Inhibition of MicroRNA-92a Protects Against Ischemia/Reperfusion Injury in a Large-Animal Model

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Background—MicroRNAs (miRs) are small noncoding RNAs that posttranscriptionally control gene expression. Small-animal studies suggest that miRs might offer novel therapeutic targets in cardiovascular diseases such as cardioprotection of murine hearts after myocardial infarction via miR-92a inhibitors. Because the functional benefits of miR-92a inhibitors in larger preclinical models are not known, we assessed the therapeutic efficacy of miR-92a inhibition in a porcine model of ischemia and reperfusion.

Methods and Results—Pigs (n=5 per group) underwent percutaneous ischemia/reperfusion (60 min/72 h or 7 days, respectively). Locked nucleic acid–modified antisense miR-92a (LNA-92a) was applied either regionally (antegrade or retrograde) with a catheter or systemically (intravenously). LNA-92a significantly (P<0.01) reduced miR-92a expression in the infarct zone regardless of the application venue. However, catheter-based delivery, but not intravenous infusion, of LNA-92a significantly (P<0.05) reduced the infarct size compared with control LNA–treated pigs, which correlated with an improved ejection fraction and left ventricular end-diastolic pressure (P<0.05). Histochemistry revealed that LNA-92a increased capillary density but decreased leukocyte influx and cardiac cell death. Complete loss of miR-92a in mice attenuated the infarct-related myocardial dysfunction to a larger extent than cardiomyocyte-specific miR-92a deletion. In vitro, LNA-92a protected against hypoxia/reoxygenation–induced cardiomyocyte cell death.

Conclusions—Regional LNA-92a delivery reduces miR-92a levels and infarct size and postischemic loss of function. LNA-92a exerts cell-protective, proangiogenic, and anti-inflammatory effects. miR-92a inhibition might be a novel therapeutic tool to preserve cardiac function after ischemia. (Circulation. 2013;128:1066-1075.)

Key Words: apoptosis • infarction • inflammation • ischemia • microRNAs • reperfusion

Myocardial infarction is caused by the sudden loss of oxygen and nutrient supply, leading to cardiomyocyte death. In addition to the oxygen deprivation–induced cardiac cell death, the inflammatory cascades that are particularly induced by ischemia and reperfusion appear to play a key role in the acute injury and the postinfarction repair process.1

Clinical Perspective on p 1075

MicroRNAs (miRNAs, miRs) are small noncoding RNAs that bind to specific target miRNAs and thereby induce degradation or translational repression. Ischemia alters the expression pattern of miRNAs in cardiac tissue,2,1 raising the possibility that miRNAs causally affect cardiac function after myocardial infarction (see the article by Small and Olson4 for a review). Moreover, apoptosis of cardiomyocytes was induced by miR-155 or inhibited by miR-246 and miR-494.7

Furthermore, several miRNAs were shown to regulate fibrosis after myocardial infarction. The upregulation of miR-21 was linked to cardiac fibrosis by some but not all studies,8,9 whereas miR-101 was recently shown to suppress cardiac fibrosis.10 In addition, members of the miR-17-92 cluster regulated neovascularization after ischemia.11,11 Particularly, antagonirs against miR-92a improved neovascularization and augmented functional recovery in a murine model of long-term ligation of the left anterior descending coronary artery.2 Despite these encouraging results from studies in small animals, only limited information is available on the preclinical development of miRNA therapeutics for cardiovascular diseases.12 Locked nucleic acid (LNA)–based inhibitors directed against miR-122 were developed for the treatment of hepatitis C and were shown to be safe and effective in nonhuman primates and phase II clinical trials.13 In contrast, evidence of therapeutic

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efficacy of miRNA inhibitors in ischemic cardiac diseases, in which timing and delivery venues are of paramount relevance, is lacking in preclinical large-animal studies.

Here, we used a pig model of acute ischemia/reperfusion injury resulting in the induction of myocardial infarction and tested the effect of miR-92a inhibition via antegrade or retrograde infusion of an LNA-based miR-92a inhibitor (LNA-92a) at the onset of reperfusion. The antegrade approach is routinely available during coronary interventions, whereas retrograde delivery was previously shown to be more effective when using gene and cell therapy.14

Methods

Animals

Pigs were purchased from Veterinary Medicine, LMU Munich (Oberschleißheim, Germany). Animal care and all experimental procedures were performed in strict accordance to the German and National Institutes of Health animal legislation guidelines and were approved by the Bavarian Animal Care and Use Committee.

Pig Ischemia/Reperfusion Model

All pig experiments were conducted at the Walter-Brendel Center for Experimental Medicine at the University of Munich. Pigs (n=5 per experimental group) were instrumented as previously described.15 Briefly, a percutaneous transluminal coronary angioplasty balloon was placed in the left anterior descending artery distal to the first diagonal branch and inflated with 6 atm (0.41 MPa) for 60 minutes. Correct localization of the coronary occlusion and patency of the first diagonal branch were ensured by injection of contrast agent. In all groups, the percutaneous transluminal coronary angioplasty balloon was deflated after 60 minutes of ischemia; the onset of reperfusion was documented angiographically.

Anti-miR Application

The LNA anti-miRs are fully phosphorothiolated oligonucleotides perfectly complementary to the 5′ region of miR-92a (base, 2–17: 5′-CAGGCCGGGACAAGUGCAAUA-3′) and were synthesized as a mixer of LNA and DNA. The LNA control oligonucleotide consists of a sequence directed against a Caenorhabditis elegans-specific miRNA with a comparable LNA/DNA content.

