, Cell Signaling), followed by incubation with goat anti-rabbit or goat anti-mouse IgG secondary antibody, horseradish peroxidase-conjugated (1:2000, Cat. No. P0448, P0447, Dako). The signal was visualized with enhanced chemiluminescence system (Sigma-Aldrich) and detected by using the ChemiDoc XRS system (Bio-Rad).

Antibody array

Human Angiogenesis Antibody Array G1 (RayBiotech) was performed according to manufacturer's instructions on antimiR-214 and ctrl-miR transfected HMEC conditioned medium.

Statistical analysis

Data are presented as mean±SEM and were compared using a Student's t-test (Microsoft Excel) or Mann-Whitney *U* test (SPSS Inc.). Differences were considered statistically significant when P-values were <0.05, indicated by an *.

miR-214 predicted targets correlate with angiogenesis-associated functions

In our previous study, we have observed the differential expression of miR-214 in compensatory arteriogenesis.[10] To understand the potential role of this miRNA, we analyzed only the most significant predicted miR-214 targets of six target prediction databases with IPA software. The miR-214 target dataset entered into IPA yielded an array of functions commonly associated with angiogenesis, like cellular assembly and organization, cellular development, cell-to-cell signaling and interaction, and cell death (Supplemental tables 1 and 2). The two most significant network functions, and affected molecular and cellular processes associated with the predicted miR-214 targets are cellular assembly and organization, and cellular development, both critical in the process of angiogenesis.

miR-214 is highly expressed in vascular cell types

To investigate if the highly conserved miR-214 (Supplementary material online, Figure SI) is indeed present in the vascular system, we examined miR-214 expression in several vascular cell types (Figure 1A). MiR-214 is highly expressed in several EC types, VSMCs, and vascular fibroblasts (vFBs), but not in CACs. We also observed that miR-214 is most abundantly present in tissues with a vast capillary bed (Supplementary material online, Figure SII). Although little is known on the role of miR-214 in the vascular system or angiogenesis, neither under physiological nor pathological conditions, the expression pattern of miR-214 suggests a significant role for miR-214 in the vascular system.

miR-214 regulates *in vitro* angiogenesis

To investigate if miR-214 functionally affects *in vitro* models for angiogenesis, we overexpressed or inhibited miR-214 in a three-dimensional EC spheroid sprouting assay. MEECs were efficiently transfected (Supplementary material online, Figure SIIIA) and transfection with either miR-214 precursor molecules (premiR-214) or a miR-214 inhibitor (antimiR-214) resulted in significant miR-214 up-regulation and repression, respectively (Figure 1B). Transfection of fluorescent control-miR was performed to confirm that small RNA molecules were still present after spheroid formation and embedding into collagen matrix (Supplementary material online, Figure SIIIB). We found that introduction of miR-214 in ECs blocked sprout formation, as shown by an 80% decrease in total sprout length, and a reduction in branching, whereas antimiR-214-mediated miR-214 inhibition resulted in a 40% increase in outgrowth of tubular sprouts and an increase in branching (Figure 1C). In addition, we modulated miR-214 levels in a HUVEC matrigel assay. In this angiogenesis assay,

miR-214 overexpression resulted in an inhibition of tubule formation as shown by a significant reduction in tubule length and size, whereas miR-214 inhibition led to an increase in tubule formation (Figure 1D). Since EC migration is an essential process of angiogenesis, we assessed the effect of modulating miR-214 levels in a scratch wound HUVEC migration assay, showing inhibition of migration by miR-214 overexpression and an induction of wound closure by antimiR-214 transfection (Supplementary material online, Figure SIV). Clearly, modulation of miR-214 expression in ECs can block or enhance vessel growth *in vitro*, suggesting a direct regulatory role for miR-214 in vessel formation. In addition, we showed that EC proliferation and viability was not affected by modulating miR-214 levels (Supplementary material online, Figure SV).

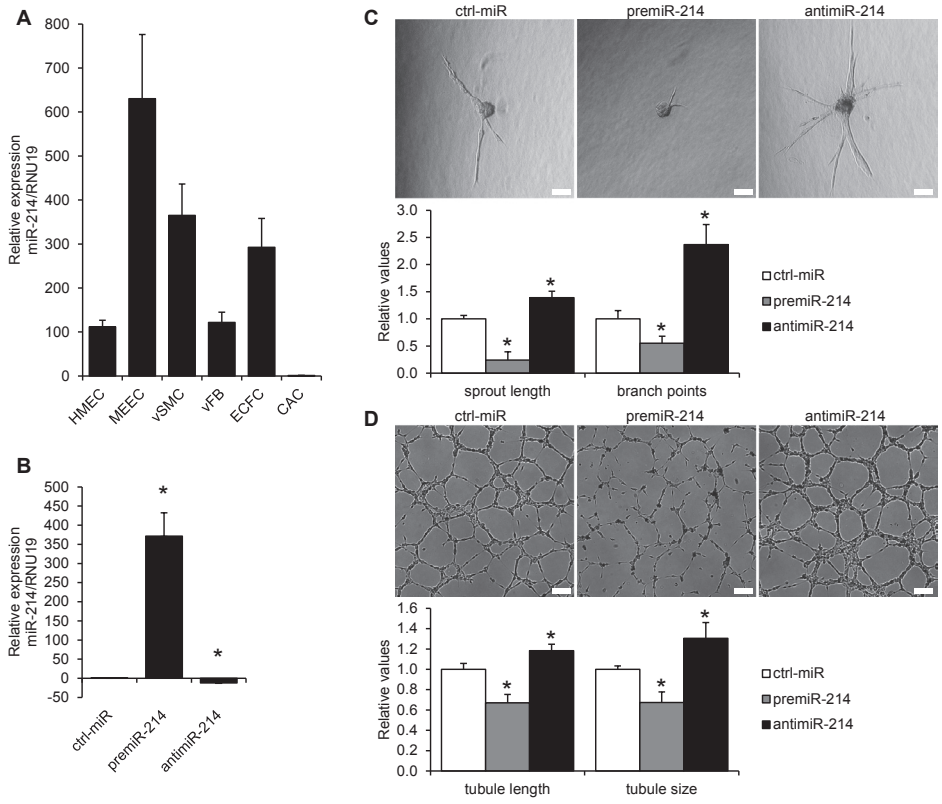

Figure 1

miR-214 is highly expressed in vascular cell types and inhibits angiogenesis *in vitro*. (A) Taqman qRT-PCR analysis of miR-214 expression in vascular cell types. Data is represented as fold induction relative to CACs. n=3/cell type. (B) Taqman qRT-PCR analysis of miR-214 expression in MEECs two days after transfection of pre- or antimiR-214, relative to ctrl-miR transfected cells. n=3. (C) Spheroid angiogenesis assay of ctrl, pre, or antimiR-214 transfected MEECs with quantification of sprout length and branch points relative to ctrl-miR. (D) Matrigel angiogenesis assay of 7000 ctrl, pre, or antimiR-214 transfected HUVECs with quantification of tubule length and size relative to ctrl-miR. n=4 for C and D.

In vivo miR-214 silencing enhances matrigel plug angiogenesis

Considering the promising effects of miR-214 modulation on angiogenesis *in vitro*, we assessed the functional involvement of miR-214 in modulating angiogenesis *in vivo*. To block miR-214 *in vivo*, we designed so-called antagomirs, which are chemically modified and cholesterol-conjugated, single-stranded RNA oligonucleotides complementary to the specific miRNAs (Supplementary material online, Figure SVIA).[14] We also designed a mismatch control antagomir (ctrl-antagomiR), containing six nucleotide substitutions compared to antagomiR-214 (Supplementary material online, Figure SVIA), which did not match any known miRNA or mRNA. We found that mice treated with antagomiR-214 displayed a significant reduction of up to 98% in miR-214 expression two weeks after initial injections (Supplementary material online, Figure SVIB). Other miRNAs were unaffected by antagomiR-214 or mismatch-antagomir treatment, as shown by the unchanged expression levels of miR-199a-3p, a miRNA with a similar seed sequence and expression pattern as miR-214 (Supplementary material online, Figure SVIC).

We used a matrigel plug EC invasion assay to assess the effect of miR-214 silencing on *in vivo* angiogenesis. Consistent with our *in vitro* data, miR-214 silencing *in vivo* enhanced the number of invading cells. While normally only little cell infiltration occurs in growth factor reduced matrigel, as shown for ctrl-antagomiR treated animals, we observed a strong increase in cell infiltration in miR-214 silenced animals (Figure 2A-2B). Additionally, erythrocyte-containing capillary structures were observed in miR-214 knockdown mice only (Figure 2A, higher magnification). To better characterize the cell infiltration and apparent capillary ingrowth, immunofluorescent staining for endothelial and smooth muscle cell markers were performed. As compared to control treated animals, a 4.3–fold increase in the number of CD31+ cells (Figure 2C-2D), and a 3.5–fold increase in the number of αSMA+ cells (Supplementary material online, Figure SVIIA-SVIIB) was observed. Moreover, a 3.5-fold increase in vessel formation was shown by CD31/αSMA+ cells that assembled into a primitive, but perfused, vascular network, consisting of ECs surrounded by SMCs (Figure 2E and 2C higher magnification). This increase in plug vascularization by antagomiR-214 demonstrates that miR-214 controls angiogenesis *in vivo*.

miR-214 silencing enhances mouse retinal developmental angiogenesis

Our previous results showed that miR-214 inhibits angiogenesis *in vivo*. To further explore the functional effect of miR-214 on angiogenesis *in vivo*, we investigated if miR-214 silencing could also promote mouse retinal vascular development, a well-characterized and robust model for *in vivo* angiogenesis. We used antagomirs to silence miR-214 during postnatal retinal vascular development. Retinas were isolated after

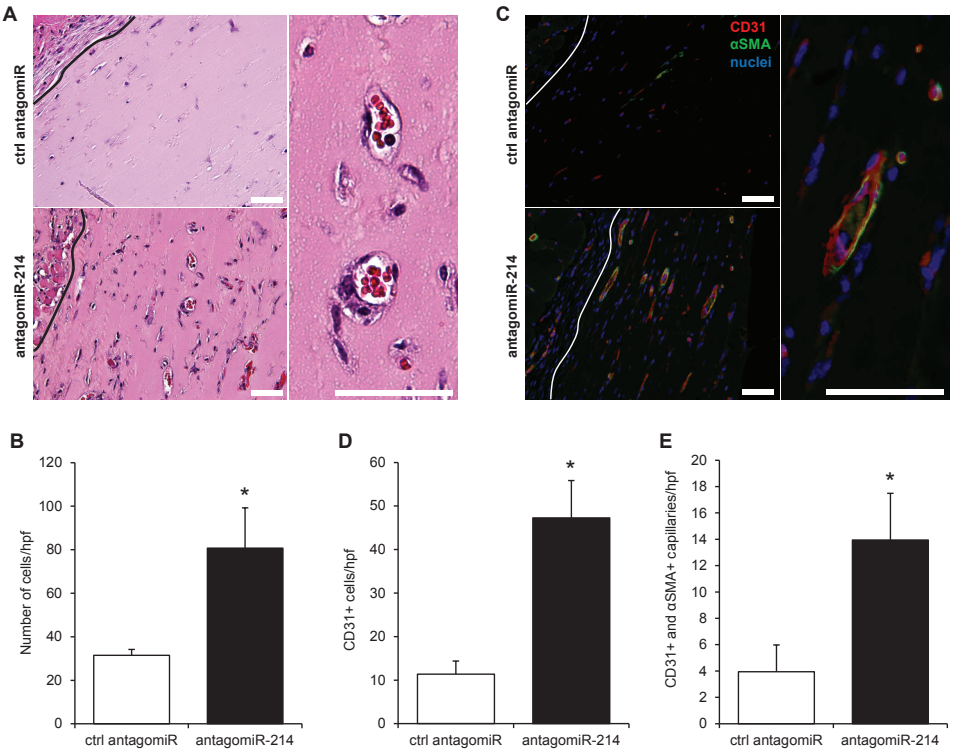

Figure 2
In vivo miR-214 silencing enhances matrigel plug angiogenesis. Effect of (A) miR-214 silencing on cell infiltration as shown by H&E staining and (C) capillary formation as shown by CD31 and aSMA staining. Quantification of (B) cell infiltration, (D) CD31+ cells, and (E) CD31+/aSMA+ capillaries in the matrigel plug. Dotted lines delimit tissue and Matrigel. n = at least 6/group.

the first postnatal week to assess the effect on the formation of the vascular network by staining with isolectin B4. We found that antagomiR-mediated knockdown of miR-214 levels in the postnatal mice resulted in enhanced capillary density as shown by an increase in retinal capillary length (Figure 3A-3B). From postnatal day seven onward, the retinal vessels start sprouting vertically into the deeper layers of the vascular plexus. Quantification of these vessels showed that this ingrowth was significantly enhanced in the miR-214 silenced mice (Figure 3A and 3C), suggesting an overall enhancement of retinal vascular development.

miR-214 targets QKI

To get insight in the mechanisms by which miR-214 regulates angiogenesis, we searched for putative angiogenesis-related mRNA targets of miR-214. Predicted by 4 out of 6 algorithms, having several miR-214 target sites, and given the essential role

in vascular development and angiogenesis, its conservation, and its expression in ECs and VSMCs, we identified QKI as a potential direct target of miR-214.[15-17] We confirmed that QKI is expressed in ECs and VSMCs (Figure 4A), and we found that QKI is directly targeted by miR-214, as we observed that the QKI 3'UTR luciferase reporter, containing two conserved target sites (Figure 4B), could be significantly repressed after co-transfection with miR-214 (Figure 4C). Importantly, mutating both target sequences complementary to the miR-214 seed region relieved the repressive effect of miR-214 on luciferase activity (Figure 4C). Although different QKI transcripts exist, they only vary in their 3'UTR lengths and aa-tails, but all hold the conserved miR-214 target

