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Inhibition of miR-92a improves re-endothelialization and prevents neointima formation following vascular injury

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Aims MicroRNA (miR)-92a is an important regulator of endothelial proliferation and angiogenesis after ischaemia, but the effects of miR-92a on re-endothelialization and neointimal lesion formation after vascular injury remain elusive. We tested the effects of lowering miR-92a levels using specific locked nucleic acid (LNA)-based antimiRs as well as endothelial-specific knock out of miR-92a on re-endothelialization and neointimal formation after wire-induced injury of the femoral artery in mice.

Methods and results MiR-92a was significantly up-regulated in neointimal lesions following wire-induced injury. Pre-miR-92a overexpression resulted in repression of the direct miR-92a target genes integrin α5 and sirtuin1, and reduced eNOS expression *in vitro*. MiR-92a impaired proliferation and migration of endothelial cells but not smooth muscle cells. *In vivo*, systemic inhibition of miR-92a expression with LNA-modified antisense molecules resulted in a significant acceleration of re-endothelialization of the denuded vessel area. Genetic deletion of miR-92a in Tie2-expressing cells, representing mainly endothelial cells, enhanced re-endothelialization, whereas no phenotype was observed in mice lacking miR-92a expression in haematopoietic cells. The enhanced endothelial recovery was associated with reduced accumulation of leucocytes and inhibition of neointimal formation 21 days after injury and led to the de-repression of the miR-92a targets integrin α5 and sirtuin1.

Conclusion Our data indicate that inhibition of endothelial miR-92a attenuates neointimal lesion formation by accelerating re-endothelialization and thus represents a putative novel mechanism to enhance the functional recovery following vascular injury.

Keywords Endothelial cells • MicroRNAs • Re-endothelialization • Neointimal formation

1. Introduction

The introduction of drug eluting (DE)-stents after percutaneous coronary intervention (PCI) has significantly reduced the rates of in-stent-restenosis.[1] However, the substances used on current stent platforms attenuate re-endothelialization and thus increase the risk of acute or late in-stent-thrombosis.[2] Lately, selective activation of re-endothelialization after endoluminal injury has been shown to attenuate neointimal lesion formation while increasing the safety at the same time.[3] Therefore, specific approaches to selectively accelerate endothelial recovery

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are promising therapeutic options to further improve current PCI-approaches.

Small non-coding microRNAs (miRs) have emerged as key regulators of gene expression by binding to target mRNAs, thus leading to translational repression or degradation of up to hundreds of targets.[4] MiR-92a is part of the miR-17∼92a cluster and inhibits endothelial cell (EC) sprouting, vessel patterning in zebrafish, and neovascularization after ischaemia by targeting protective endothelial genes such as integrin α5 (Itga5), the class III histone deacetylase sirtuin (Sirt)-1, and the flow-induced atheroprotective transcription factors Krüppel-like factor (Klf)-2 and Klf4.[5–8] While inhibition of miR-92a improved angiogenesis in models of hind limb- or myocardial ischaemia,[5] the regulation and function of miR-92a following endovascular injury remain elusive. Therefore, we tested the effects of highly specific locked nucleic acid (LNA)-based anti-miRs targeting miR-92a on re-endothelialization and neointimal lesion formation. Moreover, we determined the specific contribution of endothelial miR-92a expression for regeneration and neointimal formation by deleting miR-92a preferentially in EC using Tie2-Cre;miR-92a(fl/fl)-mice and in haematopoietic cells (HC) using Vav1-Cre;miR-92a(fl/fl)-mice. Our data show that inhibition of miR-92a