Anti-miR and control oligonucleotides were dissolved in PBS before administration. Anti-miRs were applied either systemically (intravenously) or regionally antegrade into the left anterior descending artery or retrograde into the anterior interventricular vein 5 minutes before reopening of the infarct vessel (0.03 mg/kg body weight).

Hemodynamic Measurements

Left ventricular end-diastolic pressure and ejection fraction measurements were performed before ischemia and after 72 hours or 7 days of reperfusion. Additionally, regional myocardial function was obtained after 72 hours or 7 days of reperfusion.

Infarct Size

Infarct size was assessed via methylene blue exclusion, tetrazolium red viability staining as described previously.15

Histological Analysis

Myeloperoxidase assays were performed for evaluation of leukocyte influx in the ischemic area as described.15 Capillary density (platelet endothelial cell adhesion molecule-1 staining) and cell death (terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] staining) were analyzed in the ischemic border zone (see Methods in the online-only Data Supplement).

RNA Isolation, Real-Time Polymerase Chain Reaction, and Immunoblotting

Real-time polymer chain reaction and Western blot were conducted according to standard protocols (see Methods in the online-only Data Supplement).

Generation of miR-92a–Deficient Mice

The constitutive and conditional deletion of miR-92a-1, which is expressed by the miR-17-92a cluster, was generated by homologous recombination in 129sv/Pas embryonic stem cells by genOway (Lyon, France). For this purpose, a targeting vector containing the homologous genomic miR-92a-1 sequences flanked by loxP sites and a neomycin gene flanked by FRT sites was used. For the generation of constitutive miR-92a–deficient mice (miR-92a−/−), the miR-92a recombined chimeric mice were bred with a deleter line constitutively expressing the Cre recombinase. For the generation of conditional cardiomyocyte-specific miR-92a knockout mice (miR-92a fl/fl, α-myosin heavy chain [αMHC]–Cre), the miR-92a recombined chimeric mice were first bred with C57BL/6J wild-type and Flp recombinase–expressing deleter mice to excise the neomycin selection cassette and then mated with a Cre deleter line expressing Cre recombinase under the control of the αMHC-Cre promoter.

Myocardial Infarction in Mice

Acute myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery as previously described.16 Cardiac function was determined by high-resolution echocardiography.16

Cell Culture Experiments

Cell death was induced in HL-1 cardiomyocytes by 18 hours of hypoxia/4 hours of reoxygenation in the presence of LNA-92a or control LNA. Leukocyte adhesion on human umbilical vein endothelial cell monolayer under flow (1 dyne/cm²) was performed with the iBidi system after 15 ng/mL tumor necrosis factor-α stimulation (see Methods in the online-only Data Supplement).

Statistical Analysis

The results are given as mean±SEM. Statistical analysis were performed by 1-way ANOVA. Whenever a significant effect was obtained with ANOVA, multiple-comparison tests between the groups with the Student-Newman-Keuls procedure were performed (SPSS 20.0 statistical program or Graphpad). Values of P<0.05 were considered significant differences between the groups.

Results

Inhibition of miR-92a by Systemic Versus Local Delivery of LNA-92a

First, we assessed the inhibitory potential of LNA-92a. Because of conservation of the miR-92a sequence between mouse and pig, we used the same sequence as described previously.3 For this purpose, we applied LNA-92a either systemically (by intravenous infusion) or by catheter-based antegrade or retrograde local delivery to the heart. We infused 5 mg per 1 kg heart weight on the basis of pilot studies in mice (data not shown). miR-92a expression 72 hours after ischemia/reperfusion was reduced by LNA-92a treatment in ischemic versus nonischemic zones (Figure 1A and Figures I and II in the online-only Data Supplement). Consistent with previous studies,3 miR-92a expression was upregulated in the infarcted heart tissue of both PBS– (control; 1.19-fold) and control LNA– (1.50-fold) treated pigs (Figure 1B and 1C).
Each delivery strategy resulted in a significant repression of miR-92a in both the infarcted and noninfarcted heart segments of LNA-92a–treated pigs compared with PBS- and control LNA–treated animals (Figure 1B and 1C). To determine whether the efficacy of miR-92a inhibition is limited by the LNA dose applied, we increased the administered amount by a factor of 5 and delivered 25 mg LNA-92a per 1 kg heart weight by antegrade catheter-based delivery strategy. However, the higher concentration of LNA-92a failed to induce a further significant reduction of miR-92a expression in both infarcted and noninfarcted segments (Figure 1B and 1C).

LNA-92a specificity was further analyzed by comparing expression levels of the members of the miR-17-92a cluster and paralog clusters. The sequence comparison of miR-17-92 cluster members and miR-92a family members of paralog clusters is shown in Figure 1D. LNA-92a, even when applied at the high dose, did not influence the members of the miR-17-92a cluster and miR-363 but significantly reduced the very closely resembling miR-25 (Figure 1D and 1E).

Systemic miR-92a inhibition might be disadvantageous because miR-92a is also expressed and functionally active in other tissues. Therefore, we determined whether regional catheter-based delivery of LNA-92a has fewer remote effects in noncardiac tissues. In liver, spleen, and kidney, miR-92a expression was reduced by all delivery strategies (Figure 2A through 2C). Lung expression of miR-92a appeared unaltered after antegrade and retrograde LNA-92a application and was found to be reduced after intravenous LNA-92a infusion and by antegrade delivery of the higher concentration of LNA-92a (Figure 2D).

In summary, these data demonstrate that both local catheter-based delivery and intravenous delivery of LNA-92a reduced cardiac miR-92a expression. The concomitant miR-92a repression in other tissues such as lungs was ameliorated by local LNA-92a delivery. However, when the dose was increased, antegrade delivery also fully repressed miR-92a expression in all organs tested.