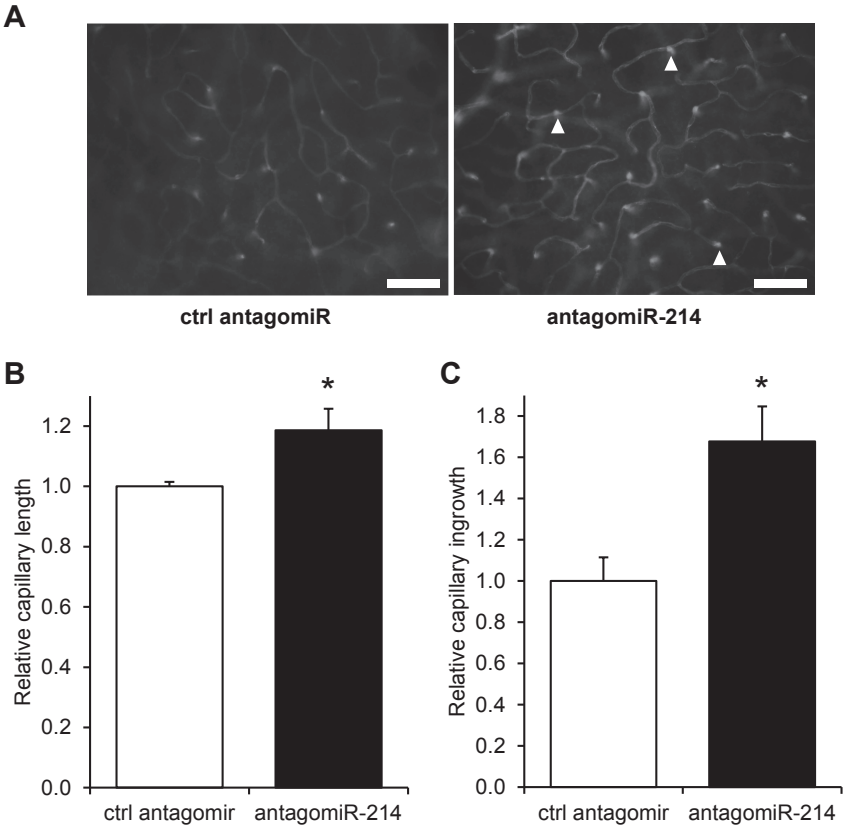

Figure 3
In vivo miR-214 silencing enhances mouse retinal developmental angiogenesis. (A) Effect of miR-214 silencing on retinal vasculature as visualized by isolectin B4 staining. Arrows mark capillary ingrowth. (B) Quantification of retinal capillary density, illustrated as relative capillary length compared to ctrl-antagomiR treated mice. (C) Quantification of retinal capillary ingrowth, relative to ctrl-antagomiR. n=3/group.

sites. This indicates that miR-214 can repress the translation of all major QKI isoforms. Since mRNA targeting by miRNAs can result in translational blockage but also in mRNA degradation, we quantified QKI mRNA expression in HMECs that had been transfected with premiR-214 or antimiR-214. We found that QKI mRNA is down- or up-regulated by 2-fold upon miR-214 overexpression or inhibition, respectively (Figure 4D), demonstrating a regulation by miR-214 not only by translational repression, but also by affecting mRNA stability. More importantly, QKI transcript levels were up-regulated in different tissues of antagomiR-214 treated mice, indicating that QKI is targeted by miR-214 *in vivo* as well (Figure 4E).

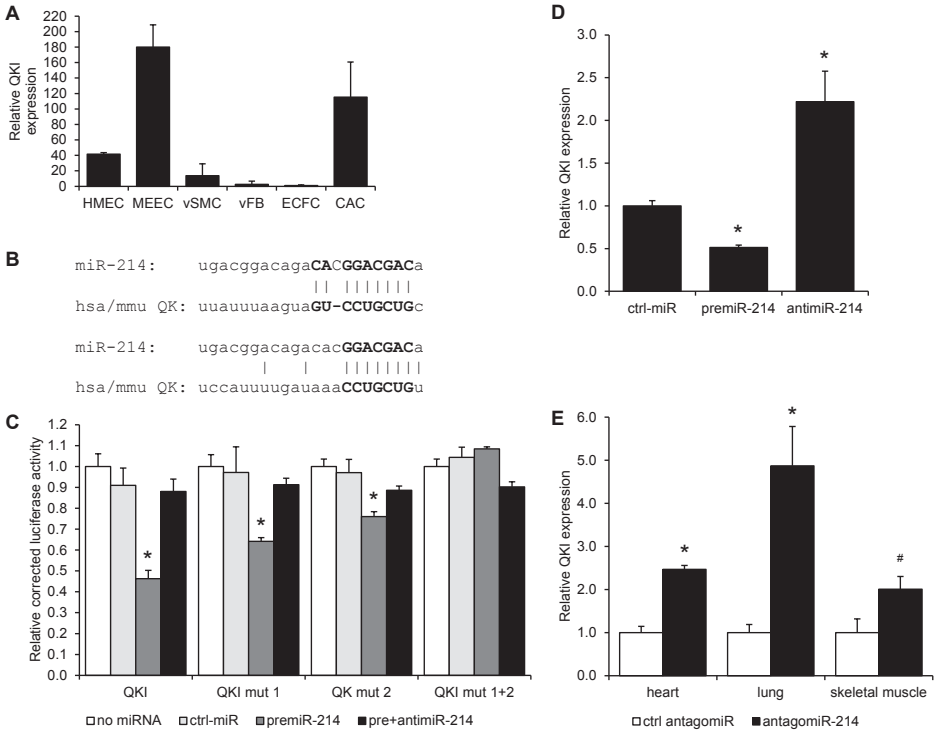

Figure 4
QKI is a target of miR-214. (A) mRNA expression of QKI in HMEC, MEEC, VSMC, ECFC, and CAC, normalized to beta-actin and represented as fold induction relative to vFB. (B) Two predicted and conserved miR-214 target sites for human and mouse QKI. (C) Luciferase reporter gene analysis shows the effect of co-transfecting ctrl-miR, premiR-214, a combination of premiR-214 and antimiR-214, with QKI-pmiR-reporter or QKI-pmiR-reporter with mutated target site 1, 2, or both. Significance is measured compared to ctrl-miR. (D) qRT-PCR analysis of QKI expression after premiR-214 or antimiR-214 transfection in HMECs, normalized to beta-actin and relative to ctrl-miR transfected cells. (E) QKI expression in heart, lung and skeletal muscle two weeks after initial antagomiR-214 injection, normalized to beta-actin and relative to ctrl-antagomiR. n=3 for A,C,D, and E, # indicates a P-value of 0.06.

QKI knockdown inhibits angiogenesis and reduces angiogenic growth factor expression

To investigate if miR-214 exerts its anti-angiogenic actions via targeting of QKI, we knocked down QKI by siRNA in ECs and performed a spheroid angiogenesis assay. SiRNA-mediated knockdown of QKI led to a similar effect on sprouting as earlier showed for miR-214 overexpression (Figure 5A-5B). A near 5-fold decrease in sprout length was observed as compared to scramble siRNA transfected cells (Figure 5B). Co-transfection of ECs with both QKI siRNA and antimiR-214 could not rescue the negative effect of QKI down-regulation on EC sprouting, as indicated by a significant decrease in tubular sprouting (Figure 5A-5B).

It has previously been shown that QKI deficiency and/or loss of function results in decreased VEGFA and bFGF expression, respectively.[17] Since VEGF and bFGF are some of the foremost regulators of angiogenesis, we investigated if the observed effect of

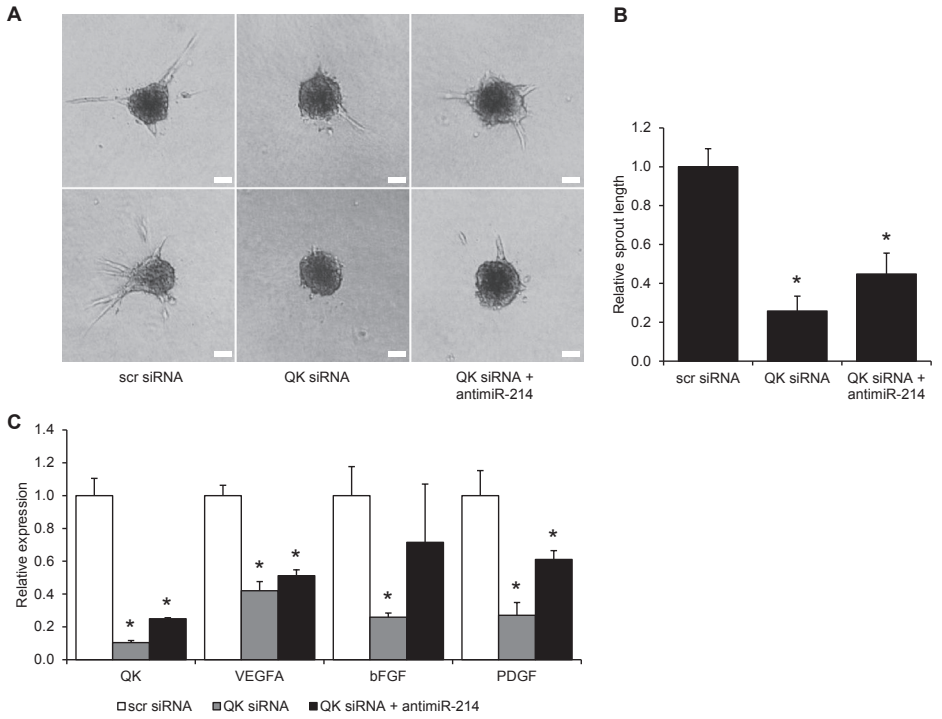

Figure 5

QKI knockdown inhibits EC sprouting angiogenesis and reduces angiogenic growth factor expression. (A) Spheroid angiogenesis assay of scramble siRNA, QKI siRNA or QKI siRNA and antimiR-214 transfected MEECs. (B) Quantification of sprout length relative to scramble siRNA. n=4. (C) qRT-PCR analysis of QKI, VEGFA, bFGF, and PDGF expression after QKI knockdown or QKI knockdown and miR-214 inhibition, normalized to p0 and relative to scr siRNA treated cells. n=3

QKI knockdown on cellular sprouting angiogenesis could be the result of decreased growth factor expression. Indeed, the efficient knockdown of QKI resulted in down-regulation of VEGFA and bFGF, but also PDGF (Figure 5C).

miR-214 negatively regulates angiogenesis by reducing pro-angiogenic growth factor expression and release

Since knockdown of the miR-214 target QKI in ECs resulted in down-regulation of VEGF expression, miR-214 up-regulation should also lead to decreased VEGF levels. Efficient overexpression or inhibition of miR-214 in ECs (Supplementary material online, Figure SVIII) led to a respective 4-fold reduction and 1.6-fold induction in VEGFA mRNA expression (Figure 6A). These results could be confirmed at the protein level (Figure 6B). Additionally, we found one predicted miR-214 target site for VEGF, but reporter gene analysis excluded the possibility that the decrease in VEGFA levels is attributable to direct targeting by miR-214 (data not shown).

Since VEGFA exerts its pro-angiogenic functions via secretion and subsequent binding to VEGF receptors, we investigated if miR-214 modulation changes the effect of CM on angiogenesis. For this, we transfected ECs with premiR-214, antimiR-214 or ctrl-miR and collected the CM after 4 days of culture, which was then used in a matrigel angiogenesis assay. The CM of antimiR-214 transfected ECs enhanced HMEC tubular formation on matrigel by increasing tubular length, the number of branch points, but most prominently by an increase in tubular size, whereas CM from premiR-214 transfected HMECs reduced tubular size (Figure 6C). This effect on tubular formation is cell death independent since HMEC proliferation and viability was not immediately affected by miR-214 overexpression or inhibition, neither directly nor via CM (Supplementary material online, Figure SIXA and SIXB). Only after ten days, miR-214 up-regulation or inhibition resulted in a minor decrease or increase, respectively, in cell number or viability (Supplementary material online, Figure SX). To investigate if the effect on the CM by miR-214 was EC-specific, we overexpressed miR-214 in CACs, a non-endothelial cell, and collected the CM, which was subsequently used in a HUVEC scratch wound assay, resulting in a significant decrease in EC migration (Supplementary material online, Figure SXI). To further investigate the mechanism by which miR-214 regulates angiogenesis, we examined the secretion of several angiogenic factors in ctrl-miR versus antimiR-214 transfected HMEC CM by protein array analysis. We found that all angiogenic growth factors, including VEGF, bFGF, PDGF, EGF, and IGF1 were up-regulated in CM of antimiR-214 transfected cells (Figure 6D). Moreover, the secreted, potent anti-angiogenic protein TSP1 was increased by miR-214 overexpression, independent of QKI (Supplementary material online, Figure SXII). The effect of miR-214 on angiogenic factor release is reflected by the

negative effect of premiR-214 CM and the positive effect of antimiR-214 CM on tubular formation as shown in Figure 6C. Taken together, our study shows that miR-214 inhibits angiogenesis via direct targeting of QKI and reducing pro-angiogenic growth factor release.

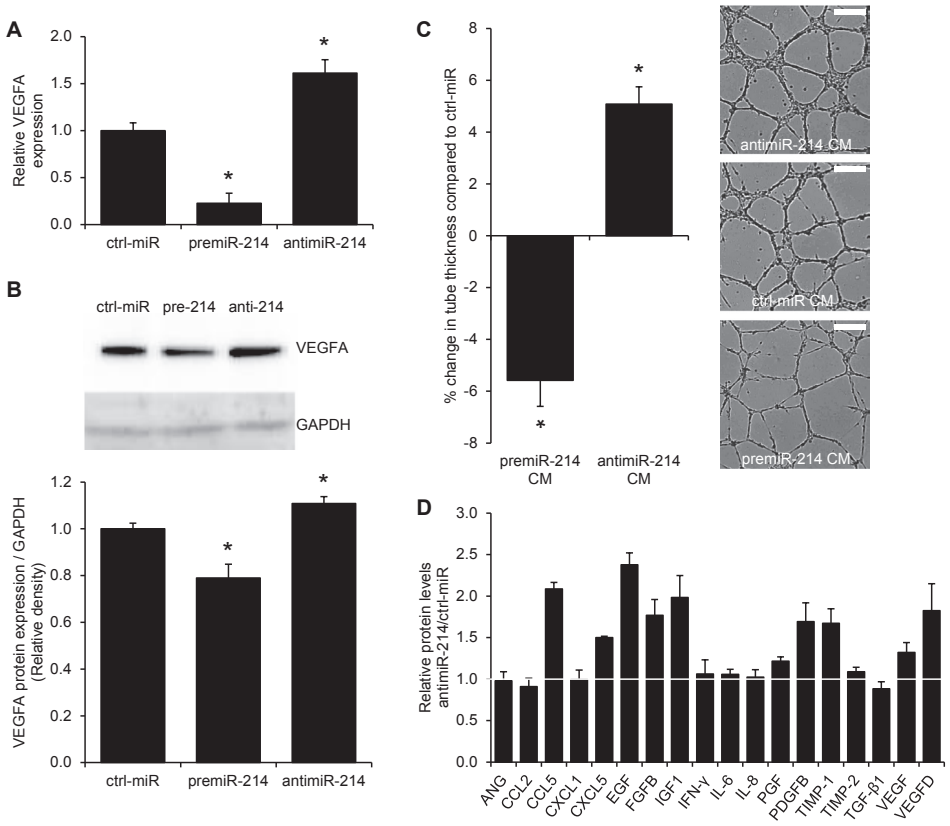

Figure 6
miR-214 negatively regulates angiogenic signaling by reducing pro-angiogenic growth factor expression and release. (A) The effect of premiR-214 or antimiR-214 transfection of HMECs on VEGFA mRNA and, (B) protein expression, normalized to GAPDH and relative to ctrl-miR transfected cells. n=4. (C) HMEC matrigel angiogenesis assay with CM of premiR-214, antimiR-214, or ctrl-miR transfected HMECs. Percentage change in tubular size is shown compared to CM of ctrl-miR transfected HMECs. n=3. (D) Angiogenic factor protein levels in miR-214-silenced HMEC CM, relative to ctrl-miR. n=2.

