Cardiac Fibrosis Revisited by MicroRNA Therapeutics

Running title: Thum et al.; Cardiac fibrosis revisited by microRNAs

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Cardiac fibrosis is a result of a variety of injurious insults of different etiologies to cardiac tissue, which ultimately culminates in destruction of physiological tissue architecture and progressive organ dysfunction. Histologically, it is characterized by activation/proliferation of fibroblasts and excessive matrix deposition including collagen\(^1\). A major role in this process has been attributed to various growth factors, proteolytic enzymes, angiogenic factors, and fibrogenic cytokines\(^1\).

MicroRNAs (miRNAs/miRs) have recently come into focus as powerful regulators of gene expression and fundamentally impact on the pathogenesis of different pathological events, including cardiac fibrosis\(^2\). MiRNAs are small non-coding RNAs (~22 nucleotides) that lead to silencing of genetic information through post-transcriptional degradation of messenger-RNA and/or translational inhibition of protein expression\(^3,4\). MiRNAs are highly conserved in different species and are thought to regulate at least 50% of the genome. MiRNAs are formed in a highly regulated process in the nucleus and are then transported into the cytosol, in which they are further processed\(^3\). Numerous studies have underlined their critical importance for disease initiation and progression by influencing distinct disease-specific signal transduction pathways\(^2,5\).

With respect to cardiac fibrosis, a number of miRNAs have been previously identified to critically impact on fibrosis regulation (Figure 1). MiR-29 was identified to target several collagens (COL1A1, COL1A2, and COL3A1) as well as fibrillin (FBN1), thus promoting extracellular matrix deposition following myocardial infarction (MI)\(^6\) (Figure 1). Specifically, following MI the miR-29 family is downregulated in vivo, thereby de-repressing its targets resulting in cardiac fibrosis. In addition, in cardiac fibrosis in response to cardiac hypertrophy and failure miR-21 is specifically enriched in cardiac fibroblasts\(^2\) and regulates the ERK-MAP kinase signalling pathway through inhibition of sprouty homologue 1 (Spry1). By this mechanism miR-21 promotes fibroblast survival and growth factor secretion. In contrast,
silencing of miR-21 with a specific antagonist (antagomir) culminated in a reduction of cardiac ERK-MAP kinase activity, inhibition of interstitial fibrosis and improvement of cardiac dysfunction. In angiotensin II-induced hypertension and cardiac fibrosis miR-133a was shown to be downregulated leading to a de-repression of its target collagen 1a1 (Col1A1)\(^7\). This promoted the extent of fibrosis development. Additionally, another study found the expression of connective tissue growth factor (CTGF), one of the major fibrosis-promoting factors, to be regulated by miR-30 and miR-133 in human and animal cardiac tissue\(^8\).

In the current issue of Circulation, Pan et al.\(^9\) add to the current knowledge of cardiac important miRNAs by investigating a novel role of miR-101 in the context of cardiac fibrosis following myocardial infarction. The expression of miR-101a and miR-101b in the peri-infarct area of rats was shown to be decreased 4 weeks after coronary artery ligation. In vitro, angiotensin II suppressed the expression of miR-101a and miR-101b in rat neonatal cardiac fibroblasts. Proliferation and collagen production in fibroblasts was abrogated by overexpression of synthetic miR-101a and b. These effects were prevented by co-transfection with a specific inhibitor of miR-101a/b. The investigators identified c-fos as a target of miR-101a by bioinformatic analysis and luciferase gene reporter assays. The expression of c-fos and transforming growth factor \(\beta\)1 (TGF\(\beta\)1) was significantly increased in cardiac fibroblasts by angiotensin II treatment. This was prevented by simultaneous overexpression of miR-101a. Through gain- and loss-of-function studies the role of c-fos was further investigated. Plasmid overexpression of c-fos in cardiac fibroblasts resulted in enhanced TGF\(\beta\)1, collagen I and III expression, while silencing of c-fos by small interfering RNA reduced their expression, thus eliciting comparable anti-fibrotic effects as miR-101a mimics. Intriguingly, the cardiac performance 4-weeks after coronary artery ligation was improved by adenovirus-mediated
overexpression of miR-101a as assessed by echocardiography and hemodynamic measurements. Interstitial fibrosis, myocyte apoptosis as well as the expression of c-fos and TGFβ1 was also decreased.

A role of miR-101 and its targets has previously been demonstrated in several other disease contexts. For instance miR-101 influences the epigenetic regulation of prostate cancer by targeting the histone methyltransferase Enhancer of Zeste homolog 2 (EZH2)\(^\text{10}\). In addition, miR-101 was shown to influence cellular self-renewal by targeting the autophagy-related genes such as Stathmin 1 (STMN1), Ras-related protein Rab-5A (RAB5A) and autophagy related 4 homolog B (ATG4D)\(^\text{11}\). Data on miR-101 and cardiac disease are also available but scarce so far. In cardiac tissue of patients with dilated cardiomyopathy and aortic stenosis the expression of miR-101 was shown to be significantly reduced\(^\text{12}\). Van Rooij et al. found the expression of miR-101a and miR-101b to be diminished in the infarct border zone of mice after myocardial infarction\(^\text{6}\). In a previous study Yang et al. reported miR-101 to be reduced in atrial tissue of patients with atrial fibrillation due to rheumatic heart disease and in a canine model of atrial fibrillation induced by atrial tachypacing for 8 weeks\(^\text{13}\). In general, most studies so far identified cardiac miR-101 to be reduced upon cardiovascular stress suggesting enhancing miR-101 strategies to be of potential therapeutic interest. The regulatory mechanisms however leading to miR-101 silencing during cardiac stress remain so far unexplored. The available studies about the role of miR-101 in cardiovascular disease are summarized in Table 1.