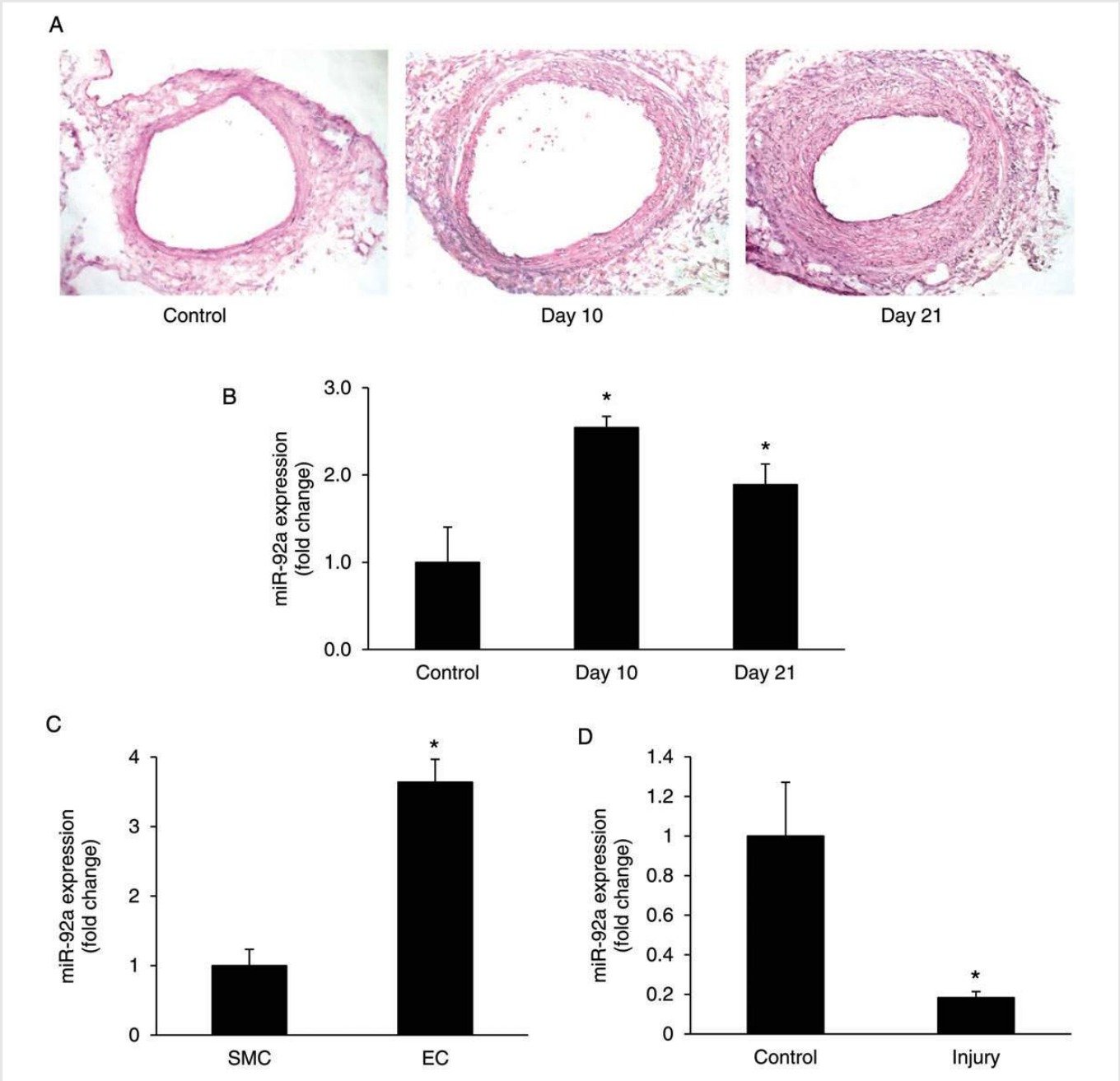

Figure 1 Regulation of miR-92a following vascular injury. (*A*) Representative H&E staining of uninjured femoral arteries and 10 and 21 days following wire-induced injury. (*B*) qPCR of neointimal lesions after wire-induced injury showed a significant up-regulation of miR-92a 10 and 21 days after injury ($n = 4$; $^*P < 0.01$). (*C*) Differential expression of miR-92a in EC and SMC *in vitro* was determined by qPCR. (*D*) MiR-92a expression in vessels before (control) and immediately after endothelial denudation (injury) was determined by qPCR ($n = 4$; $^*P < 0.01$).

in EC accelerates endothelial recovery and thus prevents leucocyte recruitment and neointimal lesion formation following wire-induced injury in mice.