DISCUSSION

Here, we have identified miR-214 as a novel regulator of angiogenesis. We have shown, both *in vitro* and *in vivo*, that modulating miR-214 levels significantly affects angiogenesis. We found that miR-214 directly targets QKI and thereby negatively regulates vascular growth factor expression and secretion.

MiR-214 is located in dynamin 3, opposite strand, a gene that is transcribed into a non-coding RNA, and of which the expression is driven by transcription factor twist-1.[18, 19] In line with our previous findings, multiple studies have shown that miR-214 is up-regulated after ischemia, although in different organs, indicating a tissue-wide role for miR-214 after ischemia.[20-22] It is interesting to speculate that the increase in miR-214 after ischemia might be due to increased levels of HIF1α, which has been shown to directly regulate the expression of twist-1, which in turn is known to regulate miR-214 expression.[19, 23] In vascular disease studies, miR-214 was found to be up-regulated after rat carotid artery angioplasty, suggesting a role for miR-214 in the process of neointimal lesion formation.[24] Additionally, miR-214 was found to be down-regulated by the angiogenesis-inducer ginsenoside-Rg1 and modulation of miR-214 levels affected the number of branch points in an *in vitro* matrigel assay.[25] However, the *in vivo* function of miR-214 in angiogenesis has not been explored. In this study, we report for the first time the essential role of miR-214 in the control of angiogenesis *in vivo*.

The high expression of miR-214 in the main vascular cell types, which is confirmed by recent studies showing that miR-214 is present in angiogenic progenitor cells, HUVECs, and human coronary arterial ECs,[26] supports our finding that miR-214 is a regulator of angiogenesis. CACs did not express miR-214, though CACs originate from a monocytic lineage and do not form or integrate into vascular networks.[27, 28] Interestingly, by transfecting miR-214 into CACs we were able to alter the angiogenic effect of CAC conditioned medium on HUVECs.

Consistent with our hypothesis that miR-214 regulates angiogenesis, *in vivo* silencing of miR-214 resulted in a strong increase in CD31+ and αSMA+ cell infiltration and capillary formation in implanted matrigel plugs in mice. The results of our ctrl-antagomiR treated mice are in agreement with previous reports as well, showing minimal cell infiltration in matrigel plugs devoid of additional growth factors.[29] The effect on *in vivo* vascularization in antagomiR-214 treated mice demonstrates the potency of the pro-angiogenic effect of miR-214 silencing, since no cells or antagomiR-214 were added to the matrigel prior to injection. In addition, we provide evidence of the formation of functional anastomoses with the host vascular system, because the extensive network of luminal structures that is formed in antagomiR-214 treated animals contained erythrocytes.

It was previously shown that miR-214 is highly expressed in the developing retina.[20] We could show that, by inhibiting miR-214 during retinal vascular development, the formation of the vascular network was enhanced, indicating a distinct regulatory role for miR-214 not only in adult angiogenesis but also in developmental angiogenesis. The effect we observed on retinal developmental angiogenesis is in support of the evident role of the direct miR-214 target QKI in mouse vascular development.

In addition to our used *in vivo* models to demonstrate the functional role of miR-214 in angiogenesis it is speculative to think on the use of miR-214 inhibition for the treatment of myocardial infarction. However, since the differential expression of miR-214 is, among others, also related to cardiomyocyte hypertrophy,[30] a clear functional correlation between miR-214 and angiogenesis is not possible.

Angiogenesis is a complex process that involves a cascade of events including basement membrane degradation, cell proliferation, migration, assembly and neovessel organization, maturation and programmed cell death, which are tightly regulated by cell-cell interactions. Several of these crucial processes in angiogenesis were found to be highly associated with the top most significant predicted miR-214 targets gathered from six different prediction databases. The two most enriched biological processes targeted by miR-214, namely cellular assembly and organization, and cellular development, are indicative of a more specific role for miR-214 in vascular development and remodeling. Interestingly, multiple studies have identified QKI, validated here as a direct miR-214 target, as a regulator of vascular development and remodeling.[15-17] Moreover, we found QKI and VEGFA to be present in the top scoring associated networks, built from each of the six datasets of predicted miR-214 targets.

QKI belongs to the signal transduction and activation of RNA (STAR) family of RNA-binding proteins, which bind mRNAs depending on specific motifs, thereby regulating pre-mRNA splicing, export of target RNAs from the nucleus, translation of proteins, and RNA stability.[31] Mouse embryos genetically deficient of QKI die due to severe defects in vascular development and failure of blood circulation in the yolk sac, the earliest site of vasculogenesis and angiogenesis.[15-17] In general, these studies have shown that QKI plays an important role in proper vascular development and remodeling by regulating interactions between ECs and SMCs. In concordance with these previous studies, we demonstrated that QKI is expressed in ECs and in VSMCs. More importantly, the vascular defects associated with QKI deficiency are reflected by the vascular sprouting defects resulting from miR-214 overexpression and QKI knockdown. By showing that co-transfection of ECs with both QKI siRNA and antimiR-214 could not rescue the negative effect of QKI down-regulation on EC sprouting, we can conclude that the major part of the negative effect seen on EC sprouting angiogenesis occurs through miR-214 targeting QKI.

Most interestingly, we and others showed that QKI deficiency results in decreased VEGFA expression, one of the most potent pro-angiogenic factors.[17] In line with these data, we show that miR-214 overexpression results in reduced VEGFA levels, which is reflected by the decreased endothelial tube organization in miR-214 CM-treated ECs. Moreover, silencing of miR-214 increased VEGF levels and VEGF secretion, as well as the secretion of several other vascular factors necessary for normal capillary tube formation. Because QKI is an RNA-binding protein, it is interesting to speculate that QKI might bind to the transcripts of genes involved in vascular development and angiogenesis, thereby post-transcriptionally regulating the expression of these genes. However, we observed that QKI did not regulate VEGFA expression by mRNA binding (data not shown). Nonetheless, it remains possible that the control of VEGFA expression can be ascribed to QKI binding other molecules, like transcription factors. Therefore, we suggest a model in which miR-214 negatively regulates angiogenic signaling via direct targeting of QKI and subsequent reduction of pro-angiogenic growth factor expression and release.

In conclusion, this is the first study to demonstrate the essential role for miR-214 in regulating *in vitro* and *in vivo* angiogenesis. The discovery that miR-214 regulates angiogenic signaling provides a new important target to potentially block pathological tissue growth or to improve therapeutic vascular growth.

Acknowledgements

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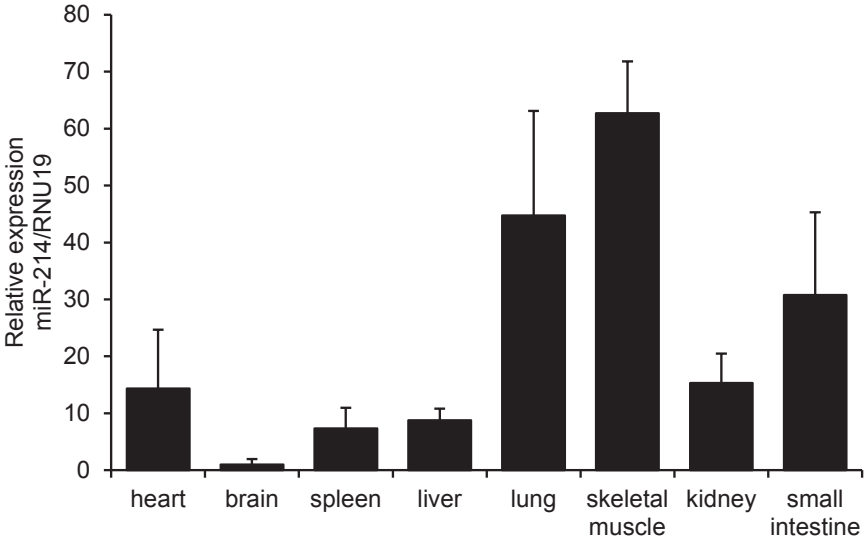
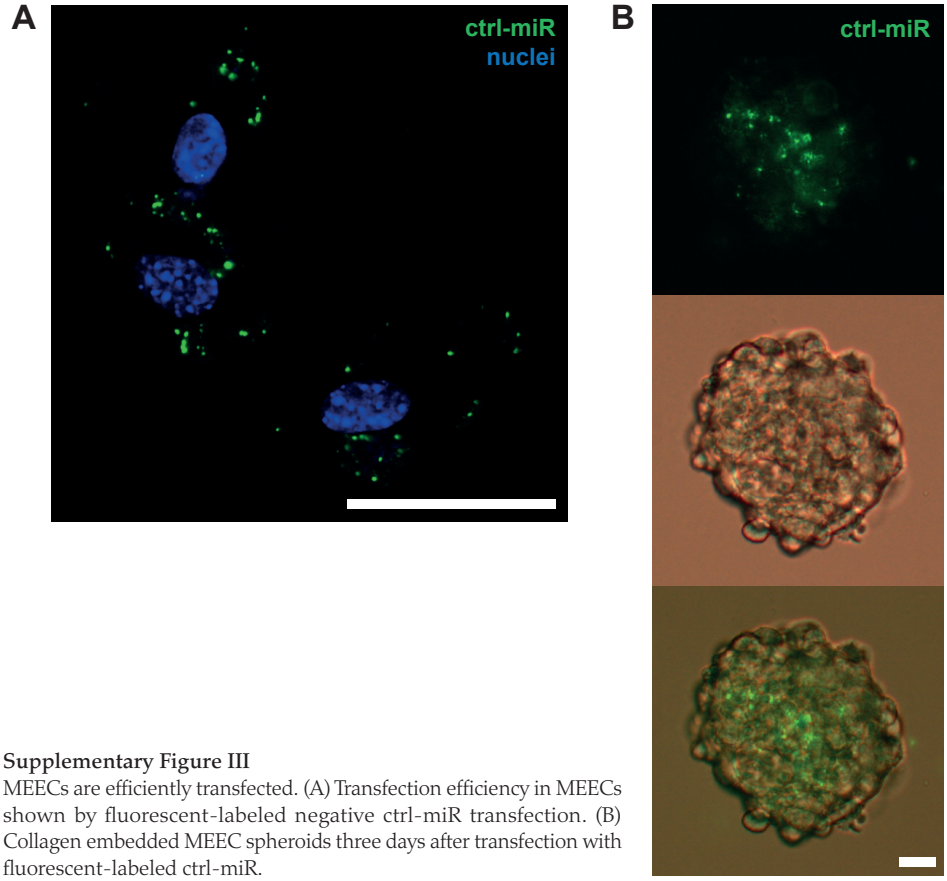
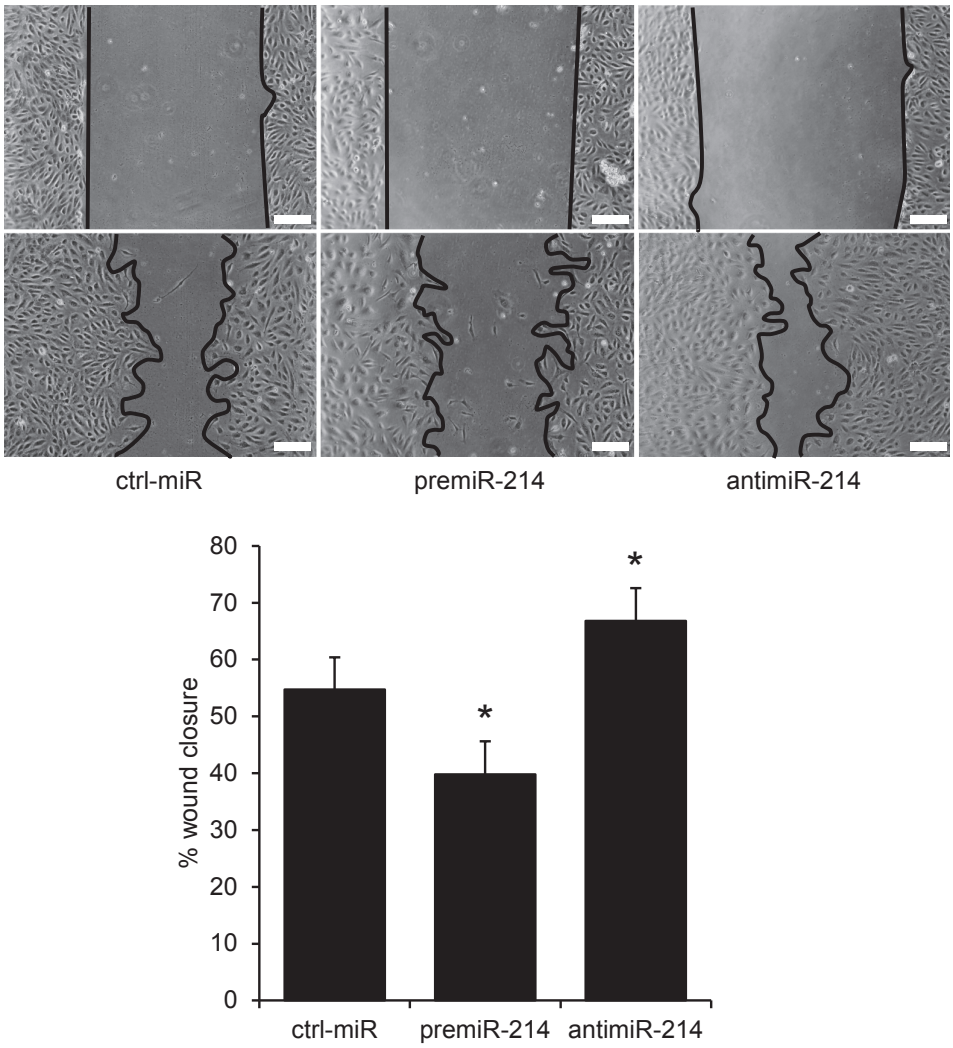
Supplementary Figure I
miR-214 is highly conserved among a wide variety of species. Aligned mature miR-214 sequences of 23 species. Variations are highlighted in grey.



