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The Therapeutic Potential of miRNAs in Cardiac Fibrosis: Where Do We Stand?

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Abstract Recent developments in basic and clinical science have turned the spotlight to miRNAs for their potential therapeutic efficacy. Since their discovery in 1993, it has become clear that miRNAs act as posttranscriptional regulators of protein expression. Their clinical potential was further highlighted by the results of miRNA-based interventions in small laboratory animals. More importantly, their therapeutic effectiveness has been shown recently in phase 2a clinical studies in patients with hepatitis C virus infection, where inhibition of miRNA-122 showed prolonged and dose-dependent viral suppression. A recent study surprisingly revealed the presence of plant-derived miRNAs in the blood of healthy humans. This finding opens up the possibility to explore miRNA-mediated therapeutics derived from (genetically modified) food. Having arrived at this point in our understanding of miRNAs, we provide an overview of current evidence and future potential of miRNA-based therapeutics, focusing on their application in cardiac fibrosis

Keywords miRNA · Fibrosis · Heart · Therapy

Introduction

Heart function depends on cardiac contraction and relaxation, which is determined both by systolic and diastolic cardiomyocyte function and by extracellular matrix (ECM) elasticity. The ECM consists of collagens, elastins, fibronectins, and basement membranes, providing both the structural backbone as well as a network for contractile force transmission [1–3]. Unlike a static framework, the ECM undergoes constant turnover through degradation and synthesis of its constituent proteins [4]. Matrix metalloproteases (MMPs) and tissue inhibitors of MMPs (TIMPs) balance the degradation of ECM components. Synthesis is facilitated by fibroblasts that produce and secrete the bulk of ECM components, a process that can be stimulated through the paracrine action of extracellular growth factors such as TGFβ and connective tissue growth factor (CTGF) [1, 3, 5]. These factors bind to cell-surface receptors and activate an array of intracellular signaling cascades, of which the ERK-MAP-kinase, Ca^{2+}-dependent signaling, and SMAD pathways are among the major ones [5]. These signaling cascades eventually converge on a limited set of transcription factors comprising SMADs, AP-1, NFkB, EGR-1, and STATs that induce the expression of the pro-fibrotic genes encoding collagens, fibronectin, and elastin [6, 7]. The secretion of the growth factors TGFβ and CTGF, both by fibroblasts and cardiac myocytes increases in response to different forms of cardiac dysfunction [5].

Whereas normal ECM turnover represents a physiological process to maintain cardiac homeostasis, excessive ECM deposition, known as cardiac fibrosis, interferes with conduction and has been shown to increase myocardial stiffness. Excessive ECM deposition is observed upon pressure overload, cardiomyocyte loss due to ischemia and during normal aging [1]. The causes of cardiac dysfunction are thus diverse, but can be classified as either external like chronic hypertension and aortic stenosis or intrinsic such as mutations in structural or metabolic genes. Excessive cardiac cell death as a consequence of myocardial infarction also represents a key factor in the development of cardiac dysfunction and hence cardiac fibrosis. This creates a particular situation in which ECM deposition on the one hand serves a biological role in damage containment, while on the other hand it impairs cardiac function, induces arrhythmia, and aggravates

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the progression towards heart failure. Limiting excessive ECM deposition therefore represents an interesting therapeutic strategy to prevent fibrosis and maintain cardiac function in heart disease.

MiRNAs

MiRNAs constitute a class of ~22-basepair-long RNA molecules that inhibit protein expression via either degradation of mRNA or interference with mRNA translation. Unlike general repressors of protein expression, miRNAs target specific mRNAs through complementary basepairing, predominantly within the 3′-UTR of a target mRNA [8, 9]. Whereas the miRNA provides target specificity, the actual mRNA degradation or translational repression is induced by components of the RNA-induced silencing complex (RISC), such as proteins of the argonaute family and Tnrc6 [10].

More than 60 % of the protein coding genes contain predicted miRNA binding sites [11, 12], indicative for miRNA regulation of almost every cellular process. As it turns out miRNAs can target multiple proteins within a biological pathway, thereby potentiating their final effect on this specific cellular process [13]. The cell- and tissue-restricted expression of many miRNAs and their mRNA targets highlights the specificity of these interactions. The observation that miRNAs are differentially expressed during disease provides additional evidence for their importance in cellular function, as do the many findings where interference with normal miRNA expression results in disease. These will be discussed later in this review with a focus on cardiac fibrosis.