2. Methods

2.1 Cell culture, transfection, detection of proliferation, and migration

Human coronary artery ECs and human coronary artery smooth muscle cells (SMC) were purchased from Lonza (Cologne, Germany) and cultured in endothelial basal medium and smooth muscle basal medium, respectively, at 37°C, 5% CO_2, air humidity until the third passage as previously described.[9] For transfection, cells were mixed with transfection complexes at numbers corresponding to 70% confluence and seeded in the respective cell-culture dishes. Transfection complexes were prepared using siPORT NeoFX Transfection Agent and Precursor-miR (pre-miR) molecules at 20 nM (Ambion, Foster City, CA, USA) according to the supplier's instructions. Sequence of 2′-*O*-methyl-oligoribonucleotides coding for pre-miR-92a was CAGGCCGGGACAAGUGCAAUA. Quantification of proliferation was assessed by using a BrdU assay as previously described (Cell proliferation ELISA, Roche, Mannheim, Germany).[10] Migration was determined in a modified Boyden chamber assay (Corning Costa Corp., Koolhovenlaan, Netherlands) in combination with a WST-assay to determine the amount of migrated cells after removal of the cells in the upper compartment of the Boyden chamber as previously described.[10] Determination of apoptosis was performed using the Cell Death Detection ELISAPlus Kit (Roche) according to the supplier's instructions. Briefly, 3500 cells per well were seeded in a 96-well microtiter plate and following transfection with pre-miRs for 24 h, cells were cultured in basal medium (EBM or SMBM, respectively) for another 24 h before quantification of apoptosis was determined using the Cell Death Detection ELISAPlus Kit.

2.2 Quantitative real-time PCR

microRNA (miR) was isolated from cells or tissue using the PureLink miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA), cDNA synthesized using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) and subjected to real-time PCR using miScript Sybr Green PCR (both: Qiagen) and MxPro 3000P (Stratagene, Cedar Creek, TX, USA). To test the efficiency of systemic injection of LNA-92a *in vivo*, total RNA was isolated with miRNeasy kits from Qiagen and real-time PCR was performed using systems from Applied Biosystems (Foster City, CA, USA). MiR levels were quantified with the $2(-\Delta Ct)$ relative quantification method using human U6 or murine sno135 as housekeeping miR. Primer sequences are available upon request.