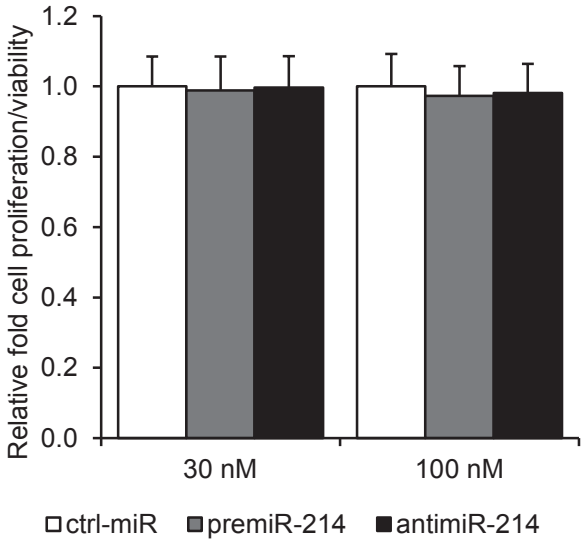
Supplementary Figure II
miR-214 is highly expressed in in tissues with a high vascular component. Taqman qRT-PCR analysis of miR-214 expression in mouse tissues. Data is represented as fold induction relative to brain. n=5/tissue.



Supplementary Figure III
MEECs are efficiently transfected. (A) Transfection efficiency in MEECs shown by fluorescent-labeled negative ctrl-miR transfection. (B) Collagen embedded MEEC spheroids three days after transfection with fluorescent-labeled ctrl-miR.



Supplementary Figure IV
miR-214 inhibits EC migration. Scratch wound cell migration assay of ctrl, pre, or antimiR-214 transfected HUVECs, quantified as percentage wound closure. n=3

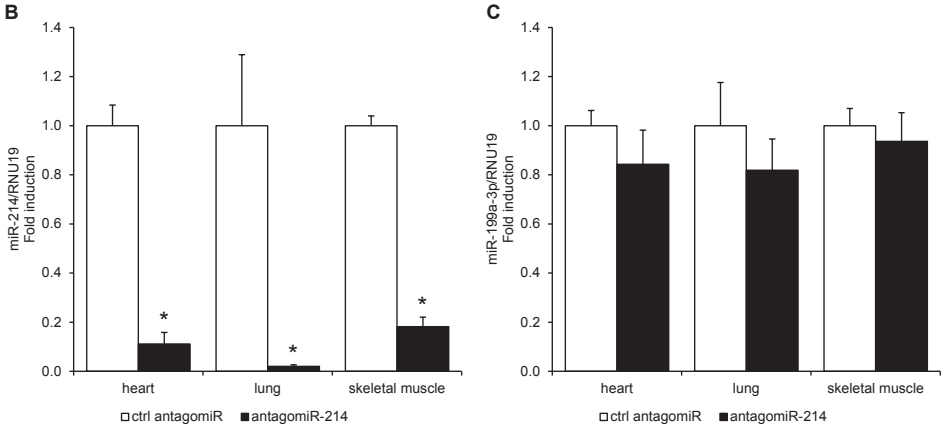


Supplementary Figure V
The effect of miR-214 on MEEC proliferation and viability. Cell proliferation/viability index of premiR-214 or antimiR-214 transfected MEECs, relative to ctrl-miR, three days after transfection. n=3.

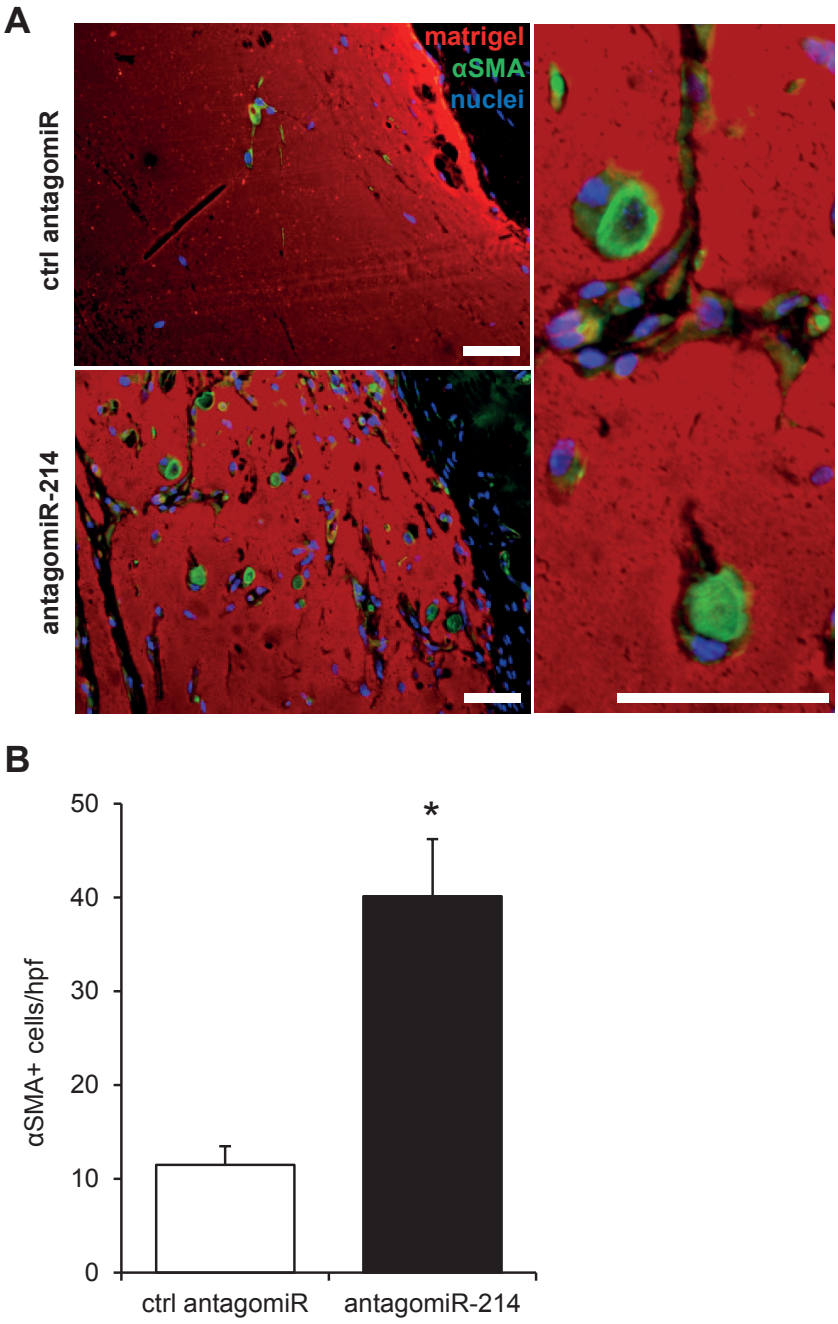
A

hsa/mmu-miR-214: acagcaggcacagacaggcagu

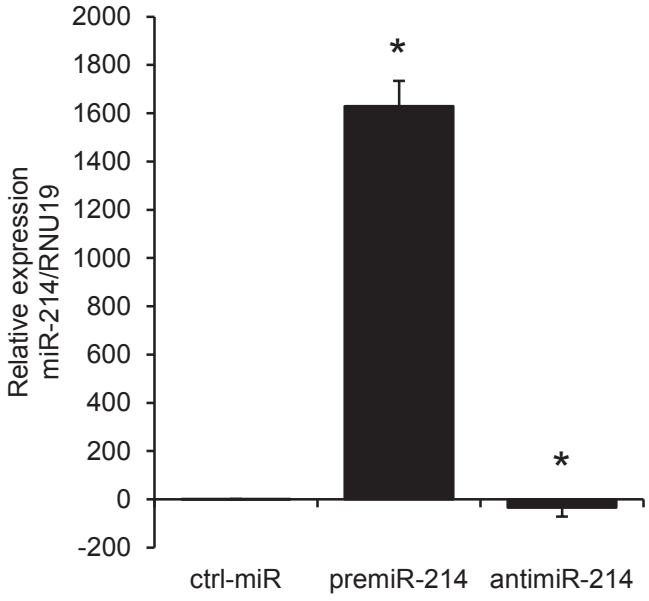
antagomiR-214: 5′-a*c*ugccugucugugccugc*u*g*u*-3′-CHOLESTEROL
control-antagomiR: 5′-a*c*u**c**ccug**c**cu**u**u**c**ccu**ua***u*g*u*-3′-CHOLESTEROL



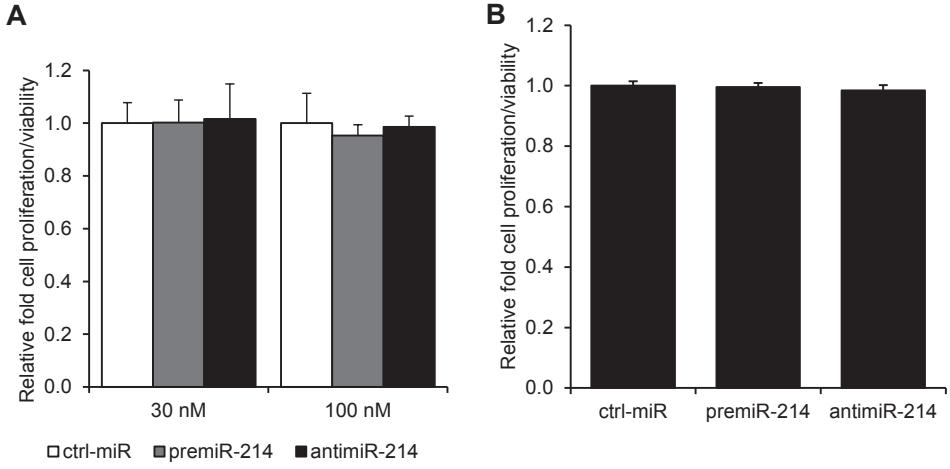
Supplementary Figure VI
miR-214 expression is diminished by antagomiR-214 treatment. (A) AntagomiR-214 consists of the inverse complementary mature miR-214 sequence. The mismatch control antagomir contains 6 mismatches (underlined). Antagomirs are 3′cholesterol-modificated, 2′O-methylated, and contain PTO-linkages (*) at the first two and last four nucleotides. Taqman qRT-PCR analysis of (B) miR-214, or (C) miR-199a-3p expression in heart, lung and skeletal muscle, two weeks after first antagomir injection. Data is represented as fold reduction relative to ctrl-antagomir. n=6/tissue.



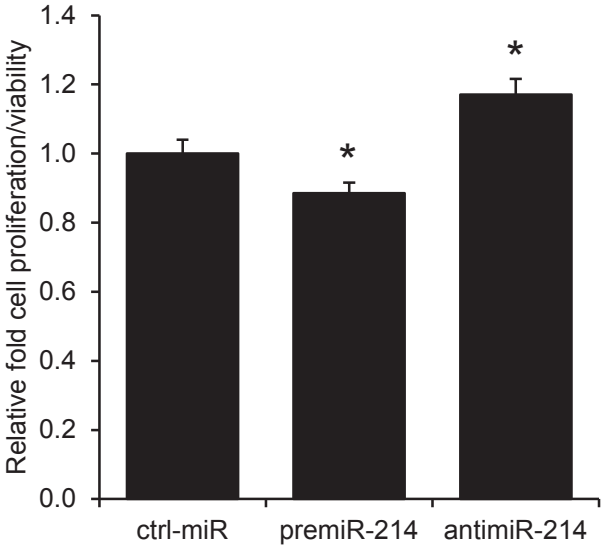
Supplementary Figure VII
In vivo miR-214 silencing enhances matrigel plug angiogenesis. (A) Effect of miR-214 silencing on capillary formation as shown by αSMA+ cell staining. (B) Quantification of αSMA+ cells in the matrigel plug. n= at least 6/group.



Supplementary Figure VIII
Efficient miR-214 up- or down-regulation in HMECs. Taqman qRT-PCR analysis of miR-214 expression two days after transfection of pre- or antimiR-214 in HMECs. Data is represented as fold induction or reduction, relative to ctrl-miR. n=3.



Supplementary Figure IX
The effect of miR-214 on HMEC proliferation and viability. (A) Cell proliferation/viability index of premiR-214 or antimiR-214 transfected HMECs, relative to ctrl-miR, three days after transfection. n=3. (B) Cell proliferation/viability index of HMECs in premiR-214 or antimiR-214 transfected HMEC conditioned medium, relative to ctrl-miR. n=3.



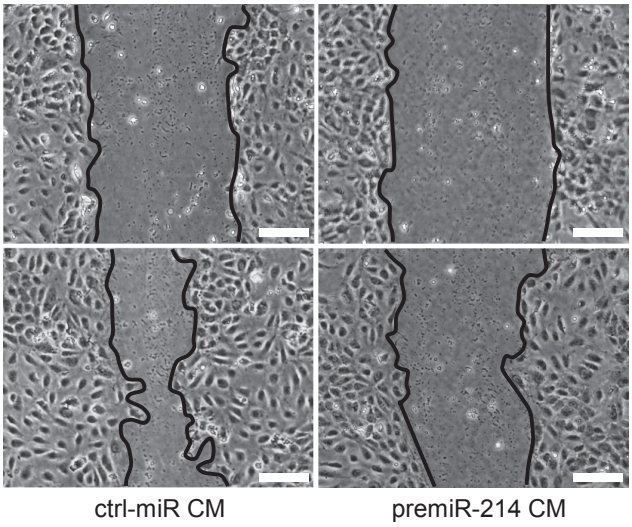
Supplementary Figure X
Long-term effect of miR-214 on HMEC proliferation and viability. Cell proliferation/viability index of premiR-214 or antimiR-214 transfected HMECs, relative to ctrl-miR, ten days after transfection. n=3.

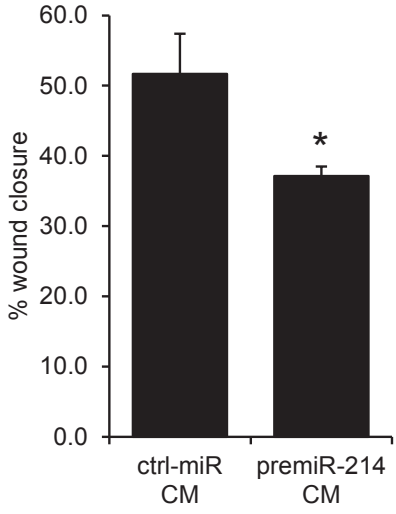
Supplemental Table 1 IPA top-scoring miR-214 predicted target associated network functions

Associated network functions	Score
Cellular assembly and organization	39
Cellular development	39
Cell cycle	35
Cancer	33
Cell signaling	33

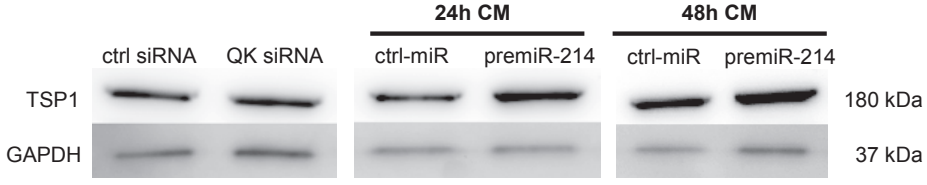
Supplemental Table 2 IPA top-scoring miR-214 predicted target associated biological functions

Molecular and cellular functions	p-value	# Molecules
Cellular development	1.12E-04 - 2.17E-02	73
Cellular assembly and organization	1.29E-04 - 2.83E-02	59
Cell death	1.35E-04 - 2.81E-02	86
Gene expression	1.51E-04 - 2.79E-02	65
Cell-to-cell signaling and interaction	2.12E-04 - 3.56E-02	56





Supplementary Figure XI
miR-214 transfected CAC conditioned medium inhibits EC migration. Scratch wound cell migration assay of HUVECs in ctrl or premiR-214 transfected CAC conditioned medium, quantified as percentage wound closure. n=4



Supplementary Figure XII
miR-214 induces anti-angiogenic TSP1 secretion independently of QK. The effect of QK siRNA, premiR-214 or antimiR-214 transfection of ECs on TSP1 protein secretion, normalized to GAPDH.