**Catheter-Based Delivery of LNA-92a Decreases Infarct Size and Apoptosis in the Heart**

To assess the cardioprotective effect of LNA-92a, we measured the infarct size (Figure 3A and 3B). Analysis of the primary ischemic region (area at risk) revealed no differences between the control and treatment groups (Figure 3C). Intravenous application of LNA-92a did not significantly decrease infarct size compared with the control groups, whereas antegrade and retrograde application of LNA-92a significantly reduced infarct size to 32±5% and 36±4% of the area at risk, respectively (Figure 3A and 3B). Increasing the antegrade LNA-92a dose did not further reduce the infarct size (34±2% of the area at risk; Figure 3A and 3B). Similar effects were seen when infarct size was determined 7 days after ischemia/reperfusion (Figure IIIA in the online-only Data Supplement). Next, we analyzed the effect of LNA-92a on cell death as measured by

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**Figure 1.** Analysis of miR-92a repression after locked nucleic acid–modified antisense miR-92a (LNA-92a) application in the heart. A through C, Scheme of the tissue sample dissection for the polymer chain reaction (PCR) analysis. The heart was cut into 5 slices perpendicular to the left anterior descending artery (LAD) from base to apex. The samples were divided into ischemic and nonischemic areas and analyzed for miR-92a expression via PCR, as shown in B and C, respectively (n>4). D, Sequence comparison of miR-17-92 cluster members and miR-92a family members of paralog clusters. E, Expression analysis of other miR-17-92a cluster members and miR-92a family members of paralog clusters after antegrade infusion of the high concentration of LNA-92a (n=4; mean±SEM; **P<0.01 vs control and control LNA [LNA-Co]).
TUNEL assay. Systemic intravenous application of LNA-92a did not reduce cell death in the ischemic area. However, local application via retrograde or antegrade delivery of LNA-92a significantly reduced cell death compared with the control groups (Figure 3D and 3E). Even the higher dose of LNA-92a had no additional effect compared with the standard dose of LNA-92a (5 mg/kg heart weight; Figure 3D and 3E).

miR-92a Inhibition Improves Global and Regional Myocardial Function

Next, we were interested in analyzing the effect of LNA-92a on heart function. Left ventricular end-diastolic pressure was significantly increased 72 hours after ischemia/reperfusion in both control groups and after intravenous delivery of LNA-92a compared with baseline values obtained before the onset.

Figure 2. Systemic effect of locked nucleic acid–modified antisense miR-92a (LNA-92a). Representative examples of expression analysis of miR-92a expression in different organs. Expression analysis was performed by polymer chain reaction in liver (A), kidney (B), spleen (C), and lung (D) in control LNA (LNA-Co) or LNA-92a–treated pigs (n=3, mean±SEM).

Figure 3. miR-92a inhibition reduces infarct size and myocardial apoptosis. A through C. Infarct size was determined by triphenyltetrazolium chloride viability and methylene blue exclusion staining. A, Examples of infarct reduction after locked nucleic acid–modified antisense miR-92a (LNA-92a) application (blue indicates nonischemic area; red, area at risk [AAR]; white, infarct; scale bar, 2 cm). B, Quantification of the infarct size as a percentage of the AAR. C, The AAR as a percentage of the left ventricle (LV). D and E, Cell death in the AAR was determined by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Representative images are shown in D (scale bar, 50 µm), and quantification is shown in E. n=3 with 4 replicates (n=5; mean±SEM; *P<0.05, **P<0.01).
of ischemia (Figure 4A and 4B). In contrast, both antegrade and retrograde delivery of LNA-92a abolished the increase in left ventricular end-diastolic pressure after ischemia/reperfusion (Figure 4A and 4B).

Measuring left ventricular ejection fraction revealed results similar to those shown for left ventricular end-diastolic pressure. Compared with controls, systemic application of LNA-92a did not significantly improve global myocardial function, whereas catheter-based application of LNA-92a improved ejection fraction after 72 hours and 7 days of reperfusion (Figure 4C and 4D and Figure IIB and IIIC in the online-only Data Supplement). Furthermore, the contraction

Figure 4. Local miR-92a inhibition improves global and regional myocardial function. A and B, Global myocardial function is displayed as left ventricular end-diastolic pressure (LVEDP) before ischemia and 72 hours after reperfusion in A. B, The change in LVEDP as delta day 3 to 0. C and D, Ejection fraction (EF) was analyzed in a left ventricular (LV) angiogram before ischemia and 72 hours after reperfusion (C) or is depicted as change day 3 to 0 (D). E, Analysis of regional myocardial function (subendocardial segment shortening [SES]) 3 days after ischemia/reperfusion under control or after pacing with 150 bpm (n=5; mean±SEM; *P<0.05, **P<0.01). LNA-92a indicates locked nucleic acid–modified antisense miR-92a.
velocity and relaxation velocity were enhanced after regional LNA-92a application compared with control and systemic application (Table I in the online-only Data Supplement). Analysis of hypokinetic and akinetic segments in the left ventricular angiography showed a significant reduction, particularly after antegrade delivery of LNA-92a, compared with controls and systemic application (Table I in the online-only Data Supplement).

Consistently, analysis of regional myocardial function showed an enhanced regional contractility after LNA-92a application under pacing. Catheter-based local application of the LNA-92a resulted in a significantly improved functional reserve of the ischemic region (at rapid atrial pacing) compared with controls or intravenous LNA-92a application (Figure 4E and Figure IIIID in the online-only Data Supplement).