c-fos together with the c-jun, ATF and JDP protein families form the transcription factor activator protein 1 (AP-1)\(^\text{14}\). The expression of AP-1 is induced by various cytokines and growth factors and activates genes responsible for cellular differentiation and proliferation\(^\text{14}\). This is partly mediated by activating cell-cycle regulators such as cyclin D1, cyclin A, cyclin E, p53,
p21^{Cip1}, p16^{Ink4a} and p19^{ARF}. Interestingly, the transcription of fibrosis-associated miRNAs such as miR-21 is also regulated by AP-1. In addition, the miR-29 promoter contains several putative binding sites for AP-1. In the case of miR-29, binding of AP-1 may result in transcriptional repression during fibrosis development. However, this has not been tested yet. A study in lung fibroblasts confirmed that tumor necrosis factor \( \alpha \) induces the expression of profibrotic TGF\( \beta \) via regulation of c-fos. Roy et al. investigated the regulation of TGF\( \beta \) in cardiac fibroblasts in response to oxygen in order to analyze the effect of reperfusion during ischemia/reperfusion injury. It was found that oxygen induced transcription of all three TGF\( \beta \) isoforms through activation of AP-1. Specifically, Fos-related AP-1 transcription factor (Fra-2) and Ask-1 (apoptosis signal-regulating kinase-1) were shown to be essential in the regulation of AP-1-dependent TGF\( \beta \) transcription in this setting. A recent study analyzed the contribution of miR-21 to cardiac fibrosis by stimulating endothelial to mesenchymal transition (EndMT).

Specifically, TGF\( \beta \) induced EndMT in endothelial cells via upregulation of miR-21. Thus, the effects of miR-101 via c-fos regulation may effect further fibrosis-related miRNAs to boost cardiac fibrosis.

The study by Pan et al. thus adds yet another dimension to the complex regulation of cardiac fibrosis by miRNAs (Figure 1). The current understanding suggests that during the development of cardiac fibrosis the AP-1 transcription factor is activated, which also turns on other profibrotic pathways such as miR-21. Pan et al. now present an additional team player in the miRNA game, that directly interferes with the expression of c-fos, a component of the AP-1 transcription factor. Thus, altered expression of this transcription factor complex by miR-101 might directly interfere with downstream signalling cascades including that of other miRNAs, thereby modulating cardiac fibrosis. This suggests an intricate feedback loop involving miR-21,
miR-101 and the AP-1 transcription factor. However, Pan et al. did not investigate effects of the other components of AP-1, namely the c-Jun, ATF and JDP protein families. Therefore, future studies have to elaborate on the possible regulation of these remaining protein families. It would also be interesting to dissect the direct effect of miR-101 modulation on the expression of other profibrotic miRNAs. The authors did not detect an effect of miR-101 on the rate of apoptosis of cardiomyocytes in vitro. The authors explain the anti-apoptotic effect of miR-101 in vivo by the observed overimprovement in cardiac function. In addition, their findings concerning apoptosis and miR-101 contradict a recent study, in which miR-101 enhanced the apoptosis rate in hepatoma cells\textsuperscript{20}. The authors attribute these varying results to the different cell types used in the respective studies. Unfortunately, the expression and effects of miR-101 on other cardiac cells such as endothelial cells or smooth muscle cells were not examined. It would be interesting to evaluate an effect of miR-101 modulation on post-myocardial neo-angiogenesis and the underlying mechanisms in cardiac vascular cells.

Importantly, the authors found a positive effect of miR-101 mimics on cardiac function and hemodynamic measurements in rats subjected to myocardial infarction. Still and in contrast to miRNA antagonists\textsuperscript{21}, the application and effectiveness of miR mimetics (with except of viral-based approaches) is difficult and needs to be improved in the future if we want to enrich cells with specific miRNAs using viral-free delivery methods.

In conclusion, Pan et al. present a detailed and innovative analysis of miR-101 deregulation in cardiac fibrosis following myocardial infarction. Unresolved points remain to be elucidated, but interfering with miR-101 expression in this setting carries future therapeutic potential.
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**References:**


Table 1. miR-101 deregulation in cardiovascular disease

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>Regulation</th>
<th>target</th>
<th>Organism</th>
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<td>not</td>
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Figure Legend:

**Figure 1.** miRNAs involved in the pathogenesis of cardiac fibrosis. AP-1, activator-protein 1; ATF = activating transcription factor; CTGF = connective tissue growth factor; JDP = Jun dimerization protein; Spry 1 = sprouty 1; TGFβ = tissue growth factor beta.
miR-21 transcription
C-Jun
C-Fos
ATF
JDP

miR-101

miR-21

CTGF
Spry1

miR-133/30c

Collagen

Fibrosis

Enhanced TGFβ and Collagen expression

Cardiac stress

AP-1

miR-29

Cardiac fibroblast