CHAPTER 8

GENERAL DISCUSSION

Heart failure is characterized by the inability of the diseased heart to provide enough force to supply sufficient blood flow to meet the body's need. Heart failure is generally caused by an ischemic event like myocardial infarction (MI). It is estimated that a typical MI results in the loss of approximately one billion functional cardiomyocytes, which are replaced by a fibrous scar [1]. The non-contractile scar prevents possible rupture but does not restore heart function, eventually leading to heart failure. The currently available surgical, drug, and device-based therapies cannot reverse the loss of functional myocardium, which is the fundamental cause of the problem. As a result of this lack of an available medical solution, heart failure has evolved into a global epidemic [2]. Therefore, the development of regenerative therapeutic strategies to halt the progression of ischemic heart disease to advanced heart failure has become one of the most urgent medical needs of this century and a holy grail of modern-day cardiovascular research.

Regenerative medicine has always captivated the fascination of scientists as it embodies an ancient human desire: everlasting youth or eternal life. The foundation of regeneration was established by Rudolf Virchow in 1858, who championed the experimental work of Robert Remak, who had discovered that cells form by division. Virchow presented his thoughts on regeneration stating that tissue regeneration is dependent on cell proliferation, which he described with the now famous words, 'Omnis cellula e cellula', saying that every cell arises from a pre-existing cell 3. This dependency of tissue regeneration on cell proliferation becomes particularly clear in the injured heart. Lower vertebrates like frogs, newts, axolotls, and zebrafish display extensive cardiomyocyte proliferation and thus possess a remarkable natural capacity for cardiac regeneration after injury [4-7]. The adult human heart has only a very limited potential for cardiomyocyte renewal, as was shown by measurements of carbon-14 integration in cardiomyocytes [8], and is therefore unable to regenerate itself after myocardial injury. While it seems that evolution has led to the loss of the cardiac regenerative capacity in mammals, the adult human heart does contain progenitor cell populations capable of self-renewal and differentiation into the different cardiac cell lineages [9-12]. In addition, various studies have shown that the limited mammalian cardiac regeneration after MI primarily occurs through differentiation of these progenitor cells, rather than by cardiomyocytes re-entering the cell cycle [13, 14]. While the adult human heart will not functionally restore itself after injury, it clearly holds a modest regenerative potential that could be exploited to achieve true cardiac regeneration. This indicates that the adult human heart is a promising target and contains a suitable cell source for regenerative medicine.

In 2007, our research group reported the successful isolation of cardiac progenitor cells from the adult and fetal human heart [12]. These cells, termed human cardiomyocyte progenitor cells (hCMPCs), were isolated by clonal culture or by Sca-1 antibody cell sorting [12, 15-17]. hCMPCs show a stem cell-like morphology and express stem cell markers such as ISL1 and c-KIT. They also express the early cardiac transcription factors, GATA4, NKX2.5, and MEF2C, but do not express any mature cardiomyocyte or hematopoietic cell surface markers. hCMPCs have telomerase activity and the capacity for self-renewal, which makes them easy to expand *in vitro*. Moreover, hCMPCs differentiate into functional cardiomyocytes without the need for co-culture with neonatal cardiomyocytes. After successful induction of cardiomyogenic differentiation, hCMPCs have sarcomeres, show gap junctional communication, display adult ventricular-like action potentials, and beat spontaneously in culture [12, 18]. hCMPCs are also able to differentiate into endothelial cells and smooth muscle cells, and are therefore capable of forming all the cells necessary for recreation of the native architecture of the heart. These characteristics and properties renders them very promising for cell-based therapy. To date, numerous pre-clinical and three phase 1 clinical studies (ALCADIA, SCIPIO, CADUCEUS) have made use of the option to transplant various cardiac-derived progenitor cells to improve cardiac regeneration and function after injury. Preliminary results of the ALCADIA and SCIPIO trials are encouraging as they have shown a reduction in myocardial scar mass, and the SCIPIO trial reported an additional improvement in left ventricular ejection fraction [19-21]. However, the preclinical studies using CPCs have shown that only a very limited number of total injected cells were found back in the heart [22-24]. This low transplantation efficiency is commonly attributed to the extremely poor survival, engraftment and functional integration of the transplanted cells, and signifies the need for novel strategies that increase the effectiveness of cell-based therapies, thereby potentiating cardiac repair in order to achieve successful cardiac regeneration.

MiRNAs have been shown to play key roles in the cardiac repair processes after MI [25] and were shown to control stem cell self-renewal, differentiation, and biology in general [26, 27]. In this thesis, we have therefore focused on the use of miRNAs to overcome the current obstacles that prevent the success of cell-based therapies. We have explored the option of improving the differentiation, survival, and engraftment of cardiac progenitor cells through specific miRNA modulation. Additionally, miRNA modulation was used to control *in vivo* angiogenesis, a critical regenerative process necessary to achieve effective cell engraftment and survival.

MiRNA regulation of cardiac progenitor cell differentiation into cardiomyocytes

Because of their eminent role in stem cell maintenance and lineage specification, in chapter 2 we investigated the expression of miRNAs in proliferating hCMPCs and in hCMPCs differentiated into beating cardiomyocytes (hCMPC-CM), with the goal to find specific miRNAs that regulate cardiomyogenic differentiation and thereby provide novel targets to enhance the generation of new cardiomyocytes. Over 40% of all investigated miRNAs were detected in proliferating or differentiated hCMPCs, suggesting that miRNAs fulfill prominent roles in hCMPCs and hCMPCs-CM. Of the 19 miRNAs that were significantly induced upon cardiomyogenic differentiation, 10 are now known to be expressed in cardiomyocytes and involved in cardiac function and development. It should be pointed out however that while 19 miRNAs are significantly upregulated upon cardiomyogenic differentiation, this does not mean that all of them are directly involved in the process of differentiation, as it is also possible that some miRNAs represent a population of miRNAs involved in cardiomyocyte homeostasis. Of all the miRNAs expressed in hCMPCs, 27 were significantly downregulated and it has been shown that seven of these (miR-146a, 155, 20a/b, 17, and 18) negatively regulate transforming growth factor beta (TGFB) signaling by directly targeting TGFB, TGFBR2, SMAD2, and SMAD4 [28-32]. Importantly, TGFB is used to induce the cardiomyogenic differentiation of hCMPCs, suggesting that the significant decrease in these miRNAs may facilitate cardiomyogenic differentiation by relieving the repressive effect on the TGFB signaling pathway. However, we did not observe any effects on differentiation upon miR-155 up or downregulation, suggesting that this miRNA regulates different processes in hCMPCs, which we described in chapter 4 and 5.

The next step was to further investigate the most highly differentially expressed miRNAs for their possible role in regulating differentiation. We found that miR-1 and miR-499 are indispensable for the cardiomyogenic differentiation of hCMPCs and significantly induce the differentiation of mESCs to beating cardiomyocyte-like cells. To date, our results have been repeated in hESCs [33], and the potent effect of miR-499 in inducing cardiomyogenic differentiation was shown by its capacity to enhance the expression of early cardiac genes even in non-cardiac cells, like bone marrow-derived MSCs [34]. The additional benefit of simultaneous upregulation of miR-1 and 499 to induce cardiomyogenic differentiation and maturation was suggested by a study showing that miR-1 and 499 play differential positive roles in cardiac differentiation of hESCs. While miR-499 promoted ventricular specification of hESCs, miR-1 served to facilitate electrophysiological maturation [35], two processes necessary for the formation of mature cardiomyocytes. In our study we could not find a clear synergistic

function of miR-1 and 499 on the myogenic differentiation of hCMPCs. However, myogenic differentiation of hCMPCs was evaluated by measuring the expression of several cardiac contractile proteins, without studying electrophysiological maturation. The combined upregulation of miR-1 and 499 was not assessed in mESCs, however, we found that miR-1 overexpression resulted in an increased percentage of beating embryoid bodies as compared to miR-499, while late cardiac marker levels did not vary significantly, indicating that miR-1 may indeed additionally induce electrophysiological maturation, at least in ESCs.

We have shown previously that hCMPCs can spontaneously differentiate into cardiomyocytes *in vivo* when transplanted into the infarcted mouse heart [23]. Pre-treating the cells with miR-1 and 499 might enhance this natural effect of the infarcted heart on hCMPC differentiation. Unfortunately, we were not able to show a clear beneficial effect of transplanting miR-1 and/or miR-499 transfected hCMPCs after mouse MI (data not shown). However, these results were likely due to the transient transfection method we used, as two other groups were able to show potent beneficial effects using more robust overexpression methods [36, 37]. These *in vivo* follow-up studies have shown the potential of pre-treating stem or progenitor cells with either miR-1 or 499 to improve the efficiency of transplantation therapy. Expression of miR-499 in human cardiac stem cells, selected based on c-KIT expression, enhanced cardiomyogenesis *in vitro* and *in vivo* after MI, leading to an increased aggregate volume of the regenerated myocyte mass and an increase in left ventricular function, as compared to untreated cells [37]. In mESCs, miR-1 overexpression enhanced cardiomyocyte differentiation following transplantation into the infarcted myocardium, reduced MI-induced apoptosis, and improved heart function [36]. These studies clearly show the benefit of overexpressing miR-1 and 499 in stem or progenitor cells to improve cardiac regeneration after MI. It also indicates that pre-treatment alone, without prior complete differentiation may be enough to facilitate cardiac regeneration. The effect of fully differentiating progenitor cells into cardiomyocytes with the help of miR-1 and 499 before transplantation remains to be investigated. However, our group has previously shown that there was no additional functional benefit from transplanting pre-differentiated hCMPCs compared to undifferentiated hCMPCs, and that similar numbers of the same cell types were formed by both cell groups [23]. However, the undifferentiated hCMPCs were shown to increase vessel density by a possible paracrine effect, which was not observed in the hCMPC-CMs treated mice, indicating that the similar functional effect seen in both cell groups had different causes. While similar numbers of cardiomyocytes were formed *in vivo* by both hCMPCs and hCMPC-CMs, it is possible that the pre-differentiated hCMPCs form more mature-like cardiomyocytes. Additionally, about 70% of the pre-differentiated hCMPCs

were fully differentiated into cardiomyocytes, indicating the need to improve the differentiation protocol. hCMPCs can be differentiated into cardiomyocytes by TGFB stimulation, however this process is inefficient and can be greatly enhanced by first treating the cells with 5-azacytidine (5-aza), a demethylating agent. However, for the clinical application of differentiated hCMPCs, 5-aza is not an option, as demethylation may result in unpredictable gene activation. Therefore it is of great interest to find other ways to improve hCMPC differentiation. While miR-1 and 499 already greatly improve differentiation, it might also be interesting to evaluate the effect of 5-aza on miRNA expression, thereby uncovering specific miRNAs that may completely substitute the use of 5-aza. Moreover, we only analyzed the role of the two most highly upregulated miRNAs in cardiomyogenic differentiation, whereas several other highly up or downregulated miRNAs may also play significant roles in this process and may offer additional targets to achieve miRNA-specific induction of differentiation. Future studies may therefore continue in evaluating the precise roles of these differentially expressed miRNAs, especially since almost no additional information in this field was published following our report.

The regeneration of dead myocardium is the key solution to reducing death from heart disease and highlights the importance of generating significant numbers of mature cardiomyocytes that are able to recreate the native architecture of the heart and electromechanically couple with the host myocardium, thereby restoring cardiac function. Our results, together with the *in vivo* follow-up studies have shown that miR-1 and 499 are potent positive regulators of cardiomyogenic differentiation and shows that miRNAs provide functional targets to enhance cardiac regeneration and function. Next to cell transplantation therapy, activation and recruitment of resident progenitor cells and subsequent induction of differentiation would be an elegant option for cardiovascular regeneration. However, because miR-1 and 499 upregulation can have adverse effects on other cell types [38, 39], this means that we would need to specifically target the local progenitor cells for miR-1 and 499 modulation. Unfortunately, local targeted miRNA therapy is currently not feasible.

MiRNA regulation of the angiogenic differentiation of cardiac progenitor cells

During MI, restoration of blood flow is critical for the survival of the myocardial tissue surrounding the necrotic infarct. In addition, regeneration of the myocardium requires reconstructing the vast capillary network that provides oxygen, nutrients, and local protective signals to every single cardiomyocyte [40]. We have previously shown that hCMPCs can differentiate into cardiomyocytes, endothelial, and smooth muscle cells

in vitro and *in vivo* [12, 23], and that miRNAs play crucial roles in determining hCMPC cell fate [41]. This, together with the important roles of miRNAs in blood vessel development led us to investigate the role of miRNAs in the angiogenic differentiation of hCMPCs. We found that miR-1 was significantly upregulated when hCMPCs differentiated into tubular structures consisting of aligned endothelial cell marker positive cells surrounded by cells expressing alpha smooth muscle actin (ASMA), and overexpression of miR-1 led to an enhanced angiogenic differentiation. Our miRNA profiling data had shown that undifferentiated hCMPCs express high levels of several miRNAs (miR-21, 126, 221, 222) which are known to be highly expressed in endothelial cells (ECs) [42-45]. The presence of these miRNAs in hCMPCs may contribute to the relative ease of differentiating hCMPCs into vascular structures, and may consequently provide attractive targets to specifically induce hCMPC differentiation into ECs. However, the angiogenic differentiation of hCMPCs constitutes of two parts, namely differentiation of hCMPCs into ECs and differentiation into vascular smooth muscle cells (vSMCs). Our data suggests that miR-1 stimulates both these processes as we found increased protein levels of CD31, KDR, and ASMA as compared to control-miR transfected cells. Even though we found that Spred1 is a direct target of miR-1, it remains to be investigated if Spred1 downregulation accounts for the observed effect on the angiogenic differentiation. It is likely that Spred1 downregulation mediates the differentiation into ECs as it controls the intracellular signaling cascade activated by different endothelial growth factors [46, 47]. Spred1 has been shown to be necessary for the myogenic differentiation of C2C12 myoblasts, a mouse muscle progenitor cell line [47], but has not been linked to vSMC differentiation, suggesting that miR-1 may target other transcripts resulting in the enhanced differentiation into vSMCs. MiR-1 has been shown to induce SMC differentiation of ESCs by targeting KLF4 [48], a regulator of SMC differentiation [49]. KLF4 is expressed in hCMPCs (unpublished data) and could therefore play a significant role in miR-1 induced SMC differentiation of hCMPCs, warranting further investigation.