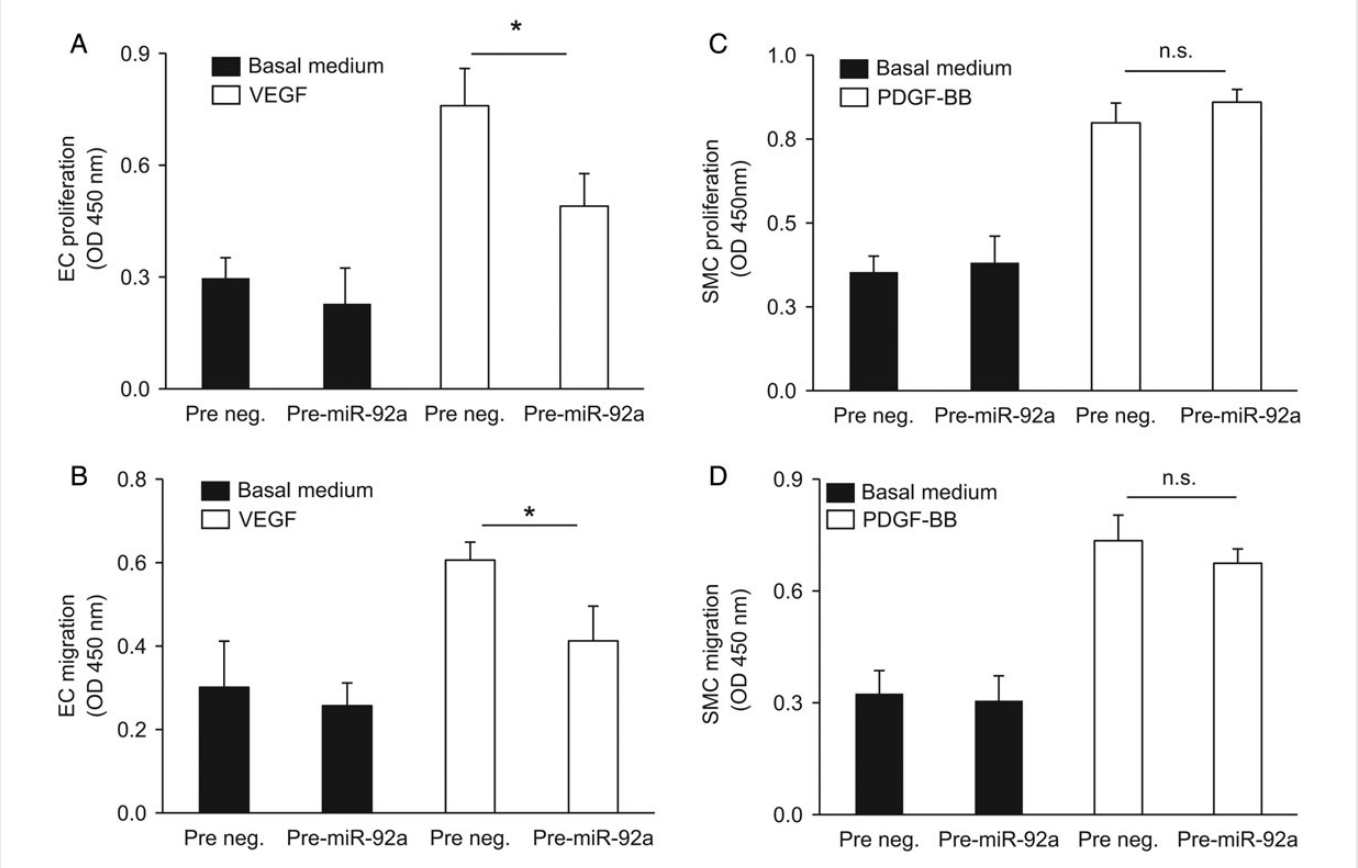

Figure 2 MiR-92a inhibits proliferation and migration of EC *in vitro*. (*A*) ECs were stimulated with VEGF after addition of pre-miR-92a or a pre-miR negative control (20 nM). Proliferation of EC was assessed at 24 h by an anti-BrdU ELISA ($n = 4$; $^*P < 0.05$). (*B*) EC migration was quantified 6 h after stimulation with VEGF after addition of pre-miR-92a or a pre-miR control by a modified Boyden chamber assay ($n = 4$; $^*P < 0.05$). (*C*) SMC were stimulated with PDGF-BB (20 ng/mL) after addition of pre-miR-92a or pre neg. Proliferation was determined at 24 h by an anti-BrdU ELISA (P = n.s.; $n = 4$). (*D*) SMC migration was assessed 6 h after stimulation with PDGF-BB (20 ng/mL) after addition of pre-miR-92a or pre neg. by a modified Boyden chamber assay. (P = n.s.; $n = 4$).

2.3 Generation of Tie2-Cre;miR-92a(fl/fl)-mice and Vav1-Cre;miR-92a(fl/fl)-mice

The conditional miR-92a-1 knockout mouse was 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. Recombined ES cell clones were injected into C57BL/6J blastocytes. Injected blastocytes were re-implanted into pseudo-pregnant females. The 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 five 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 Tie2-Cre promoter (Tie2-Cre mice) or Vav1 promoter (Vav1-Cre mice).[11] The resulting Tie2-Cre;miR-92a(fl/fl)-mice or Vav1-Cre;miR-92a(fl/fl)-mice were used as conditional knockout mice and miR-92a(fl/fl)-mice were used as controls.

2.4 Mouse femoral artery injury model

All *in vivo* experiments were performed on adult male C57BL/6J mice purchased from Charles River (Sulzfeld, Germany) as well as Tie2-Cre;miR-92a(fl/fl)-mice, Vav1-Cre;miR-92a(fl/fl)-mice, and miR-92a(fl/fl)-mice as controls. All procedures involving experimental animals were approved by the Government Animal Care Committee (GI 20/10-Nr. 110/2012) and complied with the Directive 2010/63/EU of the European Parliament.

Mice were anaesthetized with 100 mg/kg body weight ketaminehydrochloride (Ketanest®, Pfizer, Berlin, Germany) and 16 mg/kg body weight xylazinehydrochloride (Rompun® 2%, Bayer, Leverkusen, Germany). The dilation of the femoral artery was performed by inserting a straight spring wire (0.38 mm in diameter; Cook, Bloomington, IN, USA) for ~10 mm towards the iliac artery, as previously described.[12,13] Post-interventional analgesic therapy was performed using buprenorphine at 0.1 mg/kg body weight for 3 days. Inhibition of miR-92a was achieved using specific LNA-based inhibitors (miRagen Therapeutics, Boulder, CO, USA). Immediately after dilation and at Day 2 following dilation, 0.5 mg/kg LNA-92a, LNA-control, or a saline control were injected into the tail vein (0.2 mL per injection).

At the indicated time points, mice were killed by CO_2 inhalation in accordance to the AVMA guidelines for the euthanasia of animals, 2013 edition (www.avma.org) and blood samples were taken. Thereafter, mice were perfused with 4% paraformaldehyde (PFA) via the left ventricle after severing the inferior vena cava, and the femoral arteries were carefully excised.