**Inhibition of miR-92a Increases Capillary Density**
Previous studies demonstrated that inhibition of miR-92a increased angiogenesis in mice after myocardial ischemia induced by long-term ligation of the coronary artery. Therefore, we determined whether LNA-92a treatment also affects neovascularization after ischemia/reperfusion in pigs. Indeed, catheter-based delivery of LNA-92a, but not intravenous application, increased capillary density in the border zone of the infarcted hearts (Figure 5A and 5B). Because miR-92a was shown to inhibit angiogenesis by targeting integrin α5 in endothelial cells and the miR-92a target site in the 3’ untranslated region is highly conserved (Figure IV in the online-only Data Supplement), we determined the protein expression of this miR-92a target in the border zone. Consistent with the increase in capillary density after antegrade-delivered LNA-92a, the expression of the endothelial protective integrin α5 is increased compared with controls (Figure 5C and 5D).

**Inhibition of miR-92a Reduces Cardiac Inflammation**
A main effector of ischemia/reperfusion injury is overwhelming postischemic inflammation. Therefore, we additionally elucidated the effect of LNA-92a on the recruitment of leukocytes to the heart. Catheter-based LNA-92a treatment significantly reduced the number of leukocytes in the infarcted region of the heart by ≈50% compared with the control groups (Figure 6A and Figure IIIE in the online-only Data Supplement). Because no previous studies reported a pro-inflammatory property of miR-92a, we further determined the mechanism underlying this effect by in vitro studies. Therefore, we inhibited miR-92a in endothelial cells and then tested the adhesion of THP-1 cells to the endothelial monolayer after tumor necrosis factor-α stimulation under flow conditions. As shown in Figure 6B and 6C, inhibition of miR-92a in endothelial cells abolished the adhesion of THP-1 cells. Because miR-92a is also highly expressed in THP-1 cells, we additionally determined whether miR-92a inhibition...
in THP-1 cells might influence cell adhesion to endothelial cells. Indeed, pretreatment of THP-1 cells with LNA-92a also reduced THP-1 adhesion (Figure 6B and 6C), suggesting that miR-92a influences the adhesive capacity of both endothelial cells and leukocytes.

**Inhibition of miR-92a Protects Myocytes From Hypoxia/Reoxygenation–Induced Cell Death**

In addition to the vascular and anti-inflammatory effects described above, LNA-92a may act as a direct cardioprotective agent. Therefore, we investigated the influence of LNA-92a on myocyte survival. LNA-92a pretreatment of cardiomyocytes (HL-1 cells) significantly reduced hypoxia/reoxygenation–induced cell death compared with control LNA–treated cells (Figure 7A). Further analysis of the cardiomyocyte cell death revealed decreased apoptosis and necrosis after LNA-92a treatment in cells undergoing hypoxia and reoxygenation (Figure 7B).

To test whether the direct cardioprotective effect of repressing miR-92a in cardiomyocytes is indeed contributing to the improvement of heart function after acute myocardial infarction, we subjected constitutive miR-92a−/− mice and cardiomyocyte-specific miR-92a–deficient mice to acute myocardial infarction (Figure 7C–7H). Cardiac miR-92a expression was abrogated in miR-92a−/− mice and reduced by ≈30% in the cardiomyocyte-specific miR-92a–deficient mice (miR-92a fl/fl, αMHC-Cre; Figure 7D and 7G). Wall motion score index was increased in control littermates 14 days after acute myocardial infarction, indicative of an impairment of cardiac function, whereas miR-92a−/− mice showed a preserved cardiac function after acute myocardial infarction (Figure 7E).

Cardiac-specific deletion of miR-92a resulted in a partial protection of cardiac function after acute myocardial infarction (Figure 7H). Moreover, left ventricular ejection fraction was more efficiently reduced in controls (−36±10%; *P<0.05 versus day 0) compared with miR-92a−/−, αMHC-Cre mice (−27±10%; P=NS) 14 days after infarction. These data demonstrate that constitutive genetic deletion of miR-92a in all cells significantly improves cardiac function, whereas deletion of miR-92a in cardiomyocytes only partially rescues the impaired function after acute myocardial infarction.

**Discussion**

The data in the present study demonstrate that inhibition of miR-92a by an LNA-based miRNA inhibitor improves recovery after ischemia/reperfusion in a pig model. In addition, this study provides first evidence that local catheter-based delivery of the LNA-based miR-92a inhibitor more effectively augments the cardioprotective effects than systemic application. LNA-based miRNA inhibitors were tested previously in mice and large-animal models. For example, an inhibitor directed against miR-122 was shown to suppress miR-122 expression in the liver and was successfully tested in a phase II clinical trial for treatment of hepatitis C.13,17,18 In addition, LNA-based inhibitors directed against miR-15 were shown to effectively block miR-15 expression in the hearts of pigs.5 These data are consistent with our findings showing a profound inhibition of miR-92a after local delivery of LNA-92a. However, the dose used in the present study is considerably lower than the doses used in previous studies that applied 1 mg LNA-based miR-15 inhibitor per 1 kg body weight and 10 mg LNAs per 1/kg body weight to target miR-122 in monkeys.18 This may explain why intravenous infusion of LNA-92a was not fully effective in blocking miR-92a expression in the heart. In contrast, local cardiac delivery, which is estimated to deliver 5 mg/kg heart weight (=0.03 mg/kg body weight), effectively blocked miR-92a. The present study provides evidence that local catheter-based delivery more profoundly inhibits miR-92a expression in the heart compared with intravenous infusion. Thereby, both antegrade and retrograde infusion had similar effects on miR-92a inhibition in the infarct zone (Figure 1C) and on recovery of cardiac function (Figure 4).
However, antegrade delivery was slightly more effective in reducing miR-92a expression in the remote zone (Figure 1B). Of note, local cardiac delivery of viral vectors such as adenoviruses and adeno-associated viruses was more effective with the retrograde approach. However, inhibition of miR-92 by LNA-92a by antegrade intracoronary infusion appears functionally equipotent to the retrograde delivery approach, most likely because of a higher capability of the smaller molecules to transit across target cell walls. Local ante- or retrograde delivery of anti-miRs not only resulted in a higher repression of miRNAs in the heart but also had fewer effects on the expression of the miR in other organs. In particular, the expression of miR-92a in lung tissue tends to be less affected by either catheter-based delivery approach.
as opposed to intravenous application of the same amount of LNAs. However, local delivery also profoundly suppressed the targeted miRNA in liver, kidney, and spleen, indicating off-target effects resulting from coronary venous drainage of LNAs to the circulation. The partial selectivity of local LNA delivery was entirely lost when a 5-fold higher concentration of LNA-92a was used, which fully suppressed miR-92a in all tissues we analyzed. These data demonstrate that catheter-based delivery of submaximal concentrations results in preferential inhibition of miRNA expression in the heart that might be advantageous if an miRNA inhibitor is expected to have unwanted effects, e.g., in lung tissue. However, molecular strategies such as aptamers or nanoparticle-based delivery tools or viral vectors that are designed for cell-specific delivery might be better suited to obtain a cell type–specific delivery of the inhibitors.

