Cardiovascular Research (2012) **96**, 230–233
doi:10.1093/cvr/cvs222

EDITORIAL

MicroRNA 21 in tissue injury and inflammation

AUTHORS' RETROSPECTIVE

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Online publish-ahead-of-print 9 August 2012

This editorial refers to an article by S. Roy *et al.*[1] published in *Cardiovascular Research* in 2009 (see Box 1). It is accompanied by an editorial by J. Bauersachs, pp. 227–229, this issue, as part of this Spotlight on Landmark Papers in *Cardiovascular Research*.

This editorial celebrates the extraordinary citation of our recent publication[1] in *Cardiovascular Research* highlighting the emerging significance of miR-21 in the heart, particularly in the context of tissue injury, inflammation, and remodelling. The work was first submitted to a leading cardiovascular journal in January 2008. The submission was summarily rejected, citing 'paucity of novel mechanistic insight'. The work went on to be published in *Cardiovascular Research* and has now been cited 150 times in 3 years, being recognized as one of the most highly cited articles in the journal. Had the work been published in the original journal it would have been published almost a year in advance and would have today ranked third in citation score among all of nearly 300 articles published in that journal in 2009. Here, we briefly review the progress in the field since our original work was published.

1. Heart miR-21: yes, it is a validated therapeutic target

Identified as one of the first mammalian microRNAs (miRs), the miR-21 sequence is strongly conserved throughout evolution. During the early phase of development of miR-21 biology, most of the target coding genes reported were tumour suppressors. Thus, miR-21 represents one of the first 'oncomirs' named. Two concurrent works[1,2] published by the Engelhardt laboratory[2] and us[1] markedly changed the landscape by introducing the cardiac fibroblast as a major hub for miR-21 action. Taken together, these studies drew attention to the role of miR-21 in cardiovascular diseases.[1–12] Today, miR-21 is recognized as playing an important role in the development of heart disease. miR-21 expression is upregulated in failing murine and human hearts.[1,2] Induction of miR-21 by ischaemic preconditioning protects the heart against ischaemia/reperfusion injury.[6]

Could ischaemia/reperfusion-induced expression of miR-21 that we had reported be functionally futile in the heart? Indeed, a report published in 2010 concluded that miR-21 is not essential for pathological cardiac remodelling.[11] This conclusion went against the grain of both original independent studies.[1,2] So, where is the disconnect? Interestingly, the study of miR-21-deficient mice concluded that miR-21 plays no role in cardiac disease.[11] Supporting data arguing against the significance of miR-21 in heart disease were provided by studies using very short, 8-nucleotide anti-miR-21 oligonucleotides.[11] Genetic deletion of a target is commonly recognized to be compensated for during development. Therefore, this approach is limited in its ability to rule out miR-21 as a key determinant of heart function. However, the contrast in outcomes of the therapeutic trials using long[2] vs. short 8-mer[11] oligonucleotides was striking. To address this inconsistency, the Engelhardt laboratory conducted a direct head-to-head comparison of three different oligonucleotide chemistries in the same model of cardiac disease.[13] Findings from such studies dispelled the false alarm[10] and upheld the significance of miR-21 in cardiac disease by demonstrating that results from the use of 8-mer anti-miR-21 are of limited significance because the 8-mer is ineffective in suppressing miR-21 on a long-term basis. The study concluded that for long-term inhibition of miR-21 function *in vivo*, interventions based on longer anti-miRs are likely to prove to be superior due to their high potency and treatment duration.[13] Dispute and resolution aside, current work employing next-generation sequencing technologies for comprehensive murine cardiac miRNA and mRNA expression profiling in mouse left ventricle reinforces the significance of attenuating miR-21 expression in improving cardiac fibrosis and limiting pathological hypertrophy.[14]

2. miR-21: a trigger for fibrosis across organ systems

In response to tissue injury, aberrant extracellular matrix production by resident fibroblasts causes fibrotic diseases across organ systems. Both original publications introducing miR-21 to cardiac biology[1,2] pointed towards the fibroblast as a major locus of miR-21 action. In both studies, elevated miR-21 was connected to fibroblast dysfunction and fibrosis outcome. We reported the first evidence describing

The opinions expressed in this article are not necessarily those of the Editors of *Cardiovascular Research* or of the European Society of Cardiology.