2.5 Assessment of re-endothelialization

Mice were injected with 0.5 mL of 0.5% Evans blue dye (Sigma-Aldrich, Munich, Germany) in PBS 10 days after vascular injury, as previously described.[14] Mice were killed 15 min after injection of Evans blue dye and then perfused with 4% PFA via the left ventricle. The injured femoral artery was excised and opened longitudinally to expose the luminal surface. Planimetric analysis was performed by Metamorph Imaging 7.0 software (Molecular Devices, Downingtown, PA, USA) and the ratio of re-endothelialized area (defined as area not stained with Evans blue/total injured surface area) was determined.

2.6 Morphometric analysis

The whole artery was cut in 6 μm serial sections, and six cross-sections from regular intervals throughout each artery were stained with haematoxylin–eosin. For morphometric analyses, Metamorph Imaging 7.0 software (Molecular Devices) was used to measure external elastic lamina, internal elastic lamina, and lumen circumference as well as medial and neointimal area 21 days after injury.

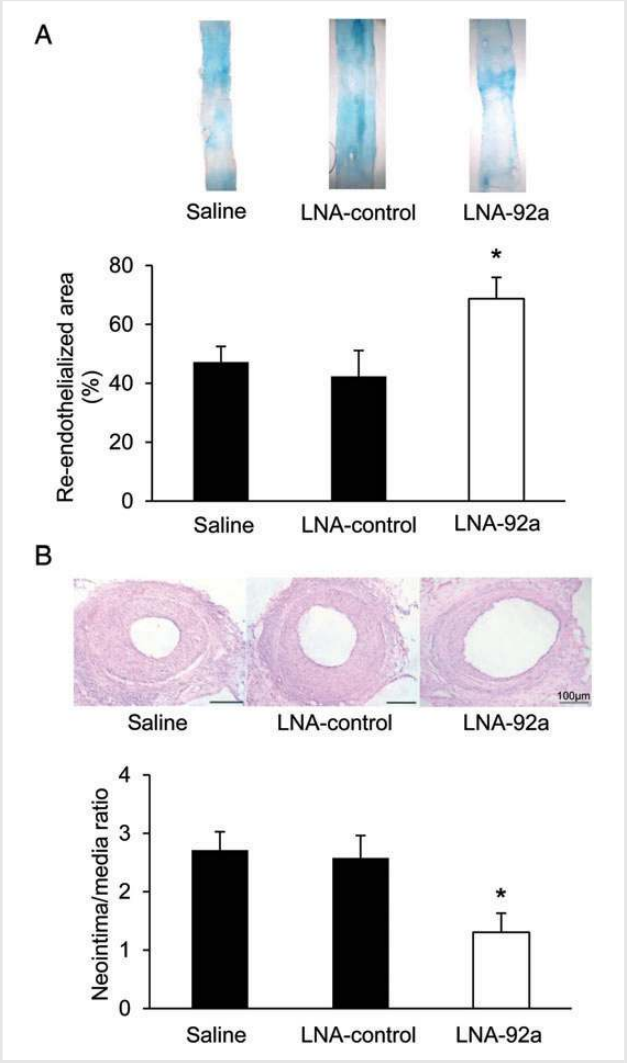

Figure 3 Enhanced re-endothelialization after targeting miR-92a. (*A*) Evans blue dye indicates non-endothelialized area by blue staining, whereas the re-endothelialized area appears white. Quantification of the areas showed increased re-endothelialization following application of LNA-92a compared with controls ($n = 6$, $^*P < 0.05$). (*B*) At 21 days after injury, neointimal lesion development of mice treated with LNA-92a was significantly reduced compared with controls ($n = 6$, $^*P < 0.05$).

2.7 Immunofluorescence and immunohistochemistry

After fixation and rehydration of 6 μm sections from the femoral artery, the slides were pre-incubated with 10% normal goat serum (Zymed® Laboratories Inc., San Francisco, CA, USA) and then incubated with antibodies against α-SMA (Sigma-Aldrich, Munich, Germany), CD31 (BD Pharmingen, Franklin Lakes, NJ, USA), CD45 (BD Pharmingen), CD68 (Serotec, Oxford, UK), Ki-67 (Abcam, Cambridge, UK), Integrin α5 (Abcam), or Sirt1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Ensuing incubations were carried out with Cy5- or Cy3-coupled secondary antibodies (Molecular Probes, Eugene, OR, USA) and counterstained with nuclear 4.6-diamidino-2-phenylindole (DAPI) (Linaris, Wertheim, Germany). Monoclonal antibodies to α-SMA were labelled directly with Cy3. Negative controls were conducted by substituting the primary antibody with an appropriate species- and isotype-matched control antibody (Santa Cruz Biotechnology).