Functionally, the local delivery of anti-miRs directed against miR-92a significantly improved the recovery of cardiac function. To the best of our knowledge, this is the first study documenting that inhibition of miRNAs can provide a functional benefit in a large-animal model of cardiac ischemia/reperfusion injury. LNA-92a reduced infarct size and augmented cardiac contractile function. Furthermore, capillary density was increased whereas inflammation was partially suppressed by miR-92a inhibition. The increase in capillary density observed after LNA-92a treatment is consistent with previous studies showing that inhibition of miR-92a improves angiogenesis and neovascularization after hind-limb and cardiac ischemia in mice. In accordance, proangiogenic factors such as endothelial nitric oxide synthase and thymosin β4 inhibit proinflammatory transcription factors of the Kruppel-like family. However, the mechanisms by which miR-92a inhibitors affect leukocyte adhesion are unknown and deserve further studies.

Inhibition of miR-92a additionally rendered cardiomyocytes resistant against hypoxia/reoxygenation–induced cell death. To determine whether a direct cardiomyocyte-protective effect might indeed contribute to the cardioprotective effects of miR-92a inhibition, we deleted miR-92a in cardiomyocytes in mice. Whereas a constitutive deletion of miR-92a completely preserved cardiac function after acute myocardial infarction, a cardiomyocyte-specific deletion only partially reduced the impairment of heart function. These data suggest that the improvement in heart function after acute myocardial infarction by miR-92a inhibition or deletion is likely attributable to the function of miR-92a in several cell types, including endothelial cells, inflammatory cells, and cardiomyocytes. Further studies in cell type–specific knockout mice are warranted to provide detailed insights into the role of miR-92a expression in endothelial cells, proinflammatory cells, and cardiomyocytes after ischemia/reperfusion.

Taken together, these results demonstrate that efficient inhibition of miR-92a exhibits pleiotropic beneficial effects, which might contribute to the improved recovery of heart function after ischemia/reperfusion injury in a preclinical pig model. In addition to endothelial protection and vascular preservation or growth, suppression of postischemic inflammation and direct cardiomyocyte protection are capable of limiting infarct size and improving regional function in the ischemic region. Thus, local antegrade delivery of LNA-92a appears to be a feasible approach to limit acute ischemia/reperfusion injury and might constitute an attractive therapeutic regimen for patients subjected to interventional treatment of acute myocardial infarction.

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Disclosures

Drs Dummeler and Zeiher hold the patent for miR-92a. Dr van Rooij is founder and shareholder of miRagen Therapeutics. The other authors report no conflicts.

References

Cardioprotection by miR-92a Inhibitors


**CLINICAL PERSPECTIVE**

Although revascularization of an infarct-related artery is state-of-the-art treatment of acute coronary syndromes, ischemia/reperfusion induces cellular stress and damage, potentially inflicting organ dysfunction. Treatment options are required to simultaneously prevent cardiomyocyte damage, endothelial dysfunction, and overwhelming postischemic inflammation. MicroRNA (miR) inhibition might meet these requirements because miRs target multiple signaling pathways, thereby providing pleiotropic effects. miR-92a, a member of the miR17-92 cluster, is upregulated after acute myocardial infarction, and its inhibition enhanced neoangiogenesis in a murine infarct model. Here, we demonstrate that regional miR-92a inhibition via locked nucleic acid–modified antisense miR-92a (LNA-92a) improved cardiac function and reduced ischemia/reperfusion–induced cardiomyocytic cell death, endothelial dysfunction, and inflammation. Regional application of LNA-92a might offer a novel therapeutic option to improve cardiac function after acute myocardial ischemia/reperfusion injury.
Inhibition of MicroRNA-92a Protects Against Ischemia/Reperfusion Injury in a Large-Animal Model

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SUPPLEMENTAL MATERIAL

Methods

Animals

German pigs were purchased from Veterinary Medicine, LMU Munich (Oberschleißheim, Germany). Animal care and all experimental procedures were performed in strict accordance to the German and National Institutes of Health animal legislation guidelines and were approved by the Bavarian Animal Care and Use Committee (AZ 55.2.1-1-54-2532-141-11). Mice experiments were performed at the University Frankfurt and were approved by the Regional Board of the State of Hessen (F88/52).