Oligonucleotide-Based miRNA Therapeutics

MiRNA mimics and antisense-based technologies now provide the opportunity to study the possible causal role of miRNAs in disease aetiology. If so, the ability to alter miRNA levels in vivo has great potential for the development of miRNA-based therapeutic interventions. MiRNA levels can be modified artificially through administration of exogenous miRNA mimics and antimiRs. MiRNA mimics are double-stranded duplexes similar to precursor miRNAs. They provide a useful tool that functions well in vitro but unfortunately has some limitations regarding in vivo application. The drawbacks mainly result from the limited capacity of cells to take up double-stranded RNA, and the need to incorporate the proper precursor strand of the duplex into the RISC to achieve functional activity [14]. The need for RISC incorporation also increases the likelihood of side effects via interference with normal miRNA processing. This is clearly illustrated by the observation that RISC complexes from miRNA overexpressing transgenic mouse models become saturated with the overexpressed miRNA [15].

Downregulation of miRNAs can be achieved more conveniently by using antimiRs, an approach that has been clearly reviewed elsewhere [16]. In brief, these short complementary sequences can bind to the miRNA, thereby competing for interaction with endogenous targets and hence interfering with normal miRNA function. In order to be therapeutically applicable antimiRs need protection against nucleases that are present in the circulation, entry into target cells and a high binding affinity for the endogenous miRNA. Many of these factors can be conferred through chemical modification of the antimiR. Phosphorothioate modification of the sugar-phosphate backbone protects against nuclease degradation and facilitates plasma protein binding, thereby also reducing its renal clearance [16]. 2-*O*-methyl cholesterol-conjugated antimiRs, known as antagomiRs, show increased stability and enhanced cellular uptake, with a preference towards the liver [17]. Chemical modifications of the ribose sugar like 2′-*O*-methyl (2′-*O*-Me), 2′-*O*-methoxyethyl (2′-MOE), 2′-fluoro (2′-F), and locked nucleic acid (LNA), provide nuclease protection and higher affinity for target mRNAs through increased duplex melting temperature [18]. This increased melting temperature also allows the length of the antimiR sequences to be shortened. That subsequently resulted in the development of tiny 8-mer LNA-based antimiRs that target the seed region and therefore confer the ability to downregulate whole miRNA families. Although this family targeting might provide stronger effects on biological pathways, it also creates a higher risk of side effects due to the broader expression pattern of an entire miRNA family compared to its individual members. Therefore, to obtain the most specific results for a given miRNA, we believe that the full-length antimiR has the highest therapeutic potential. Important with regards to off-target effects are in vitro luciferase experiments showing that translation of mRNA transcripts containing complementary sequences to the tiny antimiR is not affected by antimiR treatment [19]. Most of the approaches with miRNA-mimics and antimiRs have already been successfully applied in vitro and in small laboratory animals. They have proven valuable tools for the study of miRNA function in the heart, and cardiac fibrosis in particular.

MiRNAs in Cardiac Fibrosis

Several miRNAs have been identified as regulators of cardiac fibrosis, and some of them even target multiple proteins in fibrotic pathways. In-depth reviews of the evidence for miRNA involvement in cardiac fibrosis are provided elsewhere [5, 20, 21]. For the purpose of this review, we focus on describing only those miRNAs that affect cardiac fibrosis in vivo. Table 1 summarizes the effects of miRNA-based interventions on cardiac fibrosis. The distinction between direct and indirect effects differentiates between miRNAs

that affect the fibrotic response directly by targeting ECM components or fibrotic signaling in the fibroblast, and miRNAs that induce fibrosis secondary due to cardiac dysfunction. The direct targeting likely offers the highest therapeutic potential for treatment of cardiac fibrosis.

MiRNA-21

The discovery of the role of miRNA-21 in cardiac fibrosis is one of the most interesting examples regarding the potential of miRNA-based therapeutics. MiRNA-21 is ubiquitously expressed in many tissues as kidney, liver, lung, and the heart. Within the heart, it is expressed mainly in cardiac fibroblasts and at relatively low levels in cardiomyocytes [22]. MiRNA-21 was found to be strongly upregulated in the diseased, fibrotic myocardium. Inhibition of its effects through the use of antagomiRs was shown to prevent the development of cardiac fibrosis in β_1-adrenergic receptor transgenic mice. SPRY1 was put forward as the responsible miRNA-21 target that normally represses ERK/MAP kinase signaling. During pathological conditions, miRNA-21 becomes upregulated and further represses SPRY, thereby activating the ERK/MAP kinase signaling that leads to increased fibrosis combined with decreased levels of apoptosis. Moreover, antagomiR-21 treatment was found to be sufficient to block all these effects in vivo [22].