The number of apoptotic SMC was quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) according to the supplier's instructions (*in situ* cell death detection kit, Roche).

2.8 Intimal RNA isolation from carotid arteries and the aorta

Mice were killed and perfused with saline via the left ventricle after severing the inferior vena cava. Both common carotid arteries and the abdominal aorta were then isolated and carefully cleaned of peri-adventitial tissue. The vessel lumen of a carotid artery was flushed with 75 μL and the aorta with 150 μL of QIAzol lysis reagent (QIAGEN) using a 29-gauge insulin syringe in a microfuge tube. The eluate was used for the isolation of intimal RNA using a miRNeasy mini kit (QIAGEN) as described previously.[15] Quantitative PCR was performed as described earlier and fold changes between Tie2-Cre;miR-92a(fl/fl)-mice and miR-92a(fl/fl)-mice were normalized to vascular endothelial (VE)-cadherin.

2.9 Blood tests

Blood samples were collected 14 days after injury in mice treated with LNA-92a, control LNA, or saline ($n = 6$). The analysis was performed by the department of clinical chemistry at the Justus-Liebig-University, Giessen, based on a biochemical panel and a full blood count.

2.10 Statistical analysis

Data between the study groups were analysed with one-way ANOVA (Systat, Erkrath, Germany) followed by pair-wise multi-comparison using the Holm-Sidak method. Statistically significant differences between two groups were determined by using the Student's *t*-test. A probability value <0.05 was considered statistically significant for all comparisons.