Reagents

All chemicals and cell culture media were obtained from Sigma (Taufkirchen, Germany). Contrast agent Imeron 350 was provided by Bracco Imaging (Konstanz, Germany). LNA-modified antimiRs were kindly provided by miRagen Therapeutics (Boulder, CO, USA).

Pig Ischemia-Reperfusion Model

All pig experiments were conducted at the Walter-Brendel Center for Experimental Medicine at the University of Munich. Pigs (n= 5/experimental group) were instrumented as previously described. Briefly, a percutaneous transluminal coronary angioplasty (PTCA) balloon catheter was advanced via guiding catheter and the balloon was placed in the left anterior descending artery (LAD) distal to the first diagonal branch and inflated with 6atm (0.41MPa) for 60 minutes. Correct localization of the coronary occlusion and patency of the first diagonal branch were ensured by injection of contrast agent via guiding catheter. In all groups, the PTCA balloon was deflated after 60 minutes of ischemia; the onset of reperfusion was documented angiographically. After either 72h or 7 days of reperfusion, hemodynamic measurements were performed and the pigs were sacrificed for further analysis of the infarct size, inflammation and apoptosis as described below.

AntimiR Application

AntimiRs were delivered either systemically by intravenous infusion (“LNA-92a iv”) or regional delivery, e.g. antegrade (“LNA-92a ante”) or retrograde (“LNA-92a retro”). For systemic application, LNA-92a was applied intravenously 5 min before reopening of the occluded vessel (5 mg/kg heart weight = 0.03mg/kg body weight). Local antegrade application was performed via the “over the wire” PTCA balloon catheter (either 5 mg/kg
heart weight “LNA-92a ante”, or 25 mg/kg heart weight “LNA-92a high ante”). Here the vessel was reopened after 60 min of ischemia, the “over the wire” PTCA balloon was slightly inflated again and the infusion of the LNA-92a ante and LNA-92a high ante was performed for 5 min. Retrograde application was performed via selective pressure-regulated retroinfusion as described earlier 1,2. Briefly a retroinfusion catheter was introduced to the anterior interventricular vein (AIV) before placement of the LAD occlusion. After 55 min of ischemia selective retroinfusion of 5 mg/kg heart weight LNA-92a was performed for 10 min. For control, either PBS (“control”) or control LNAs (“LNA-Co”) were applied by antegrade infusion as described above for the LNA-92a ante group at a concentration of 5 mg/kg heart weight. For 60 min ischemia and 7 days reperfusion a control groups as well a LNA-92a high ante group was performed.

**Hemodynamic Measurements**

Left ventricular enddiastolic pressure (LVEDP) and ejection fraction (EF) measurements were performed before ischemia and after 72 hours / 7 days of reperfusion. Additionally, regional myocardial function was obtained at 72 hours / 7 days of reperfusion via ultrasound crystals (Sonometrics, USA). Here the ultrasound crystals were implanted in the ischemic and non-ischemic tissue after sternotomy and opening of the pericard. Subendocardial segment shortening (SES) was assessed in the ischemic and non ischemic region at rest and under increased heart rated (150 bpm).

**Infarct size**

Infarct size was assessed by methylene blue exclusion, tetrazolium red viability staining as described earlier 1. Briefly, before explantation of the heart the LAD was ligated at the side of infarct induction and methylene blue was injected into the left ventricle. After excision of the heart, tetrazolium red was injected into the LAD distal of the occlusion site, thereby staining the viable myocytes in the ischemic area. Then the heart was cut into 5 slices and digital pictures were taken for planimetric analysis of the infarct size and the area at risk (AAR). Tissue samples of the infarct area, the AAR and the control region were harvested for TUNEL assay, capillary staining and myeloperoxidase assay. Furthermore, for detecting miR-92a expression, each of the 5 slices was further cut in pieces as representatively shown in Figure 1A.

**Histological analysis**

Myeloperoxidase assay were performed for evaluation of leukocyte influx in the ischemic area as described before 1. Tissue from area at risk (AAR) was analyzed for capillary density (PECAM-1 (Santa Cruz, California) staining). For quantification, 5 fields were
counted/region/heart Kupatt 2007. Apoptosis detection (ApopTag Kit, Millipore, Schwalbach, Germany) was performed according to the manufacturer's guidelines in the area at risk (AAR).

**RNA isolation and real-time PCR**

Tissue samples from infarcted and non-infarcted heart, as well as lung, liver, spleen and kidney were collected, total RNA was isolated with miRNeasy kits from Qiagen following the manufactures protocol and real-time PCR was performed with Applied Biosystems (Carlsbad, CA) microRNA assays for microRNAs on a StepOnePlus device (Applied Biosystems).

**Western blot**

Immunoblot analysis was performed by mixing 50 µg of protein sample with 4x protein loading dye and denaturing the proteins by heating to 95 °C for 5 min. Next, samples were loaded on a precast 8-16% gradient SDS-polyacrylamide gel for separation by electrophoresis. The proteins were then blotted onto an activated (in methanol for 1 min) polyvinylidene fluoride membrane (wet transfer for 1.5 h at 20 W). To prevent unspecific binding of antibodies to the membrane, it was blocked by incubation in 5% milk + 2% donkey-serum in TBS with 0.1% Tween-20 (TBS-T) for 30 min at RT. Next, the membrane was incubated with the primary antibody (Itga5, Cell Signaling) diluted 1:3000 in 5% BSA in TBS-T overnight. The membrane was washed three times for 10 min in TBS-T at RT and incubated with the horseradish peroxidase-conjugated secondary antibody (anti-rabbit, diluted 1:4000 in 2.5% milk in TBS-T) at RT for 2 h. For visualization, the membrane was washed 3 times with TBS-T and incubated with enhanced chemiluminescence (ECL) reagent according to the protocol of the manufacturer (Amersham), before images were acquired on the Fluorchem M (Proteinsimple, Santa Clara, CA, USA).