For the therapeutic application of miR-1, one can think of treating hCMPCs with miR-1 prior to injection or fully pre-differentiating the cells, or even treating resident progenitor cells. While the last option is an attractive approach for stimulating vascularization without cell transplantation, this option is hampered by the lack of cell-specific miRNA delivery methods. Pre-treatment may represent the most powerful strategy for improving cardiac regeneration in general, because miR-1 can induce both the cardiomyogenic as well as the angiogenic differentiation of hCMPCs dependent on environmental cues like TGFB and pro-angiogenic growth factors. When miR-1 pre-treated hCMPCs are transplanted into an area high in angiogenic growth factors, Spred1 downregulation may favor the differentiation into new vasculature, whereas

TGFB-rich areas will likely induce cardiomyogenic differentiation, although TGFB is also known to induce SMC differentiation [50]. This theory is strengthened by our previous finding showing that injection of hCMPCs into the infarcted mouse myocardium induced spontaneous differentiation into cardiomyocytes, endothelial cells and smooth muscle cells, indicating that the injured heart itself provides distinct signals that trigger hCMPC differentiation and myocardial repair. These data indicate that the local environment may direct hCMPC differentiation into the required cell types, which shows the advantage of pre-treating hCMPCs instead of fully differentiating them. A recent study has shown that transplantation of miR-1 pre-treated mESCs in the infarcted mouse heart led improved heart function [36]. Strikingly, vessel density was not evaluated in this study, while being one of the most important processes known to improve cardiac function after MI in cell transplantation studies. Next to advancing our understanding of how the angiogenic differentiation of hCMPCs is regulated, these new insights may help to develop novel therapeutic strategies to enhance the formation of new vasculature and improve cardiac regeneration.

MiRNA regulation of cardiac progenitor cell survival and migration

A major obstacle to the success of cell transplantation therapy is the extremely limited survival and engraftment of transplanted cells. To be able to survive and engraft, the cells need to cope with the hostile environment after MI. This has prompted scientists to come up with ways to protect the transplanted cells. One of the potential solutions to cell death is to prepare the cells so they are able to escape apoptosis and necrosis. The broad regulatory role of miRNAs in stem and progenitor cell biology led us to explore the option of improving the survival and engraftment of cardiac progenitor cells through specific miRNA modulation. MiRNA profiling in hCMPCs showed that miR-155 is expressed in hCMPCs and several studies had shown that miR-155 reduces apoptosis [51-53]. We therefore induced oxidative stress in hCMPCs, mimicking the ischemic nature of the infarcted myocardium and found that the expression of miR-155 was significantly increased, indicating a potential role of miR-155 in increasing hCMPC survival. We found that oxidative stress selectively induced hCMPC necrosis, which could significantly be reduced by miR-155 overexpression, without affecting apoptosis. The specific effect on necrosis led us to investigate a previously proposed target of miR-155, RIP1 [54], a death domain receptor protein specifically involved in a non-apoptotic death pathway, termed necroptosis [55]. We found that RIP1 is a direct target of miR-155, and showed that RIP1 inhibition by siRNA, or by the RIP1-specific inhibitor Nec-1 [56], led to a specific reduction in necrosis and increased survival,

comparable to miR-155 overexpression. After overexpression of miR-155 in hCMPCs, we analyzed the expression of 84 key genes involved in the apoptosis or pro-survival pathway, and found that only three genes were significantly deregulated, indicating that miR-155 specifically blocks necrosis, independent of pro-survival and apoptosis pathways. Although oxidative stress is a major factor in the infarcted heart, it remains to be investigated if necroptosis is the main death pathway for hCMPCs after cell transplantation. However, if apoptosis is a major contributor to cell death after cell transplantation, miR-155 preconditioning may still be effective. Several studies, supporting our findings showing the positive role of miR-155 on cell survival, have shown that the increase in cell survival was due to reduced apoptosis [51-53, 57-65]. This effect of miR-155 on apoptosis was simply not observed in hCMPCs as we did not see any caspase activity in oxidative stress-induced hCMPCs, indicating that the apoptosis pathway is not activated in hCMPCs. One of the studies showing the positive role of miR-155 on cell survival found that miR-155 could directly target FADD and caspase-3 [60]. FADD has been shown to be indispensable for necroptosis [66], and could therefore be an additional interesting target in the regulation of hCMPC necroptosis by miR-155. Intriguingly, miR-155 inhibition did not result in enhanced necrosis, which may be explained by the possibility that necrosis had already reached a maximum level due to the oxidative stress and could not be increased any further. Another possibility is that endogenous miR-155 fulfills other roles in the hCMPC.

In chapter 5, we uncovered an additional role of miR-155 in the regulation of hCMPC migration. The success of cell transplantation therapy is currently hindered by poor cell engraftment. One of the factors contributing to poor cell retention is the unrestricted migration of cells, away from the myocardium in need [23, 67, 68]. Limiting hCMPC migration after transplantation may therefore provide a means to overcome limited cell engraftment. By increasing miR-155 in hCMPCs we showed that migration could be significantly reduced with concomitant reduction in MMP2 and 9 activities, two key enzymes commonly associated with cell migration. However, we found that MMP2 and 9 protein expression was not affected, pointing to a possible role for miR-155 in regulating the expression of MMP16, an activator of MMP2 and 9. MMP16 was indeed a direct target of miR-155, and by siRNA or blocking antibody mediated inhibition of MMP16, we demonstrated the functional effect of MMP16 inhibition on hCMPC migration. The observed decrease in cell migration by MMP16 inhibition was in agreement with a previous report showing an inhibitory role of miR-146b on MMP16 expression and cell migration [69]. The negative effect of miR-155 on cell migration is in agreement with two other studies [30, 70], whereas another report showed an increase in migration, however, in this study the enhanced migratory capacity was a result of a phenotypic transition towards a more motile cell type [71]. Recently, it was

shown that hCMPCs have the capacity to modulate their own matrix environment by MMP2 and 9 expression [72]. This observation is in line with our finding that hCMPCs regulate their migration by regulating the expression of MMP16, and suggests that pre-treatment with miR-155 may increase cell retention after transplantation.
While Nec-1 may be used to specifically inhibit hCMPC necrosis after transplantation, pre-treatment with miR-155 has the advantage of simultaneously increasing cell survival and retention of the transplanted cells at the site of injection. MiR-155 preconditioning may therefore provide a novel opportunity to improve the efficiency of cell transplantation. In fact, a pilot experiment using bioluminescence imaging to track living cells has shown that pre-treating hCMPCs with miR-155 increased the number of viable cells, measured at day seven post transplantation. Whether increased cell survival, cell retention, or both were the cause of the observed effects remains to be investigated. Other groups have also proven the potential of using miRNAs to increase cell survival and engraftment. A study using MSCs showed that miR-210 was significantly upregulated in MSCs which had undergone several cycles of ischemia/reoxygenation [73]. The increased miR-210 levels led to increased cell survival, which could be abrogated by miR-210 inhibition. When the preconditioned MSCs were subsequently transplanted in a rat model of MI, more cells survived [73] and a follow-up study showed an increase in functional recovery, possibly induced by transfer of miR-210 to native cardiomyocytes [74]. Another group has demonstrated the combined use of three miRNAs (miR-21, 24, 221) to increase CPC survival after transplantation [75]. Lentiviral overexpression of these miRNAs led to longer survival, as measured by bioluminescence imaging, and improved functional recovery, which was in part attributed to increased wall thickness.

MiRNA regulation of *in vivo* angiogenesis

Enhancing the restoration of vasculature after MI has a dual beneficial role as it may improve survival of endogenous tissue as well as transplanted cells. Hypoxia in the ischemic myocardium is suspected to be one of the critical factors contributing to the poor survival and engraftment of transplanted cells. Therefore, to allow successful regeneration of the damaged myocardium, it is of key importance to restore myocardial perfusion by generating new blood vessels, enabling free availability of oxygen and nutrients to sustain the transplanted progenitor cells and newly formed cardiomyocytes. Although the exact mechanisms involved in angiogenesis after MI have not been fully elucidated, several studies have attempted to induce blood vessel formation via pro-angiogenic growth factor delivery strategies [76]. However, the translation of these

strategies to clinical trials involving patients with chronic coronary artery disease was unsuccessful [77]. One of the reasons that accounts for this negative result is that delivery of a single growth factor may not be adequate for the establishment of functional vasculature. MiRNAs inhibit the expression of multiple genes, which often belong to one specific functional network. This provides broad and robust transcriptional regulation which suggests the potential of using miRNAs to induce *in vivo* angiogenesis. This led us to evaluate the role of specific miRNAs in the regulation of angiogenesis. We have shown, both *in vitro* and *in vivo*, that inhibition of miR-214 significantly enhanced angiogenesis. We found that miR-214 directly targets the RNA-binding protein QKI and thereby negatively regulates the expression and secretion of several pro-angiogenic growth factors. QKI has been shown to control VEGF protein expression [78], however, it remains to be investigated how exactly QKI regulates the expression of the various angiogenic growth factors. Although we showed that QKI knockdown resulted in the downregulation of VEGF, FGF, and PDGF, several other miR-214 regulated factors were not investigated in relation to QKI. It is possible that miR-214 may also directly target some of these factors, instead of indirectly controlling their expression via QKI. A recent study showed that miR-214 directly targets the pro-angiogenic growth factor HDGF, and the secretome-related anti-angiogenic function of miR-214 was supported by the finding that miR-214 inhibition led to increased HDGF secretion and enhanced tumor angiogenesis [79]. We did not analyze the expression of HDGF in our study, however, HDGF is closely related to FGF [80], which is regulated by miR-214 and QKI. This study also supported our finding that miR-214 has the ability to regulate angiogenesis in different tissues and different developmental stages, as we showed that next to enhancing *in vivo* matrigel angiogenesis in adult mice, antagomir-mediated inhibition of miR-214 also resulted in enhanced vascular development in the retina of newly born mice. MiR-214 has been shown to be highly expressed in the choroid [81, 82], the vascular layer of the eye, which supports our finding that miR-214 tightly controls retinal vascular development. The indispensable role of QKI in mouse vascular development [78, 83, 84] warrants further investigation of the role of miR-214 in developmental angiogenesis.

Several studies have shown an increase in miR-214 expression upon ischemia in different organs, including the heart [82, 85, 86]. Ischemia itself induces angiogenesis, which is inhibited by miR-214. Therefore, during ischemia, miR-214 might have a role in keeping angiogenesis in check, which suggests that inhibiting miR-214 in the ischemic area may provide a novel approach to enhance the formation of new blood vessels. Additionally, many studies have presented evidence that after MI, angiogenesis is stimulated by VEGF and FGF [76]. We have shown that the expression of these two growth factors is regulated by miR-214, indicating the potential benefit of inhibiting

miR-214 after MI. However, since the differential expression of miR-214 is also related to cardiomyocyte hypertrophy [87], we need ways to target only the ECs in the myocardium, and not the cardiomyocytes. We have obtained promising results in a pilot experiment using various doses of a fluorescent labeled antagomir, which indicated that specific dosing led to localization of the antagomir primarily in the vasculature of the myocardium.

Future directions

We have shown that miRNAs can regulate several aspects required to achieve successful cell transplantation therapy and cardiac regeneration. Without a doubt, miRNAs have the potential to become one of the most important therapeutic modalities in the coming years. Since 2001, there has been an explosion of interest in miRNAs, which is ever-growing as indicated by an almost exponential increase in the number of miRNA papers every year. In order to make reasonable predictions as to where the future of miRNA research is headed it is essential to know where we are departing from. For the field of miRNA research this is rather difficult as this is a very young field, which is growing rapidly and spreading to every crook and corner of scientific research. One thing that is evident is that miRNA research is still in its infancy and many hurdles need to be conquered to fully comprehend the extraordinary role of these tiny powerful regulators.

MiRNA research has to deal with a variety of difficulties and gaps in our knowledge on fundamental miRNA biology. MiRNA knockout animal studies have taught us that only few developmental processes are absolutely dependent on single miRNAs [88-90]. The lack of embryonic lethality results from significant miRNA redundancy, as other miRNAs may have the same or similar target profiles, thereby functionally compensating for the loss of other miRNA. Therefore, it is difficult to discover the function of a specific miRNA, which emphasizes the importance of determining miRNA function in the absence of miRNAs related in sequence and function. Moreover, miRNA redundancy also suggests that therapeutic modulation of a single miRNA may not be sufficient. Another difficulty in determining miRNA function is that the actions of miRNAs often only become pronounced under conditions of pathological signaling, which necessitates the use of more intricate experiments using genetic modifications or pathway activators/inhibitors. In our studies we have used various differentiation protocols, oxidative stress, and the activation of specific cellular processes to determine the role of specific miRNAs. We have also performed a miRNA profiling study to find associations of specific miRNAs with the process of

cardiomyogenic differentiation. Researchers have collectively performed these miRNA profiling studies, which led to an almost exclusive investigation of the extremely highly expressed miRNAs that are differentially expressed during disease or other processes. Future studies may therefore focus on the previously discarded miRNAs, as those might fulfill important general roles needed for normal cellular function. Additionally, many of the profiling studies were performed in the earlier years of miRNA research, when significantly fewer miRNAs were described. High-throughput sequencing keeps adding many new miRNAs with potential novel roles in cardiac regeneration, justifying the repeat of profiling studies. Currently, one of the major gaps in our knowledge of miRNA biology lies in the regulation of their expression. To advance our understanding of how cells direct miRNA regulated processes, it is essential to know what genetic and epigenetic elements are responsible for their expression. Additionally, very little is known on the impact of miRNA mutations or mutations in their target 3'UTRs. Mutations in the seed regions of evolutionarily conserved miRNAs are rare, whereas SNPs within miRNA target sites are more common [91, 92]. Due to the imperfect complementary miRNA-mRNA binding, together with miRNA redundancy in mRNA regulation, it seems unlikely that miRNA or target site mutations will cause significant phenotypic effects. However, it was recently shown that a mutation outside the seed region of miR-499 had a favorable impact on cardiac function in mutated-miR-499 transgenic mice compared to wild type miR-499 transgenic animals [93]. Additionally, two other studies have shown functional effects caused by mutations in the 3'UTR of mRNAs, which resulted in either creation or loss of a functional miRNA binding site [94, 95]. As miRNAs exert their effects through mRNA binding, an understanding of their targetome is of outstanding importance to identify their exact roles. Consequently, a much pursued goal in miRNA research is the establishment of a method that accurately and efficiently identifies the direct targets. Our method of using several miRNA target prediction databases combined with the examination of gene expression data was very successful in finding direct miRNA targets in the different studies described in this thesis. While our studies are primarily performed with human cells, the translation of miRNA regulatory networks from mice to human needs to incorporate the consideration that the often highly conserved miRNAs show dissimilarities in the genes they target in different species [96].