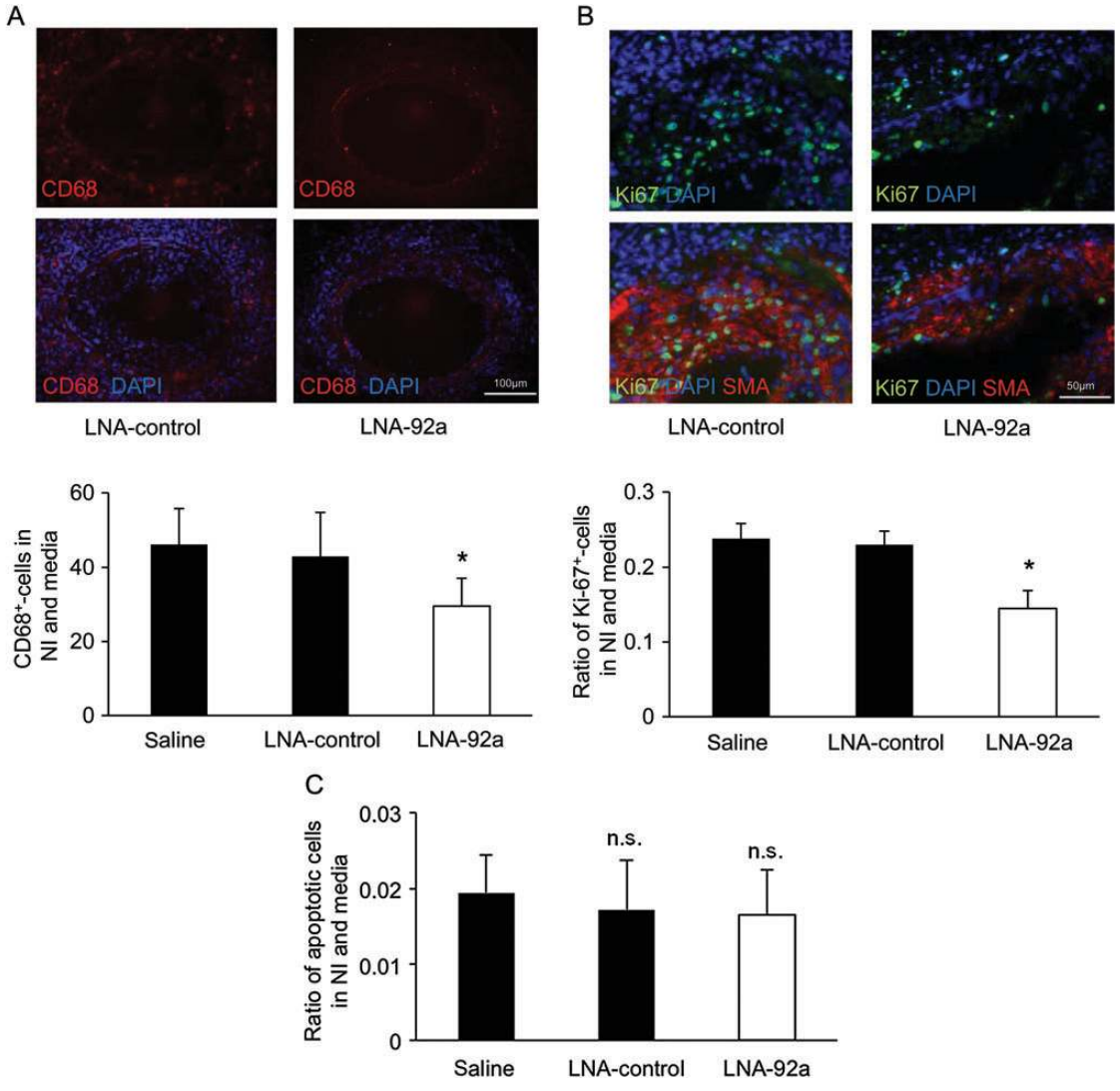

Figure 4 Systemic application of LNA-92a attenuates the inflammatory response and inhibits SMC proliferation following vascular injury. (*A*) Representative staining and quantification of CD68 (red) and DAPI (blue) positive cells within the neointimal lesion and medial layer 14 days after injury is shown. ($n = 6$, $^*P < 0.05$). (*B*) Representative staining and quantification of proliferating cells within the neointimal and medial layer is indicated by Ki-67 (green). Co-staining was performed with DAPI (blue) and α-smooth muscle actin (SMA, red). The number of proliferating cells within the neointima (NI) and media was determined by dividing the number of Ki-67 positive cells per section by the total cell number per section ($n = 6$, $^*P < 0.05$). (*C*) TUNEL staining of dilated arteries was performed 14 days after injury. Treatment with LNA-92a had no effect on apoptosis within neointimal lesion and the medial layer compared with controls.

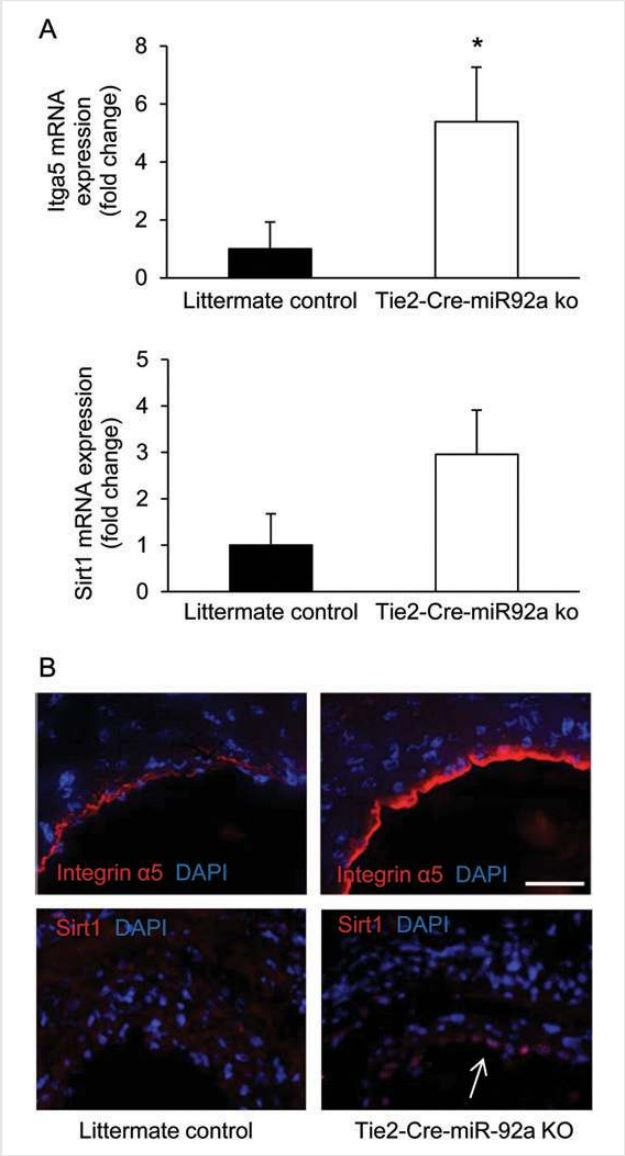

Figure 5 Conditional knockout of miR-92a in EC induces up-regulation of miR-92a target genes. (*A*) mRNA levels of Itga5 and Sirt1 were assessed in freshly isolated and selected EC from the carotid artery and the aorta of Tie2-Cre;miR-92a(fl/fl)-mice and compared with controls ($n = 3$, $^*P < 0.05$ for Itga5 and $P = 0.09$ for Sirt1). (*B*) Immunohistochemistry indicates a robust increase in Itga5 and Sirt1 expression levels in conditional miR-92a knockout mice compared with controls in the regenerating endothelium following vascular injury. The arrow indicates the luminal side of the neointimal lesion which is lined by ECs expressing Sirt1.