**Generation of miR-92 deficient mice**

The constitutive and conditional miR-92a-1 knockout mouse models were generated by homologous recombination in 129Sv/Pas embryonic stem (ES) cells by genOway (Lyon, France). For this purpose, a targeting vector containing the homologous genomic miR-92a-1 sequences flanked by loxP sites and a neomycin gene flanked by FRT sites was used. The sequence of the microRNA miR-92a exists on two Chromosomes, Chr-13 (human) resp. Chr-14 (mouse) and Chr-X. The sequence on Chr-13 (human) resp. Chr-14 (mouse) was named miR-92a-1 and the one on Chr-X is named miR-92a-2. In the KO mice the predominant miR-92a form miR-92a -1 was deleted, whereas miR-92a-2 is still intact. Recombined ES cell clones were injected into C57BL/6J blastocysts. Injected blastocysts were re-implanted into
pseudo-pregnant females to generate miR-92a recombined chimeras, which were then used either for generation of constitutive or conditional miR-92a knockout mice.

For the generation of constitutive miR-92a knockout mice (miR-92a -/-), the miR-92a recombined chimeric mice were bred with a deleter line constitutively expressing the Cre recombinase. After backcrossing for at least 5 generations with C57BL/6J wild type mice miR-92a -/- reached a background with >99% C57BL/6J. Wild type littermates were used as controls.

For the generation of conditional miR-92a knockout mice (miR-92a fl/fl, αMHC-Cre), the miR-92a recombined chimeric mice were bred with C57BL/6J wild type and Flp recombinase expressing deleter mice to excise the neomycin selection cassette. The generated miR-92a fl/+ mice were backcrossed with C57BL/6J wild type mice for at least 5 generations. miR-92a fl/fl (>99% C57BL/6J) were then mated with a Cre deleter line expressing the Cre recombinase under the control of the αMHC-Cre promotor. The resulting miR-92a fl/fl, αMHC-Cre mice were used as cardiomyocyte conditional knockout mice and miR-92a fl/fl mice were used as controls.

**Genotyping:**

DNA isolation of tails from offspring derived from mating of miR-92a +/- mice was done using the DNeasy Blood & Tissue Kit (Qiagen, Hidden, Germany) following the manufactures protocol. PCR was performed using the Novagen® KOD Xtreme™ Hot Start DNA Polymerase (Merck KGaA, Darmstadt, Germany) and the primers 28632cre-DIM1 and 28633cre-DIM1.

DNA isolation and PCRs for genotyping of miR-92a fl/fl, αMHC Cre mice were done using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, Hamburg, Germany) following the manufactures protocol. PCR primers for amplification of the miR-92a floxed allele are 28614flp-DIM1 and 28615flp-DIM1. PCR for amplification of the Cre recombinase was done using primers 4045 Cre-forw and 4046 Cre-rev in combination with an amplification of Cpxm1 as an internal control with the primers 4029 Cpxm1-forw and 4030 Cpxm1-rev.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ – 3’)</th>
<th>Fragment length</th>
<th>Application</th>
</tr>
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<tr>
<td>28632cre-DIM1</td>
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<td>miR-92a +/+ miR-92a +/- miR-92a -/-</td>
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<td>28633cre-DIM1</td>
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<tr>
<td>28614flp-DIM1</td>
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<td>28615flp-DIM1</td>
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<td>4045 Cre-forw</td>
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<td>281 bp</td>
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<td>4046 Cre-rev</td>
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<td>4029 Cpxm1-forw</td>
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<td>Cpxm1 [internal control for Cre-PCR]</td>
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**Myocardial infarction in mice**
Acute myocardial infarction (AMI) was induced by permanent ligation of the left anterior coronary artery under mechanical ventilation and anaesthesia with isoflurane and analgesia with bupivacaine (1 mg/kg, 0.25% bupivacaine 1 to 3 drops on the incision area) and carprofen (5 mg/kg subcutaneously) perioperatively and every 24 h for 3 days post-operatively. Cardiac function was determined by high resolution echocardiography (Vevo770 or Vevo2100, VisualSonics, Toronto, Ontario, Canada) at day 0, 7 and 14. Wall motion score index (WMSI) was analyzed using the 16-segment model based on 3-short axis views (4 segments apical, 6 middle, and 6 basal), with wall motion scored 1 for normal, 2 for hypokinetic, 3 for akinetic, 4 for dyskinetic, and 5 for aneurismal, according to the guidelines of the American Society of Echocardiography. WMSI was calculated as the ratio of the sum of wall motion scores over the total segments scored.

**Micro-Array:**
Transcriptional profiling was performed by ATLAS Biolabs GmbH using GeneChip Mouse Genome 430 2.0 Arrays (mRNA, full genome). Data were analyzed by use of the AltAnalyze software.

**Cardiomyocyte Cell Culture**
HL-1 cardiomyocytes were cultivated in Claycomb Medium. Cells were plated on 24well plates. Pre-treatment with either control LNA (LNA-Co) or LNA-92a was performed 24h before hypoxia as indicated in the experiments in a concentration of 500nM. After medium exchange, cells underwent 18h of hypoxia and 4h of reoxygenation. Cells death analysis were performed directly after the experiment via Trypan blue staining and 3 representative pictures per well were taken. Results are given as % of living cells. Furthermore, FACS analyses of these HL-1 cells were performed with the FITC Annexin V Apoptosis Detection Kit (BD Bioscience, Heidelberg, Germany) according to the manufactures protocol for detection of necrosis (PI) and apoptosis (Annexin V).