The precise role of miRNA-21 in cardiac fibrosis gets complicated when considering other findings where miRNA-21 knockout mice display a normal hypertrophic and fibrotic response upon stimulation by TAC, chronic calcineurin activation, AngII infusion, and myocardial infarction. Additionally, there was no effect of LNA-based tiny antimiR-21 treatment on cardiac fibrosis both upon TAC and AngII infusion [23]. Subsequent follow-up studies have however revealed the importance of antimiR chemistry, as the tiny LNAs are quickly cleared by renal filtration. Recent findings also underline the importance of timing and the exact model system that is used for antimiR experiments [24]. Other groups have further substantiated the evidence of miRNA-21's role in cardiac fibrosis, both in vivo and in vitro [25–27]. In this regard, the finding that miRNA-21 regulates MMP2 via a PTEN-dependent pathway supports the notion of miRNA-21 as a key player in the fibrotic response [28]. Combining these findings with the observation that miRNA-21 plays a role in kidney fibrosis [29] it can be concluded that this miRNA regulates fibrosis, although the effect of different model systems has to be further explored.

MiRNA-29

MiRNA-29 is highly expressed in lung, kidney, heart, and many other tissues [13]. Target prediction analysis revealed a remarkably large number of fibrosis-related genes for the miRNA-29 family (29a, 29b, and 29c), of which collagens (COL1A1, COL1A2, and COL3A1), fibrillin-1 (FBN1), and elastin (ELN1) were identified as direct targets by in vitro luciferase assays. The targeting of so many ECM components by the miRNA-29 family underlines the potential of miRNAs to target entire cellular processes. Furthermore, this study shows that in vivo treatment of mice with cholesterol-based antimiR-29b resulted in increased collagen expression in liver and to a lesser extent the heart, an effect probably due to hepatic antimiR sequestration and hence predominant

Table 1 Overview of in vivo effects on cardiac fibrosis by miRNA-based interventions

miRNA	Intervention	Effect	Target(s)	Direct/indirect[a]
miRNA-21	AntagomiR-21 (systemic) [22]	Prevents fibrosis	SPRY1	Direct
	miRNA-21 knockout [23]	Does not prevent fibrosis	–	–
	Tiny-LNA-antimiR-21 (systemic) [23]	Does not prevent fibrosis	–	–
miRNA-29	AntagomiR-29b (systemic) [13]	Increased cardiac collagen expression	COL1A1, COL1A2, COL3A1, FBN1, ELN1	Direct
miRNA-24	AntagomiR-24 [34]	Improved cardiac function after MI	GATA2, PAK4	Indirect
	miRNA-24 over-expression (lentiviral) [33]	Decreased ECM gene expression	Furin	Direct/indirect
	miRNA-24 mimic (local delivery) [35]	Improved cardiac function	Bim	Indirect
miRNA-22	miRNA-22 knockout [38]	Increased isoproterenol-induced fibrosis	PPARa, Sirt1, HDAC4	Indirect
miRNA-133	miRNA-133a transgene [40]	Protected against TAC-induced fibrosis	CTGF?	Direct?
	miRNA-133a-1/2 double knockout [42]	Extensive cardiac fibrosis	Cyclin D2, SRF	Indirect
	AntagomiR-133 [41]	Cardiomyocyte hypertrophy	RhoA, Cdc42, Nelf-A	Indirect?
miRNA-101	Adenoviral over-expression [44]	Decreased MI-induced fibrosis	c-Fos	Direct
miRNA-206	HMGB1-induced miRNA-206 over-expression [48]	Decreased MI-induced fibrosis	TIMP3	Direct?
miRNA-132	AntimiR-132 pericyte transplantation [49]	Decreased fibrosis	Ras-GAP, MCBP2	Indirect
miRNA-214	miRNA-214 knockout [50]	Increased MI-induced fibrosis	NCX1, Ppif, CamkIId	Indirect
miRNA-1	miRNA-1 AAV9 over-experssion [51]	Decreased TAC-induced fibrosis	NCX1, Fbln2	Direct