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MicroRNA expression in response to murine myocardial infarction: miR-21 regulates fibroblast metalloprotease-2 via phosphatase and tensin homologue

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Received 29 April 2008; revised 5 January 2009; accepted 11 January 2009; online publish-ahead-of-print 15 January 2009

Time for primary review: 24 days

KEYWORDS
Reactive oxygen;
Non-coding gene;
Gene expression;
Genomics;
Redox

Aims MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level by either degradation or translational repression of a target mRNA. Encoded in the genome of most eukaryotes, miRNAs have been proposed to regulate specifically up to 90% of human genes through a process known as miRNA-guided RNA silencing. For the first time, we sought to test how myocardial ischaemia–reperfusion (IR) changes miR expression.

Methods and results Following 2 and 7 h of IR or sham operation, myocardial tissue was collected and subjected to miRNA expression profiling and quantification using a Bioarray system that screens for human-, mice-, rat-, and Ambi-miR. Data mining and differential analyses resulted in 13 miRs that were up-regulated on day 2, 9 miRs that were up-regulated on day 7, and 6 miRs that were down-regulated on day 7 post-IR. Results randomly selected from expression profiling were validated using real-time PCR. Tissue elements laser-captured from the infarct site showed marked induction of miR-21. *In situ* hybridization studies using locked nucleic acid miR-21-specific probe identified that IR-inducible miR-21 was specifically localized in the infarct region of the IR heart. Immunohistochemistry data show that cardiac fibroblasts (CFs) are the major cell type in the infarct zone. Studies with isolated CFs demonstrated that phosphatase and tensin homologue (PTEN) is a direct target of miR-21. Modulation of miR-21 regulated expression of matrix metalloprotease-2 (MMP-2) via a PTEN pathway. Finally, we noted a marked decrease in PTEN expression in the infarct zone. This decrease was associated with increased MMP-2 expression in the infarct area.

Conclusion This work constitutes the first report describing changes in miR expression in response to IR in the mouse heart, showing that miR-21 regulates MMP-2 expression in CFs of the infarct zone via a PTEN pathway.

Box 1 Title page including abstract from the original 2009 publication in *Cardiovascular Research*.[1] Used with permission of Oxford University Press on behalf of the European Society of Cardiology.

changes in miR expression in response to ischaemia/reperfusion in the murine heart, demonstrating that miR-21 regulates matrix metalloprotease-2 (MMP-2) expression in cardiac fibroblasts of the infarct zone via a phosphatase and tensin homologue (PTEN) pathway. Tissue elements laser-captured from the infarct site showed marked induction of miR-21 in fibroblasts. *In situ* hybridization studies using a locked nucleic acid miR-21-specific probe identified that ischaemia/reperfusion-inducible miR-21 was specifically localized in the infarct region of the reperfused heart. Studies with isolated cardiac fibroblasts identified PTEN as a direct target of miR-21. A marked decrease in PTEN expression was observed in the infarct zone. This decrease was associated with increased MMP-2 expression in the infarct area.[1] Our observation that miR-21 silences PTEN in the infarcted heart is now known to have additional implications. Endothelial-to-mesenchymal transition (EndMT) is emerging as a significant contributor to transforming growth factor-β (TGF-β)-dependent cardiac fibrosis. miR-21 contributes to, at least in part, TGF-β-mediated EndMT in the heart via silencing of PTEN.[15] Thum *et al.*[2] demonstrated that miR-21 regulates the ERK–MAP kinase signalling pathway in cardiac fibroblasts. miR-21 levels were noted to be specifically upregulated in fibroblasts of the failing heart, augmenting ERK–MAP kinase activity through inhibition of sprouty homologue 1 (Spry1). In this way, miR-21 contributed to cardiac fibrosis.[2] The significance of miR-21 in causing fibrosis seems to transcend the heart as an organ. In mice with bleomycin-induced pulmonary fibrosis as well as in the lungs of patients with idiopathic pulmonary fibrosis, miR-21 is elevated.[16] Here again, miR-21 expression was primarily localized to myofibroblasts. Even when the intervention was performed 5–7 days after the initiation of pulmonary injury, suppression of miR-21 diminished the severity of experimental lung fibrosis in mice. Interestingly, TGF-β1, a common driver of fibrotic response across organ systems, induced miR-21 expression in primary pulmonary fibroblasts. Importantly, miR-21 was recognized as being the mediator of pro-fibrogenic activity of TGF-β1 in fibroblasts. miR-21 regulates the