**Leukocyte Adhesion**
For detection of leukocyte adhesion shear stress experiments were performed as follows: Ibidi-slides (ibiTreat VI0.4, Martinsried, Germany) were seeded with HUVEC which were pretreated either with LNA-co or LNA-92a (500nM). After 24h, when cells layer was confluent, HUVECs were stimulated with TNFα (15ng/ml) for 18h. Then leukocytes, THP-1 cell line, were superfused at a concentration of 750000 cells per ml and at a flow rate of 0.57
ml/min (=1dyn/cm², Harvard apparatus, South Natick, USA). After 8 min of cell superfusion and 1 min of washing with medium only, adherent cells were counted.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LNA-co</th>
<th>LNA-92a i.v.</th>
<th>LNA-92a Retro</th>
<th>LNA-92a ante</th>
<th>LNA-92a high ante</th>
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<td>arrhythmias</td>
<td>0.33±0.21</td>
<td>0.33±0.22</td>
<td>0.33±0.20</td>
<td>0.20±0.18</td>
<td>0.16±0.16</td>
<td>0.20±0.18</td>
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<td>81±1.85</td>
<td>78±1.93</td>
<td>84±2.41</td>
<td>86±3.82</td>
<td>81±3.96</td>
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<tr>
<td>AP (72h reperfusion)</td>
<td>102±5.61</td>
<td>97±3.10</td>
<td>104±2.5</td>
<td>99±2.95</td>
<td>102±3.10</td>
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<td>dP/dt max (rest 72h reperfusion)</td>
<td>1208±80</td>
<td>1270±119</td>
<td>1202±50</td>
<td>1404±32*</td>
<td>1667±112*</td>
<td>1601±100*</td>
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<tr>
<td>dP/dt min (rest 72h reperfusion)</td>
<td>-1054±63</td>
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<td>-845±72</td>
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<td>dP/dt max (Pacing 130 72h reperfusion)</td>
<td>1273±94</td>
<td>1313±53</td>
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<td>1529±105*</td>
<td>1627±202*</td>
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<td>dP/dt min (Pacing 130 72h reperfusion)</td>
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<td>-1092±106</td>
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<td>Hypokinetic segments</td>
<td>23.3±5.0</td>
<td>25.0±6.5</td>
<td>19.3±6.0</td>
<td>10.0±2.1*</td>
<td>7.6±1.5*</td>
<td>10.0±2.9*</td>
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<tr>
<td>Akinetic segments</td>
<td>20.0±4.5</td>
<td>17.0±2.9</td>
<td>19.3±7.0</td>
<td>7.3±2.2</td>
<td>4.3±1.76*</td>
<td>8.0±2.9</td>
</tr>
</tbody>
</table>
Suppl. Figure 1

Slice 2

Region 9

Region 10

Region 11

Region 12

Region 13

Region 14

Region 15

Region 16

rel. expression miR-92a vs. U6
Suppl. Figure 3

A

Infarct [% AAR]

control  |  LNA-92a high ante

B

EF [%]

pre ischemia  |  7 days reperfusion

C

LVEDP [mmHg]

pre-ischemia  |  7 days reperfusion

D

SES [% non-ischemic region]

Rest  |  150 bpm

E

Leukocytes / g tissue

non-ischemic  |  infarct
Conservation of miR-92a:
ssc-miR-92a: 5’- UAUUGCACUUGUCCCGGCCUGU -3’
hsa-miR-92a: 5’- UAUUGCACUUGUCCCGGCCUGU -3’
mmu-miR-92a: 5’- UAUUGCACUUGUCCCGGCCUG -3’

Seed sequence

Conservation of ITGA5 in the 3’UTR:

ssc-miR-92a 3’- UGUCCGGCCCGCUUCACGUUAU -5’
Pig ITGA5 3’UTR 5’- AAACUCUGUUGCAAGUGCAAAAC -3’
Human ITGA5 3’UTR 5’- AAACUCUGUUGCAAGUGCAAAAU -3’
Mouse ITGA5 3’UTR 5’- AAACUCUGUUGCAAGUGCAAAAC -3’
Legends supplemental material

Table 1: Arrhythmias, hemodynamic and contractility after 60min of ischemia and 72h of reperfusion. (n=5, MEAN ± SEM, *p<0.05 vs. control and LNA-co)

Supplemental Figure 1: Scheme of the tissue sample dissection and results of the corresponding PCR analysis detecting miR-92a expression. N>4

Supplemental Figure 2: Scheme of the tissue sample dissection and results of the corresponding PCR analysis detecting miR-92a expression. N>4

Supplemental Figure 3: (A) Quantification of the infarct size as percentage of the area at risk (AAR) after 60 min of ischemia and 7 days of reperfusion (B) Global myocardial function is displayed as left ventricular enddiastolic pressure (LVEDP) before ischemia and 7 days after reperfusion in panel. (C) Ejection fraction (EF) was analyzed in LV-angiogram before ischemia and 7days after reperfusion. (D) Analysis of regional myocardial function (subendocardial segment shortening (SES)) 7 days after ischemia/reperfusion under control or after pacing with 150 beats per minutes. (E) Leukocyte influx was determined by myeloperoxidase measurement of ischemic and non-ischemic heart tissue. (n=3, MEAN ± SEM, *p<0.05)

Supplemental Figure 4: Sequences of miR-92a and the miR-92a target site within the 3’UTR of ITGA5.