[a] Direct and indirect differentiates between miRNAs that affect the fibrotic response directly by targeting ECM components or fibrotic signaling in the fibroblast, and miRNAs that induce fibrosis secondary due to cardiac dysfunction

inhibition of liver mRNA transcripts. Unfortunately, this study did not investigate the levels of cardiac fibrosis on histological sections after in vivo antimiR-29 treatment of mice. Additional supporting evidence is provided by in vitro studies on cultured cardiac fibroblasts which show a modest decrease on collagen expression upon treatment with miRNA-29 mimics [13]. Studies in kidney and lung confirmed this correlation between miRNA-29 expression and fibrosis [30, 31]. More direct in vivo evidence is provided by the observation that overexpression of miRNA-29 via transposon-mediated transfer decreased the amount of bleomycin-induced pulmonary fibrosis in mice [31]. These studies also confirmed the repressive effects on expression of collagens and fibronectin. Promising as this may be, caution should be taken to use miRNA-29 mimics as therapy for cardiac fibrosis since other studies observed increased cardiomyocyte apoptosis upon miRNA-29 treatment, a highly undesirable side effect [32].

MiRNA-24

Initially identified as being downregulated in a mouse model of myocardial infarction, the role of miRNA-24 in cardiac fibrosis is less well characterized. Cardiac miRNA-24 was found to be expressed in both endothelial cells, fibroblasts, and cardiomyocytes, although not all reports agree on this distribution [33–35]. Inhibition of miRNA-24 by antagomiRs in mice has been reported to improve cardiac function after MI. It improves capillary network formation and inhibits endothelial cell apoptosis via increased expression of the endothelial transcription factor GATA2 and the serine/threonine kinase PAK4 that induces JNK- and MAP-signaling in endothelial cell [34]. Contrastingly, Wang et al. found that cardiac function after MI also improved after intramyocardial lentiviral miRNA-24 overexpression [33]. This improvement coincided with decreased collagen and fibronectin expression in addition to lower α-SMA (a myofibroblast marker) levels. In vitro work confirmed the correlation between miRNA-24 levels and collagen expression and identified furin as the fibrosis-regulating miRNA-24 target [33]. Furin is a Ca^{2+}-dependent protease that is involved in the processing of TGF-β1 and MMP1. Both the lentiviral and the antagomiR approaches are however not specific for any cardiac cell type and it is therefore difficult to distinguish effects on cardiomyocytes, fibroblasts, and endothelial cells. Yet another study found a role for miRNA-24 in cardiomyocyte apoptosis, where local mimic-delivery improved cardiac function [35].

With these contrasting findings it becomes difficult to pinpoint the exact role of miRNA-24 in cardiac fibrosis. This gets even further complicated by the fact that miRNA-24 is not only expressed in the heart but plays a role in the progression of cancer and keratinocyte differentiation [36, 37]. While the in vivo lentiviral and antagomiR findings look promising, the identification of furin is solely based on correlating expression analysis with miRNA-24 and collagens. The mechanistic explanation remains therefore far from conclusive and requires further investigation.

MiRNA-22

A recent report describes studies which show that isoproterenol-induced cardiac fibrosis is exacerbated in miRNA-22 null mice compared to wildtype littermates [38]. Since miRNA-22 expression is not restricted to the heart, further studies were performed in conditional cardiac miRNA-22 knockout mice. These studies confirmed the initial finding in the full knockout that loss of cardiac miRNA-22 results in the accelerated development of fibrosis upon isoproterenol stimulation. Luciferase assays identified PPARα, Sirt1, and HDAC4 as direct targets of miRNA-22. Additional experiments indicated Sirt1 and HDAC4 as the main effectors on cardiac hypertrophy, thereby making the development of cardiac fibrosis likely secondary to general cardiac dysfunction [38].