Smad pathway. Boosting miR-21 levels enhanced, whereas knocking down miR-21 attenuated, Smad2 phosphorylation in response to TGF-β1 stimulation. Furthermore, Smad7 is a direct target of miR-21. Smad7 plays an essential role in the negative-feedback regulation of TGF-β signalling by inhibiting TGF-β signalling at the receptor level. Silencing of Smad7 by miR-21 may therefore support fibrogenic effects of TGF-β1.[16] miR-21 is also implicated in vascular remodelling.[17] This effect is partly mediated via silencing of programmed cell death 4 (PDCD4). Inhibition of miR-21 reversed vascular remodelling induced by balloon injury.[17] In the skeletal muscle, fibrosis is directly implicated in the death of patients suffering from Duchenne muscular dystrophy. Recently, it was demonstrated that extracellular plasminogen activator inhibitor-1 (PAI-1)/urokinase-type plasminogen activator balance regulates miR-21 biogenesis. In addition, it controls age-associated muscle fibrosis and progression of dystrophy. Age-associated fibrogenesis was successfully intercepted by miR-21 inhibition, whereas miR-21 overexpression aggravated the severity of disease. The PAI-1-miR-21 fibrogenic axis is recognized as a target to treat fibrosis and muscular dystrophies.[18] In the kidney, fibrosis is a final stage of many forms of disease leading to compromised organ function. Unilateral ureteral obstruction (UUO)-induced renal fibrosis is associated with changes in expression of miRNAs that are responsive to stimulation by TGF-β1 or TNF-α. Among these miRNAs, miR-21 demonstrated the greatest increase in UUO kidneys. Inhibition of miR-21 *in vivo* attenuated UUO-induced renal fibrosis, pointing towards a causative role of miR-21.[19]

3. Anti-inflammatory macrophage miR-21

Successful mounting of a pro-inflammatory response and timely resolution of inflammation are both required for successful wound healing.[20–22] Macrophages are immune cells involved in various biological processes including tissue repair and host defense. The first evidence supporting the significance of miRNA in governing macrophage function was published in 2007 when profiling studies were performed to identify miRNAs induced in primary murine macrophages after exposure to pro-inflammatory conditions. miR-155 was recognized as a common target of a broad range of inflammatory mediators.[23] Ever since, interest in understanding the role of miRNA in regulating the inflammatory response to injury has sharply risen.[24–26] In one of the first works that reported the anti-inflammatory properties of miR-21 in macrophages, it was noted that miR-21 silences pro-inflammatory interleukin (IL)-12.[27] The IL-12 family is composed of three heterodimeric cytokines with overlapping pro-inflammatory and immunoregulatory functions.[28] In human airway epithelial cells, IL-13 induces miR-21. In the lungs, miR-21 inhibits toll-like receptor 2 agonist-induced lung inflammation in mice.[29] Resolvins, including D and E series resolvins, are endogenous lipid mediators generated during the resolution phase of acute inflammation from the omega-3 polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid.[30] miR-21 is inducible by resolvin D1 and may play a role in resolving acute inflammation.[31] PDCD4 has pro-inflammatory properties. Translational inhibition of PDCD4 by miR-21 therefore has anti-inflammatory consequences in the context of sepsis.[32]