Overall, the development of miRNA research techniques is barely keeping up with the demand, especially in the field of *in vivo* miRNA modulation, which is necessary for the development of safe miRNAs therapeutics. While hCMPCs can easily be pre-treated with pre or antimiRs *in vitro*, and subsequently transplanted, the *in vivo* modulation of miRNAs to induce specific effects on cardiac regeneration is more complicated. Fortunately, there is an enormous interest in exploiting miRNAs for

therapeutic purposes, and much research is focused on developing techniques for *in vivo* miRNA modulation. For cardiac regeneration purposes, miRNAs-based therapeutics could represent a true revolution for the reason that miRNAs have been shown to robustly regulate many repair processes by simultaneously targeting multiple pathways, and because miRNAs can very successfully be modulated *in vivo*. The relative ease of either decreasing or increasing individual miRNA levels *in vivo* has been shown by using oligonucleotide based antagomiRs [97, 98], LNA antimiRs [99-101], miR-decoys [102], miR-sponges [103], or miR-mimics [104, 105], thereby providing many possibilities for the therapeutic manipulation of miRNAs (Figure 1). In chapter 7, we have chosen to use antagomiRs because of their high specificity due to complete complementarity to a specific miRNA. However, the shorter LNA-antimiRs have the advantage of targeting entire miRNA families, which might be helpful to reduce the effects of miRNA redundancy in mRNA targeting. LNA-antimiRs have successfully been used to treat a disease in non-human primates [100], and are currently being evaluated in the first human clinical trials (Santaris Pharma, ClinicalTrials.gov). Intriguingly, nature itself may provide additional targets to manipulate miRNA levels,

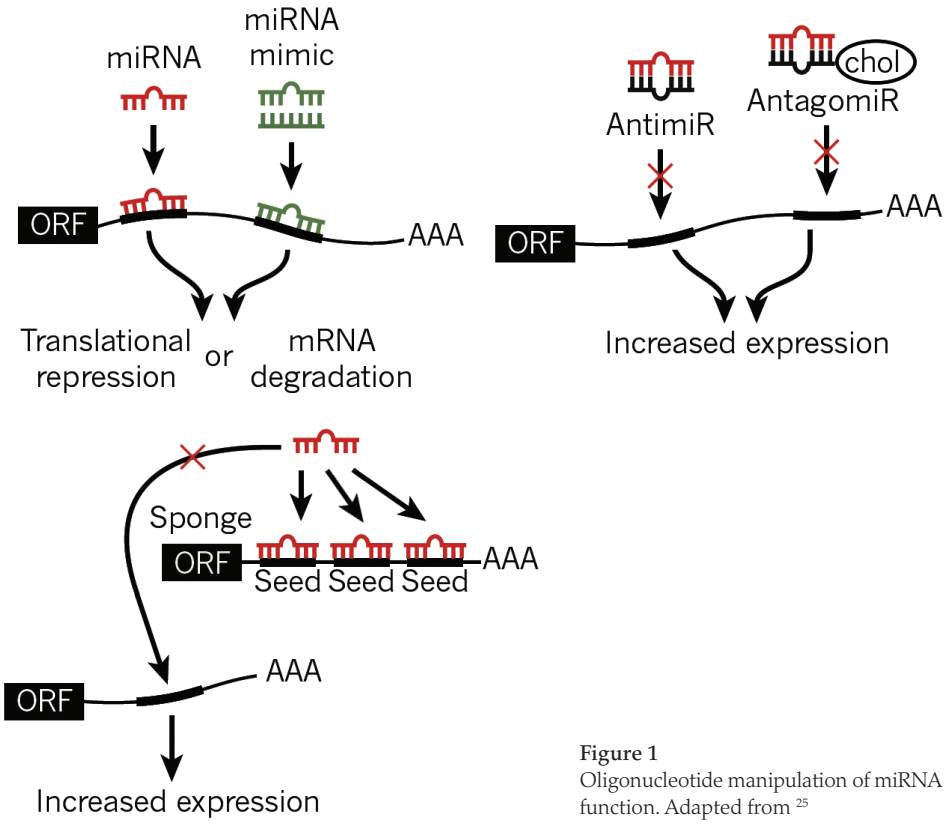


Figure 1
Oligonucleotide manipulation of miRNA function. Adapted from [25]

as it was shown that so-called competitive endogenous RNAs, which include mRNAs, pseudogenes, or long non-coding RNAs, can regulate the levels of biologically active miRNAs, and therefore provide natural miRNA decoys [106]. While the currently available small RNA chemistries have great stability, cell-permeability, specificity and affinity, progression of miRNA-based therapeutics is hindered by the lack of approaches for the delivery to specific tissues or target cells.

Next to the development of miRNA-based therapeutics, miRNAs have emerged as circulating biomarkers for cardiovascular disease, thereby providing a novel diagnostic tool [107]. How these miRNAs actually end up in the circulation remains unclear. However, it has recently become clear that miRNAs are a main component of extracellular vesicles termed exosomes [108], and that the majority of serum miRNAs is concentrated in these exosomes [109]. We have previously observed that the number of surviving hCMPCs after transplantation in a mouse model of MI is too low to explain the improvement in cardiac function [23]. These results suggested that hCMPCs may promote myocardial healing through a paracrine mechanism. The presence of a paracrine effect indicates that the intrinsic regenerative capacity of the heart is stimulated, thereby ascribing an additional beneficial effect to cell transplantation therapy. The high miRNA content of exosomes implies that miRNAs may be the key players in the paracrine mechanism. In support of this notion, a study using pericyte progenitor cells has shown that after transplantation, activation of cardiac repair was mediated by secreted miR-132 [110]. Using miRNA modified exosomes may therefore provide a valid option to improve cardiac function.

Because of their great potential to generate all cell types needed for myocardial regeneration we have focused on the use of hCMPCs. Moreover, we have recently shown that hCMPCs can be used for cardiac tissue engineering purposes, as they retain their viability and functional properties after printing in a tissue graft [111]. However, next to CPCs, a number of other cell types may have the potential to become important contestants in the pursuit of achieving clinically relevant cardiac regeneration. Cardiomyocytes themselves might provide one of these players, as researchers are focusing on re-activating endogenous regenerative mechanisms, such as cardiomyocyte dedifferentiation and proliferation, that exist only early in life. Rat cardiomyocytes have been shown to dedifferentiate and proliferate *in vitro* [112], and growth factors from embryonic cardiac development, such as periostin [113], and neuregulin1 [114], and other growth inducing factors such as oncostatin M [115], can induce subpopulations of cardiomyocytes to reenter the cell cycle *in vitro*. MiRNAs may also have the potential to regulate cell cycle reentry of cardiomyocytes, as miR-133 and the miR-15 family have been shown to regulate mitotic arrest of cardiomyocytes in zebrafish or postnatal

mice, by targeting several cell cycle controlling proteins [116 117]. Another promising source for new cardiomyocytes also comes from the heart itself in the form of cardiac fibroblasts. Remarkable progress has been made in the field of cellular reprogramming, as it was recently shown that cardiac fibroblast could be directly reprogrammed to cardiomyocytes *in vitro* and *in vivo*, with a specific set of factors, without conversion to an intermediate pluripotent state [118-120]. MiRNAs were also shown to be able to directly reprogram cardiac fibroblasts to cardiomyocytes, which is indicative of the power of miRNAs in controlling cell fate and identity [121]. Certainly, major issues concerning the efficiency of conversion remain, but reprogramming the scar-forming fibroblasts into functional cardiomyocytes within the infarcted heart is undeniably very appealing.

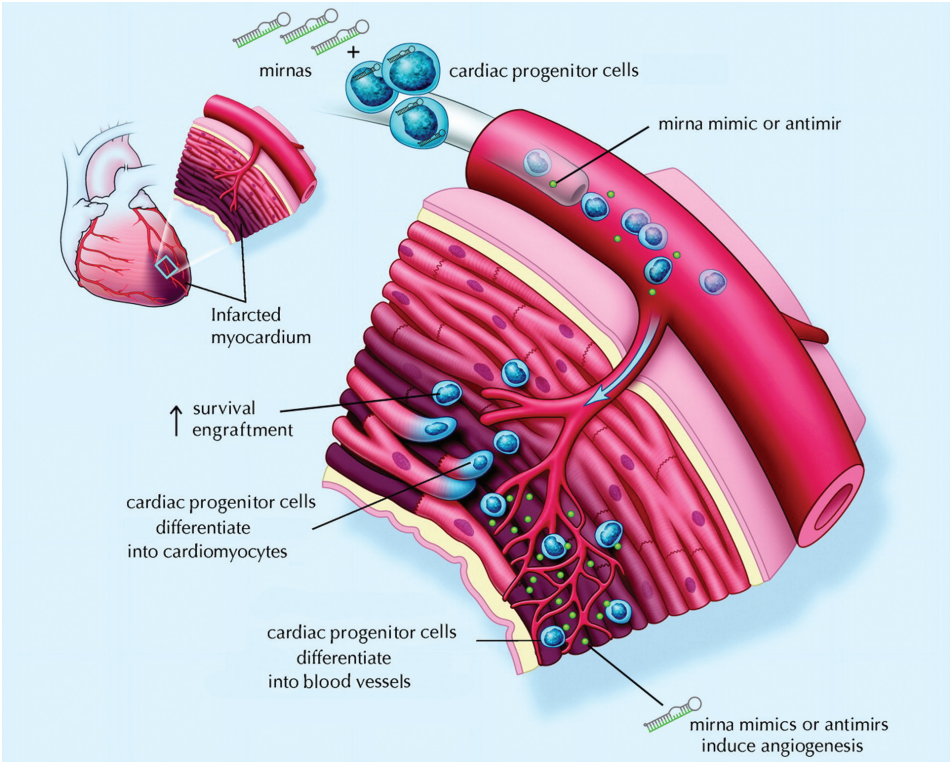


Figure 2
Cardiac regeneration by CPC transplantation and combined miRNA therapy. Adapted from [122]

CONCLUSION

Taken together, this thesis should provide the reader with an overview of an exciting area of biology that is likely to lead to new approaches for the treatment of heart failure. We have provided novel insight in how to improve cardiac regeneration therapies by exploring innovative methods to enhance cardiac progenitor cell differentiation, and to increase the efficiency of cell transplantation therapy by increasing cell survival, engraftment and angiogenesis. Our study results indicate that miRNAs provide novel therapeutic targets to promote cardiac regeneration. However, while significant steps have been made to improve the efficiency of cell transplantation therapy, we still have to figure out ways to produce cells on a clinically relevant scale, find the optimal delivery methods and timing of therapies, and develop strategies to accurately assess the physiological and anatomic changes to evaluate the success of the therapy. Ultimately, we envision a specific miRNA therapy, tailored to the hostile environment of acute MI. This therapy might be composed of an initial treatment that modifies specific miRNA levels directly following MI, followed by transplantation of miRNA modified CPCs. However, before miRNA-based therapeutics may safely enter the clinical arena, a more detailed understanding of the role of miRNAs in the complex biological event of cardiac regeneration is required. Nevertheless, we may conclude that miRNAs represent one of the clues leading to the holy grail of modern-day cardiovascular research; the development of a clinically successful regenerative therapy to halt the progression of ischemic heart disease to advanced heart failure.

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LETTER BY VAN MIL ET AL REGARDING ARTICLE

DYNAMIC MICRORNA EXPRESSION PROGRAMS DURING CARDIAC DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS: ROLE FOR MIR-499

Circ Cardiovasc Genet 2011;4;e3

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With interest, we read the recent article by Wilson et al[1] in Circulation: Cardiovascular Genetics describing the role for miR-499 in dynamic microRNA (miRNA) expression programs during cardiac differentiation of human embryonic stem cells (hESCs). The authors demonstrated that a signature group of miRNAs is present in hESCs, whose expression is significantly altered after cardiomyogenic differentiation. We agree that improved understanding of cardiomyogenic differentiation is important, thereby including miRNA regulation. However, we believe that to understand general miRNA regulation and to interpret in silico prediction analysis, further studies and confirmations are needed, including other available cell sources.

In our previous study, we used undifferentiated human cardiac-derived progenitor cells (hCMPCs) and hCMPCs fully differentiated into cardiomyocytes (hCMPC-CM).[2] We were pleased to read that the authors found several cardiac-related miRNAs, like miR-1, -133, -208, and -499, to be upregulated in hESC-CM (Figure 2f[1]), which supports earlier findings showing that these miRNAs are highly upregulated in hCMPC-CM[2] and in cardiac-differentiated ESCs.[3] Moreover, miR-125, -143/-145, -199a/-214, and -27b (Figure 2d) were also highly increased in hCMPC-CM2, and miR-18a, -19, -20, -25, -663, -92, and -93 (Figure 2e[1]) were significantly decreased in hCMPC-CM.[2] Interestingly, the embryonic-associated miRNAs (Figure 2c[1]) were not observed in our undifferentiated hCMPCs[2], suggesting that these miRNAs are related to an ESC pluripotent state and not expressed in progenitor cells that are predestined, like the hCMPCs. We believe that by comparing different cell sources and the induction of cardiomyogenic differentiation, a more complete understanding and selection of potential interesting miRNAs can be made.

Wilson et al investigated whether predicted targets for miR-1, -208, and -499 were reduced on cardiac differentiation in hESC. Their data show that the target expression is actually higher in beating embryoid bodies and hESC-CM than in hESCs (Figure 3b[1]). Only in fetal heart the target expression is lower than in hESCs. We therefore believe that it is not justified to suggest that target gene expression is gradually reduced during cardiac differentiation. In the authors' discussion, they stated to be surprised by the continued target expression in hESC-CM; however, it is important to understand that these targets are in silico predictions, of which the majority is not validated by reporter gene analysis.

Previously, we reported the cardiac-specific expression of miR-499 and its co-expression with and location within the MYH7B gene.[2] As Wilson et al,[1] we demonstrated that miR-1 and miR-499 overexpression greatly enhanced cardiomyogenic differentiation but, more importantly, that miR-1 or miR-499 inhibition

could completely prevent cardiac differentiation of hCMPCs[2], thereby establishing the indispensable role for miR-1 and miR-499 in cardiomyogenic differentiation. Our group validated SOX6 as a target of miR-499, and by small interfering RNA inhibition of SOX6, we could greatly enhance hCMPC cardiac differentiation.[2] We therefore believe that SOX6 is a crucial myogenic differentiation factor that deserves attention. By in silico prediction analysis, Wilson et al confirmed that the most significant pathway targeted by miR-499 is the Wnt/β-catenin pathway, including SOX6 (Table 11).[1] It would be interesting, therefore, to investigate whether cardiomyogenic differentiation of hESCs is governed by targeting of SOX6 as well, thereby answering important mechanistic questions about cardiac differentiation.