MiRNA-133

The role of the muscle-specific miRNA-133 in cardiac fibrosis was initially established by in vitro experiments that identified CTGF as a direct target [39]. in vivo studies with αMHC miRNA-133a transgenic mice subsequently showed that the transgenes are protected against TAC-induced cardiac fibrosis. Besides this protective effect, cardiomyocyte apoptosis was also inhibited in the transgene [40], while in vivo antagomiR-133 treatment induced cardiomyocyte hypertrophy [41]. Furthermore, RNA-immunoprecipitations confirmed that CTGF mRNA directly binds to miRNA-133a loaded RISC complexes in the same transgenic mouse model [15]. Double knockout mice of miRNA-133a-1 and miRNA-133a-2 show dilated cardiomyopathy with extensive cardiac fibrosis, whereas the individual deletion of one miRNA-133 isoform does not lead to a phenotype. The effect of knocking out all miRNA-133 isoforms seems however to be twofold, as half of the mice die as neonates due to ventricular-septal defects and the surviving ones develop a dilated cardiomyopathy that progresses towards heart failure. This phenotype can be partially explained by increased cell proliferation and hypertrophic growth due to the lack of repression by miRNA-133 on the cyclin D2 and SRF respectively [42]. Unfortunately this study provides no information on CTGF expression, which might be partly responsible for the observed fibrotic response.

Evidence for the direct targeting of fibrosis by miRNA-133a is provided by the identification of COL1A1 as a direct target by luciferase assays. Subsequent in vivo studies in AngII treated rats show that miRNA-133a expression

correlates with COL1A1 expression and the level of cardiac fibrosis [43].

MiRNA-101

Acute myocardial infarction in rats leads to downregulation of miRNA-101a and -101b in the infarct zone, an area that subsequently develops massive fibrosis. In vitro treatment of cardiac fibroblasts with miRNA-101 mimics was found to inhibit AngII induced proliferation and expression of collagens, fibronectin, MMP2, and MMP9. C-fos was identified as a miR-101 target that mediates the proliferative effects through the induction of TGF-β expression. Moreover, adenoviral overexpression of miRNA-101a in vivo decreased the amount of MI-induced fibrosis, as well as mRNA levels of collagens, MMP2 and MMP9 [44]. The observed downregulation of miRNA-101 during myocardial infarction might therefore be causal in the development of cardiac fibrosis. MiRNA-101 is however widely expressed in other tissues than the heart, ranging from lung to a wide range of carcinomas [45, 46].

MiRNA-206

Another miRNA implicated in cardiac fibrosis is miRNA-206, a muscle-specific miRNA involved in skeletal muscle differentiation [47]. This miRNA is upregulated upon high mobility group box 1 protein (HMGB1) treatment of a murine MI model. HMGB1 acts as chromatin-modifying factor that allows transcriptional complexes to access the DNA and has been found to promote tissue repair and cardiac regeneration after myocardial infarction. Luciferase assays show direct miRNA-206 targeting of TIMP3, an inhibitor of MMPs. Increased miRNA-206 levels would therefore be expected to increase MMP activity and thereby decrease the amount of fibrosis. The study fails to show a direct effect in vivo, but HMGB1 treatment correlates with increased cardiac miRNA-206 expression and decreased cardiac fibrosis in the MI model [48]. Although the mechanism of action by which HMGB1 increases miRNA-206 expression remains unclear, the miRNA-206 effect on TIMP3 seems convincing.

MiRNA-132

Pericytes in the vasculature play a role in angiogenesis and show high expression levels of miRNA-132. The paracrine secretion of miRNA-132 by vascular pericytes transplanted from the saphenous vein to the heart was found to improve cardiac function in a mouse model of myocardial infarction [49]. In vitro experiments revealed increased endothelial tube formation and decreased myofibroblast differentiation upon culture in pericyte-conditioned, miRNA-132 enriched medium. The effects are likely conferred via Ras-GTPase activating protein and methyl-CpG-binding protein 2. Regarding cardiac fibrosis, it was found that in vivo inhibition of miRNA-132 via transfection of transplanted pericytes with antimiR-132 decreased the capacity of these pericytes to improve cardiac contractility, angiogenesis, and interstitial fibrosis [49]

MiRNA-214

MiRNA-214 is expressed in the heart, but it has much higher expression in lung and is also present in kidney, skeletal muscle, and intestine. Deletion of miRNA-214 in mice revealed no abnormalities in cardiac function and morphology at baseline. Myocardial infarction through a permanent LAD ligation showed increased mortality of miRNA-214 knockout mice. Transient ligation of the LAD however revealed increased fibrosis, possibly secondary to increases in cardiomyocyte apoptosis. Slc8a1 (NCX1) was identified through luciferase assays as a direct miRNA-214 target, probably causing the phenotype via impaired Ca^{2+}-handling. Additionally, Ppif and CamkIId, both regulators of Ca^{2+}-homeostasis, were also identified as direct targets that were regulated in vivo at the protein level [50].