Beyond its direct effects on macrophages, miR-21 has a list of biological targets validated in a variety of cell types that point to hypotheses that could provide additional anti-inflammatory mechanisms. Analysis of predicted target genes of miR-21 on the basis of resources available in TargetScan4.0, PicTar, and miRanda resulted in the identification of a total of 930 candidates.[33] Additional characterization of these candidates through target-pathway analysis pointed towards the following two specific signalling pathways that are significantly ($P < 0.01$) regulated by miR-21: (i) Janus kinase (JAK) and signal transducer and activators of transcription (STAT) signalling pathway (target count = 16; CSF3R, SPRY2, IL23R, CNTFR, IL15, IL7, IL13RA1, STAT3, SOS2, SPRY1, PIK3R1, LIFR, IL9, JAK3, IL12A, IL13RA2); (ii) cytokine–cytokine receptor interaction (target count = 20; CSF3R, SPRY2, IL23R, CNTFR, IL15, IL7, IL13RA1, STAT3, SOS2, SPRY1, PIK3R1, LIFR, IL9,JAK3, IL12A, IL13RA2).[33] Collectively, these pathways represent the core of the cytokine response system. Dysregulation of cytokine signalling is known to be a root cause of inflammation. From a therapeutic standpoint, targeting cytokine networks is viewed as a promising strategy to control chronic inflammatory diseases.[34] JAK/STAT signalling helps elicit transcriptional response to external stimuli.[35] Specifically, it directly links ligand binding to a membrane-bound receptor with the activation of a transcription factor. Cytokines specifically bind to their corresponding receptors, leading to the activation of receptor-associated JAKs. The receptor-bound JAKs activate STAT transcription factors that translocate into the nucleus to induce target gene expression. JAK-STAT signalling is directly implicated in cytokine-mediated inflammation.[36,37] Inhibition of this pathway by small compounds is recognized as a potential therapeutic target for various inflammatory diseases.[38] The silencing effects of miR-21 on the JAK-STAT pathway may be utilized as an anti-inflammatory strategy.

miR-21 may exercise anti-inflammatory effects also through silencing of PTEN. Myeloid PTEN is known to promote inflammation.[39] Furthermore, PTEN silencing strengthens PI3K-Akt signalling, which is known to suppress inflammation.[40–42] Akt is also known to coordinate macrophage transitions and resolution of inflammation during tissue repair.[43] DOCK (dedicator of cytokinesis) guanine nucleotide exchange factors activate the Rho-family GTPases Rac and Cdc42 to control phagocytosis. Phagocytosis, in addition to clearance of unwanted cells, may influence inflammatory outcomes.[44,45] miR-21 may thus influence phagocytosis and inflammation through silencing of DOCK.[46] MCP-1, a key driver of inflammation, is also a biologically validated target of miR-21.[47] Silencing of MCP-1 represents a potentially influential mechanism that can be added to the anti-inflammatory mechanisms of miR-21. Although in endothelial cells macrophage miR-21 may possess pro-inflammatory functions,[48] macrophage miR-21 plays a key role in the resolution of inflammation. In specific cases, pro-inflammatory stimuli are reported to induce miR-21,[49] leading to the hypothesis that inflammation is initiated with a built-in provision to resolve. In support of this hypothesis, it is noted that activation of NF-κB, a major driver of inflammation, leads to the induction of anti-inflammatory miR-21.[50] Induced miR-21 may exert anti-inflammatory properties by suppressing NF-κB signalling.[51] Likewise miR-21 is also induced by TNFα, a landmark pro-inflammatory cytokine.[52] Further studies on macrophage miR-21 are likely to shed new light on the resolution of inflammation.

4. Conclusion

miR-21 has established itself as a key regulator of fibroblast function with potent implications in tissue injury and inflammation. miR-21 plays an important role in the pathogenesis of heart disease, making it a therapeutic target. In macrophages, miR-21 seems to play a major role in helping resolve inflammation. Understanding of such

cell-specific functions of miR should help develop effective miR-based therapeutic strategies.

Conflict of interest: none declared.

Funding

Relevant work in the authors' laboratory is supported by the following grants from the National Institutes of Health: HL073087, GM069589, GM077185, NS42617, and DK076566.

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