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NEDERLANDSE SAMENVATTING

Elk jaar overlijden wereldwijd miljoenen mensen aan de gevolgen van hart- en vaatziekten. Vooral de gevolgen van een hartinfarct hebben hierin een groot aandeel. Een hartinfarct ontstaat door een afsluiting van één of meer van de bloedvaten die het hart van bloed voorzien (de kransslagaders). Hierdoor krijgt een deel van de hartspier langdurig te weinig bloed en dus ook te weinig zuurstof, waardoor de getroffen hartspiercellen (cardiomyocyten) afsterven. Een hartinfarct wordt vrijwel altijd veroorzaakt door slagaderverkalking (atherosclerose). Wanneer een atherosclerotische plaque in de kransslagader scheurt, zal zich een bloedstolsel vormen welke de bloedstroom door de kransslagader volledig kan blokkeren. Een hartinfarct resulteert in het afsterven van ongeveer een miljard cardiomyocyten, welke worden vervangen door littekenweefsel. Dit stugge litteken voorkomt dat het hart scheurt, maar zorgt er in combinatie met het verminderd aantal cardiomyocyten ook voor dat het hart minder goed kan samentrekken. De hartfunctie gaat hierdoor in de loop van de tijd flink achteruit, waardoor er niet meer voldoende bloed rondgepompt kan worden en de patiënt uiteindelijk kan sterven aan hartfalen.
Het lichaam zelf is niet in staat om de afgestorven cardiomyocyten te vervangen. Helaas zijn de huidige medische therapieën na een hartinfarct ook niet in staat om het fundamentele probleem, het verlies aan hartspier, terug te draaien. Door het gebrek aan een dergelijke therapie is hartfalen uitgegroeid tot een wereldwijde epidemie en is het ontwikkelen van nieuwe regeneratieve therapieën voor het voorkomen van hartfalen van zeer groot belang. Een van de mogelijke therapieën, waar de laatste jaren veel onderzoek naar is gedaan, is stamceltherapie. In 2007 werd door onze onderzoeksgroep een stamcel geïsoleerd uit het foetale en volwassen mensenhart. Deze cellen worden human cardiomyocyte progenitor cells (hCMPCs) genoemd, omdat ze in staat zijn te differentiëren tot functionele cardiomyocyten. Echter, de hCMPCs bleken ook te beschikken over de capaciteit om endotheelcellen en gladde spiercellen te vormen, welke beiden voorkomen in bloedvaten. Ook al is

het hart niet in staat om zich functioneel te herstellen na een infarct, blijkt het toch stamcellen te bevatten met de potentie voor hartregeneratie. Omdat de hCMPCs uit het hart afkomstig zijn en alle voor hartregeneratie belangrijke celtypen kunnen vormen, werd onderzocht of deze veelbelovende cellen gebruikt konden worden voor cel transplantatie therapie om het beschadigde hart te herstellen. Uit deze en andere studies met hart progenitor cellen bleek dat de vermindering van de hartfunctie kon worden voorkomen. Er werd echter maar weinig nieuw hartweefsel gevormd en van volledig herstel was geen sprake. Een mogelijke verklaring hiervoor is dat maar zeer weinig van de getransplanteerde cellen konden worden teruggevonden in het hart. Dit lijkt het gevolg te zijn van meerdere factoren, zoals het slecht overleven van de getransplanteerde cellen in het schadelijke milieu van het beschadigde weefsel, het migreren van de cellen naar onbeschadigde plekken in het hart en daarbuiten, de inefficiënte differentiatie naar de verschillende benodigde hartcellen en de daaraan gekoppelde integratie in het te herstellen hartweefsel. De effectiviteit van celtherapie en regeneratie van het hart kan daarom aanzienlijk verbeterd worden wanneer er nieuwe strategieën ontwikkeld worden om deze obstakels te overbruggen.

De afgelopen jaren is gebleken dat zogenaamde microRNAs (miRNAs) een cruciale rol spelen in de ontwikkeling van het hart en ook in hartziekten (hoofdstuk 1). MiRNAs zijn kleine stukjes RNA (qua chemische structuur vergelijkbaar met DNA), die niet coderen voor een eiwit, maar wel de expressie van genen beïnvloeden. De mens heeft ongeveer 20.000 genen die coderen voor eiwitten, welke via een tussenstap van messenger RNA worden samengesteld uit aminozuren. Naast deze circa 20.000 stukken coderend DNA heeft de mens nog véél meer DNA in het genoom waarvan lang werd gedacht dat het geen functie vervulde omdat het niet codeert voor eiwit. We weten nu dat een groot deel hiervan wel wordt vertaald in stukjes RNA. Dit RNA leidt echter niet tot aanmaak van eiwitten maar vervuld zelf een rol in de cel. MiRNAs vallen onder deze klasse van niet-coderend RNA. MiRNAs kunnen de expressie van messenger RNAs beïnvloeden door te interfereren met hun translatie naar eiwit (hoofdstuk 1) en bepalen zo het verloop van allerlei processen in de cel.

Recent onderzoek heeft aangetoond dat miRNAs ook belangrijke functies vervullen in stamcellen en dat ze een rol spelen in de verschillende herstelprocessen na een hartinfarct (hoofdstuk 1). Het doel van het onderzoek dat beschreven is in dit proefschrift is dan ook om nieuwe miRNAs te vinden welke celtherapie kunnen verbeteren en daardoor de regeneratie van het hart na een hartinfarct kunnen bevorderen. Vanwege de capaciteit om alle celtypen te kunnen vormen welke nodig zijn voor het herstel van het hart en de hoopgevende resultaten met hCMPC transplantatie hebben we het merendeel van onze studies uitgevoerd met deze cardiale progenitor cellen. We hebben onderzocht of de differentiatie, overleving en migratie

van hCMPCs kon worden gemanipuleerd door middel van specifieke miRNA modulatie. Daarnaast werd miRNA modulatie gebruikt voor het bevorderen van nieuwe vaatvorming in het lichaam (angiogenese), een belangrijk regeneratief proces dat nodig is voor de overleving en innesteling van getransplanteerde cellen.

Voor volledig functioneel herstel van het beschadigde hartspierweefsel na een infarct zijn grote aantallen nieuwe cardiomyocyten nodig. De differentiatie van hCMPCs in cardiomyocyten verloopt echter niet gemakkelijk. Daarom is in hoofdstuk 2 onderzocht of miRNAs betrokken zijn bij de differentiatie van hCMPCs tot cardiomyocyten en of dit proces verbeterd kan worden door middel van specifieke miRNA modulatie. We laten zien dat miRNA-1 (miR-1) en miR-499 onmisbaar zijn voor de differentiatie van hCMPCs tot cardiomyocyten en dat na het toevoegen van deze twee miRNAs aan hCMPCs de vorming van cardiomyocyten aanzienlijk sneller en efficiënter verloopt. We tonen aan dat dit gebeurt via de repressie van Sox6, een eiwit dat de expressie onderdrukt van verschillende genen welke vereist zijn voor de ontwikkeling van spierweefsel. Zowel miR-1 als miR-499 zijn sterke positieve regulatoren van cardiomyogene differentiatie en vormen zo functionele targets voor het bevorderen van de regeneratie van het hart.

Het regenereren van hartweefsel betekent ook dat er nieuwe bloedvaten gevormd moeten worden zodat er zuurstof en voedingstoffen naar de cardiomyocyten vervoerd kunnen worden. In hoofdstuk 3 is daarom onderzocht of miRNAs ook de angiogene differentiatie van hCMPCs kunnen stimuleren. Door het kweken van hCMPCs op een soort extracellulaire matrix vormen deze uit zichzelf vaatachtige structuren bestaande uit endotheel en gladde spiercellen. Tijdens dit proces neemt de hoeveelheid miR-1 in de cellen toe. Het toevoegen van miR-1 aan hCMPCs tijdens dit proces zorgt voor de vorming van meer ontwikkelde vaatachtige structuren. Zowel de vorming van endotheel als gladde spiercellen wordt gestimuleerd door miR-1. We laten zien dat miR-1 Spred1 target, een eiwit dat het effect van verschillende pro-angiogene groeifactoren blokkeert. MiR-1 lijkt dus ook geschikt om de vorming van nieuwe bloedvaten te stimuleren en zou zo kunnen bijdragen aan een verbeterd herstel na hartschade.

Een groot probleem bij cel transplantatie is dat het merendeel van de getransplanteerde cellen niet weet te overleven in het ischemische gebied van het infarct. Een mogelijke oplossing hiervoor is om de cellen hierop voor te bereiden, zodat ze niet dood gaan door apoptose of necrose, twee vormen van celdood. In hoofdstuk 4 beschrijven we de rol van een specifieke miRNA in het tegengaan van celdood in hCMPCs. Eerdere

studies hebben aangetoond dat miR-155 een rol speelt in het reduceren van apoptose. In hCMPCs die blootgesteld worden aan oxidatieve stress, waarmee we het ischemische milieu van het infarct nabootsen, blijkt miR-155 aanzienlijk toe te nemen. Door het toevoegen van miR-155 aan de hCMPCs wordt echter niet apoptose, maar alleen necrose geremd, wat verreweg de voornaamste vorm van celdood is in hCMPCs. Dit effect wordt gemedieerd door directe remming van RIP1, een eiwit dat vereist is voor niet-apoptotisch celdood, waardoor de overleving van hCMPCs onder oxidatieve stress sterk verbeterd.

Naast het tegengaan van celdood is het ook van belang om de getransplanteerde cellen op de vooraf gedefinieerde injectieplekken te houden. Het tegengaan van ongewenste celmigratie kan de integratie van de getransplanteerde cellen in het te herstellen hartweefsel bevorderen. Door het toevoegen van miR-155 aan hCMPCs in hoofdstuk 4 merkten we op dat naast celdood ook de motiliteit van de hCMPCs geremd leek. In hoofdstuk 5 laten we zien dat een toename van miR-155 resulteert in een vermindering van celmigratie, welke veroorzaakt wordt door een afname in de activiteit van MMP2 en MMP9, twee enzymen die celmigratie mogelijk maken. Deze twee enzymen worden niet direct door miR-155 getarget, maar miR-155 target wel een andere MMP, MMP16, een activator van MMP2 en 9. Door middel van directe remming van MMP16, remt miR-155 dus indirect de activatie van MMP2 en 9 waardoor de celmigratie wordt beperkt. Naast het remmen van celdood, remt miR-155 ook celmigratie waardoor deze miRNA mogelijk zeer geschikt is voor het verbeteren van celtherapie.

Het moduleren van specifieke miRNAs in cellen buiten het lichaam is relatief eenvoudig, waardoor we alle mogelijkheid hebben om hCMPCs voor te behandelen alvorens we ze transplanteren in het hart. Echter het moduleren van miRNAs in het lichaam (in vivo) en in het hart zelf is gecompliceerder. In hoofdstuk 6 beschrijven we de verschillende tot nu toe onderzochte technieken voor het therapeutisch gebruik van kleine regulerende RNAs in vivo. De momenteel beschikbare technieken zorgen voor een grote stabiliteit en cel-permeabiliteit, en de affiniteit en target-specificiteit is alleen maar beter geworden. Hierdoor kunnen miRNAs momenteel al op verschillende manieren met succes in vivo worden gemanipuleerd. Echter, de ontwikkeling van op miRNA gebaseerde geneesmiddelen wordt nog belemmerd door het gebrek aan technieken om specifieke weefsels en cellen te bereiken. Gelukkig is er een enorme belangstelling voor het gebruik van miRNAs voor therapeutische doeleinden, en richt veel onderzoek zich op het ontwikkelen van dit soort technieken.

In hoofdstuk 7 hebben we gebruik gemaakt van een techniek waarmee we miRNAs in vivo kunnen inhiberen. Voor succesvolle regeneratie van de beschadigde hartspier is het van essentieel belang om de bloedtoevoer te herstellen. De vorming van nieuwe bloedvaten na een hartinfarct heeft een dubbel positief effect; zowel de overleving van het hartweefsel als van de getransplanteerde cellen wordt bevorderd. In hoofdstuk 7 beschrijven we de rol van een specifieke miRNA in het reguleren van angiogenese. We tonen aan dat miR-214 een remmer is van angiogenese in vivo in verschillende weefsels. Door miR-214 in vivo te inhiberen met een specifieke miR-214 inhibitor, wordt angiogenese gestimuleerd. De anti-angiogene rol van miR-214 wordt gemedieerd door directe inhibitie van QKI, een RNA-bindend eiwit met een essentiële rol in de ontwikkeling van bloedvaten. De afname van QKI zorgt voor een daling in de expressie en secretie van verschillende pro-angiogene groeifactoren, welke het anti-angiogene effect van miR-214 verklaart.

Bij elkaar genomen toont deze studie aan dat miRNAs in staat zijn om verschillende aspecten te reguleren die van groot belang zijn voor het succes van celtherapie en de regeneratie van het hart. Echter, vóórdat op miRNA gebaseerde therapieën veilig gebruikt kunnen worden in de kliniek is een meer gedetailleerde kennis van de rol van deze kleine krachtige regulatoren in de complexe biologie van hartregeneratie noodzakelijk.

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CURRICULUM VITAE

Alain van Mil was born on June 11, 1980, in Zeist, the Netherlands. After completing his secondary education in 1999 at the Maurick College in Vught, he studied Biology at Utrecht University for three years after which he specialized in molecular biology at the University of Applied Sciences Utrecht, where he obtained his bachelor's degree in Biology & Medical Laboratory Research. He performed two internships during his bachelor studies: the first was conducted at the Hubrecht Institute where he worked on identifying novel gene interactions in Wnt signaling; for his second internship he studied HLA class I mutations in leukemia at the department of Pathology of the University Medical Center Utrecht. During these internships he became interested in disease-related molecular mechanisms. In 2006 he enrolled in the master's program 'Biology of Disease' at Utrecht University. During his master, he completed his first internship at the department of Cardiology of the University Medical Center in Utrecht, exploring the role of microRNAs in cardiac progenitor cell differentiation. He performed a second internship at the department of Farm Animal Health at the Faculty of Veterinary Medicine, where he worked on inducing pluripotency in unipotent stem cells. After graduating cum laude in 2008, Alain went back to the department of Cardiology where he started his PhD on the role of microRNAs in cardiac regenerative medicine, which resulted in the present thesis. Since September 2012, Alain is working as a post-doc at the department of Cardiology at the University Medical Center in Utrecht. Recently he received an ICIN Fellowship, which provides him with the opportunity to go abroad and gain further knowledge in therapeutic heart regeneration in the Mercola Laboratory of the Sanford Burnham Medical Research Institute.