MiRNA-1

The muscle-specific miRNA-1 has been shown to play a key role in the development TAC-induced heart failure and cardiac remodeling. AAV9-mediated overexpression of miRNA-1 under control of the CMV promoter was able to decrease cardiac hypertrophy and improve cardiac function. The levels of cardiac fibrosis were also strongly reduced in this model. The study confirmed direct targeting of NCX1 and additionally identified fibulin2 (Fbln2) as a new direct target of miRNA-1 [51]. Fbln2 is a secreted protein involved in ECM remodeling, and loss of Fbln2 was found to attenuate the progression of remodeling after MI [52].

MiRNAs as Therapeutic Targets

Given the number of miRNAs involved in cardiac fibrosis it becomes interesting to investigate their potential as therapeutic targets in humans. As evident from small animal studies, knockdown of miRNAs proves both a feasible and effective approach to decrease fibrosis and improve cardiac function [13, 22, 33, 38, 40, 44, 50]. Recent studies using LNA-based antimiRs have shown that these compounds are also functional and well-tolerated in primates and humans. AntimiR-122 has recently entered phase 2 clinical trials for treatment of hepatitis C infections and thereby provides the

first example of the successful path from discovery to therapeutic application in humans.

MiRNA-122 and Hepatitis C

The liver-specific miRNA-122 was found to regulate propagation of the hepatitis C virus (HCV) in the liver. HCV consists of a single RNA molecule that gets fully translated into one long polypeptide and subsequently cleaved into functional viral proteins. In contrast to the conventional function in mRNA degradation or translation inhibition, MiRNA-122 binds to the 5′-UTR of the virus and prevents viral degradation and activation of the host immune response [53]. Therapeutic silencing of miRNA-122 is thus expected to decrease virus stability and improve the host immune response. Initial experiments using LNA-based antimiR-122 (miravirsen, Santaris) on chimpanzees with a chronic HCV infection showed viral suppression [54], while subsequent phase 1 studies in healthy volunteers revealed no adverse effects of antimiRs. Phase 2a trials with HCV patients show the efficacy of antimiR-based therapy since viral RNA levels are decreased in a dose-dependent manner, while no adverse side effects were observed. Additionally, the viral genome did not show any signs of selective pressure to change the sequence of the miRNA-122 sites during the 14 week follow-up after treatment [55]. The antimiR-122 approach is thereby approaching real-life clinical application.

The anti-viral effects of antimiR-122 are likely to be independent of its effects on the biological function of miRNA-122 on liver metabolism and cholesterol homeostasis [56]. The observation of decreased cholesterol levels upon miravirsen treatment supports the hypothesis that endogenous biological processes can be regulated by miRNA-based since miRNA-122 targets several genes involved in cholesterol homeostasis. This effect on an endogenous biological pathway might actually represent the truly interesting finding with regards to future clinical applications for antimiRs in general.

With regard to side effects of antimiR treatment, miravirsen treated patients showed a reduction of aminotransferase levels in the blood [55], which might be counter-indicative for the presence of liver damage reported in previous studies with phosphorothioate-based antimiRs More concerning is the finding that miRNA-122 knockout mice exhibit increased progression of hepatic carcinoma [57]. This potential side effect has not been observed in the clinical studies, although the 14 week follow-up period is too short for carcinomas to develop.

Targeting Strategies

For any therapy to be effective, it has to target the proper tissue and cell type. One advantage that contributes largely to the success of antimiR-122 is the confined expression of this miRNA to the liver. Systemic knockdown is therefore less likely to cause side effects, a factor that certainly contributes to the success of miravirsen. Not every miRNA has however such a specific expression pattern, as illustrated by most of the miRNAs involved in cardiac fibrosis. Directed targeting strategies for specific cells and tissues could therefore greatly improve the potential of miRNA-based therapeutics. Many ingenious approaches are being developed in experimental small laboratory animals, and although human data remains scarce it is worthwhile to discuss the most promising targeting and delivery strategies.

The application of adeno-associated-virus (AAV) mediated genetic transfer in small rodent animals and sheep proves to be feasible, adding some degree of tissue-specific targeting by the availability of different viral serotypes [58, 59]. Additional tissue specificity can be achieved by using a tissue-specific promoter to drive cardiac expression. The field got a big impulse from the first clinical trials in which recombinant-AAV1 achieved overexpression of SERCA2 in the heart. The initial results of the phase 1 CUPID trial revealed no major adverse effects in patients with heart failure [60, 61]. AAV can therefore also be used to express antimiR sequences or precursor miRNA in the heart. Additionally, experiments with genetically engineered AAV9 underline the feasibility of improving cardiac targeting, thereby attaining higher specificity [62].

Ultrasound echography proves another interesting field for novel delivery strategies. Microbubbles can be applied as contrast agent to greatly improve the ultrasound imaging of blood flow. Interestingly, these tiny gas bubbles can be forced to collapse upon specific ultrasound stimulation. The possibility of loading these microbubbles with pharmacological agents cleared the way for their application in therapeutic drug delivery [63, 64]. Systemic delivery of drug-loaded microbubbles can now be combined with local release through ultrasound induced microbubble collapse [65]. By applying the ultrasound to the heart, the bubbles will release their content locally. When loaded with antimiRs, this local release may improve targeting of the heart. Although some specificity will be lost through systemic diffusion of released antimiRs, the targeting efficiency is expected to remain much higher compared to systemic administration, simultaneously decreasing the risk of side effects. We excitingly look forward to the first study that applies antimiR-loaded microbubbles in vivo.

Another similar though challenging approach to achieve cell type specific miRNA uptake may be available through the use of microvesicles. Microvesicles or exosomes are cell-derived vesicles that carry proteins, mRNAs, and miRNAs in the blood. Whereas microbubbles depend on external stimulation by ultrasound for release of their content, protein-coated microvesicles may facilitate their own uptake through

receptor interactions [66]. Cells naturally contain mechanisms for receptor-induced endocytosis, and vesicles coated with the proper receptor substrates provide a means to deliver mimics and antimiRs to other cell types and tissues. This might be an important approach especially regarding miRNA mimics that encounter difficulties in their cellular uptake. Restricted tissue distribution of these receptors even provides a means for specific targeting. In vitro proof of principle has been provided by the functional transfer of mRNA by microvesicles from the cardiomyocyte-like HL-1 cells to fibroblasts [67]. Since the antimiR has to be eventually delivered to the cardiac fibroblast or myocyte, crossing the endothelial barrier in vivo constitutes a major barrier to this approach. Biogenesis also poses a challenge on the use of microvesicles, since it requires miRNA or antimiR overexpressing packaging cells.

Another study adds exciting perspectives to targeting and delivery strategies. Deep sequencing of human blood samples in a Chinese population detected several non-human miRNAs. Further analysis showed that one of the most abundant miRNAs was the rice-derived miRNA-168a. Studies with rice-fed mice showed that the miRNAs were actually taken up via the digestive tract [68]. The presence of plant-derived miRNAs in the human circulatory system therefore shows the ability of miRNAs to cross the gastrointestinal barrier. The therapeutic implications of this finding with regard to systemic miRNA delivery are very promising. A recent publication however contests these findings as miRNA-21$^{-/-}$ mice fed a miRNA-21-supplemented diet showed no increase in plasma levels of this miRNA [69]. It therefore remains to be determined to what extend food-derived miRNAs actually enter the circulation. Dosage and the specific miRNA might turn out to be important determinants for this route of delivery.

With the exception of AAVs the actual supportive evidence for directed targeting in humans remains limited. Small rodent models have however provided useful and promising insights in the feasibility of several diverse strategies. Additional research will provide the answer to which approach holds the biggest therapeutic potential.

Perspectives and Limitations

As the experiments with animal models and the findings of miRNA-122 in relation to HCV illustrate, miRNAs regulate cardiac fibrosis and can act as targets for therapeutic intervention. Before proceeding to discuss the potential of miRNA-based therapies, it is worth to mention the most important characteristics of an effective therapeutic strategy to treat cardiac fibrosis. Inhibition or prevention of the fibrotic response constitutes the main goal, and unwanted side effects represent the major limiting factors for clinical application. Pharmacokinetic properties like uptake, excretion, stability, and tissue distribution are also important determinants that affect therapeutic application.

It is no coincidence that antimiR-122 was the first to reach clinical trials. The initial experiments and clinical studies with miRNA-122 in relation to HCV clearly illustrate the therapeutic potential of miRNA-based interventions. This miRNA however has two features that contribute to its success that are absent from the set of miRNAs involved in cardiac fibrosis and thereby complicate their applicability for miRNA-based interventions. First, off-target effects by antimiR-122 treatment are less likely to occur due to its tissue-restricted expression in the liver. Second, the high viral expression levels are also likely to be more susceptible to changes in miRNA-122 levels compared to endogenous mRNA targets.

Unlike miRNA-122, most miRNAs involved in cardiac fibrosis show a broad spectrum of cell and tissue expression. Possible exceptions are the muscle-specific miRNA-133, miRNA-206, and miRNA-1 which besides the heart are also expressed in skeletal muscle. Since a broad expression pattern increases the risk for off-target effects, a good therapeutic target benefits from restricted expression, making miRNA-133, miRNA-206, and miRNA-1 good candidates for further development.

Whether the anti-fibrotic effect results from increased or decreased miRNA levels represents another factor of importance in therapeutic targeting. At this moment, miRNA knockdown by using antimiRs seems to be the most feasible therapeutic approach. Most tested antimiR interventions however increase cardiac fibrosis, with the possible exception of miRNA-21. Other antimiR interventions even yield opposing results, as illustrated by miRNA-24. Since most fibrosis-regulating miRNAs seem to decrease the level of cardiac fibrosis upon overexpression, this approach holds the biggest promise for miRNA-based therapies. Overexpression comes however with a set of limitations that have to be overcome before therapeutic applications can be developed. One of the major issues with miRNA mimics is that they have to be incorporated into the RISC complex. Besides difficulties with cellular uptake, a high level of overexpression therefore also results in RISC saturation, which interferes with endogenous miRNA function [70]. Unwanted off-target effects pose another limitation to the use of miRNA mimics. Whereas antimiRs in the worst case only affect other tissues that express a given miRNA, systemic administration of mimics will affect any cell that expresses its mRNA targets. With each miRNA having multiple potential mRNA targets, systemic delivery of miRNA mimics will probably not be applicable for therapy. Directed targeting remains however an interesting option.

Although microvesicle-mediated delivery still has to prove its feasibility and microbubble-mediated approaches

do not solve the problem of efficient cellular uptake, AAV-mediated transfer of precursor miRNAs has good potential for local delivery, especially when combined increased cardiac targeting and the use of cardiac specific promoters. The finding of a useful promoter poses a challenge by itself, since in contrast with cardiomyocytes there is a lack of cardiac fibroblast specific promoters. Future experiments have to confirm the application of AAV as miRNA carriers for human targeting, but the technology seems promising.

Another issue arises from one basic but important principle: we only know what we have been looking for. The sheer number of predicted targets for any given miRNA hints to the multitude of potential functions, of which only a limited number has been explored properly. In the case of miRNAs affecting cardiac fibrosis, most of them have been initially identified as regulators of fibrosis in the heart or another organ. Follow-up studies are therefore likely to focus on fibrosis in other tissues, confirming its postulated role. This however overlooks many other potential functions of these miRNAs, creating a bias in our knowledge that requires additional investigation or dealing with unforeseen findings during the progression towards clinical application. This is illustrated by the miRNA-122 example where several studies found effects on cell proliferation [57]. In short-term studies, these effects can easily be overlooked, as the detection of tumor growth requires a longer follow-up period. Many other miRNAs discussed in relation with cardiac fibrosis have been shown to affect apoptosis, cell proliferation, and other cellular processes that might be involved in cancer progression, which requires longer follow-up periods, and careful investigation to reveal their effects.

Improved targeting strategies have the potential to overcome many of the issues raised above. Especially the clinical trials with AAV are very promising. When combined with minimal off-target effects, the adverse side effects of miRNA-based therapies can be overcome. This opens the way for the development of new approaches to disease treatment and cardiac fibrosis in particular. Not all in vitro and in vivo findings show consistent patterns and the discrepancy in observations regarding miRNA-21 and miRNA-24 reveals an important prerequisite regarding therapeutic application: the eventual effect depends on a delicate interplay between the chemistry of the therapeutic miRNA, the underlying disease, and many other unknown factors. It is however the charting of these unknowns that leads to new breakthroughs, and the first results on miRNA-based therapeutics are promising. Taking into account that out of more than thousand miRNAs, only a very limited subset has been characterized for their role in cardiac fibrosis, we currently might have been discussing only the very tip of a therapeutically interesting iceberg.

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