3. Results

3.1 MiR-92a expression is increased in neointimal lesions

After wire-induced injury of the femoral artery, miR-92a expression was significantly increased at a time point of partial re-endothelialization at Day 10 (fold change 2.5 $\pm$ 0.13; $n = 4$; $^*P < 0.01$) and remained significantly elevated up to Day 21 until complete re-endothelialization after injury, as determined by qPCR (*Figure 1A* and *B*). In vascular cells *in vitro*, miR-92a was found to be predominantly expressed in human coronary ECs and to a much lesser extent in human coronary artery SMC (*Figure 1C*). *In vivo*, wire-induced denudation of the endothelial layer resulted in significantly lower levels of miR-92a expression within whole artery lysates when compared with uninjured arteries still containing the endothelial layer (*Figure 1D*) as determined immediately after denudation.

3.2 Pre-miR-92a inhibits proliferation and migration of EC but not SMC

Treatment of vascular cells with pre-miR-92a prevented proliferation of EC but not SMC, as shown by a BrdU incorporation assay (OD 450 nm: 0.490 $\pm$ 0.087 vs. 0.759 $\pm$ 0.099; $n = 4$; $^*P < 0.001$). Moreover, pre-miR-92a specifically inhibited EC but not SMC migration, as determined by a modified Boyden chamber assay (OD 450 nm: 0.412 $\pm$ 0.083 vs. 0.606 $\pm$ 0.042; $n = 4$; $^*P < 0.05$) (*Figure 2A–D*). In contrast, pre-miR-92a had no effect on EC or SMC apoptosis (Supplementary material online, *Figure S1*). Following overexpression of pre-miR-92a in EC, a significant down-regulation of Itga5, Sirt1, and eNOS mRNA expression levels (which previously have been described as direct and indirect miR-92a target genes) was detected 24 h after transfection (Supplementary material online, *Figure S2*).

3.3 Inhibition of miR-92a enhances re-endothelialization and reduces neointimal formation

Systemic injection of LNA-92a into the tail vein significantly decreased the expression levels of miR-92a in various tissues, including heart, aorta, and muscle of healthy mice in a dose-dependent manner, as determined by qPCR (Supplementary material online, *Figure S3*, data not shown). Immunohistochemical analysis of the previously validated miR-92a target genes Itga5 and Sirt1 showed an enhanced expression of both proteins in dilated arteries following treatment with LNA-92a compared with controls 14 days after injury (Supplementary material online, *Figure S3*).

Systemic application of LNA-92a (0.5 mg/kg body weight) immediately after vascular injury and 48 h later significantly enhanced the recovery of the endothelial layer compared with controls 10 days after injury (69 $\pm$ 7 vs. 42 $\pm$ 9% of denuded area; $n = 6$; $^*P < 0.05$) (*Figure 3A*). Moreover, neointimal lesion development was significantly reduced by LNA-92a treatment compared with the control groups 21 days after injury (neontima/media ratio: 1.30 $\pm$ 0.33 vs. 2.58 $\pm$ 0.39; $n = 6$; $^*P < 0.001$) (*Figure 3B*). Concomitantly to the accelerated re-endothelialization, the number of accumulating $CD68^+$ monocytes/macrophages within the vascular wall was significantly reduced following LNA-92a treatment 14 days after injury (29 $\pm$ 8 vs. 43 $\pm$ 12 cells/section; $n = 6$; $^*P < 0.01$) (*Figure 4A*). Furthermore, the number of proliferating $Ki67^+$ SMC within the medial layer and neointimal lesion was reduced 14 days after injury (15 $\pm$ 2 vs. 23 $\pm$ 2% of SMC/section; $n = 6$; $^*P < 0.01$) (*Figure 4B*). In contrast, treatment with LNA-92a had no effect on apoptosis of neointimal and medial cells compared with controls (*Figure 4C*). Analysis of blood panels of mice treated with LNAs did not reveal any significant changes 14 days after injury (Supplementary material online, *Table S1*)

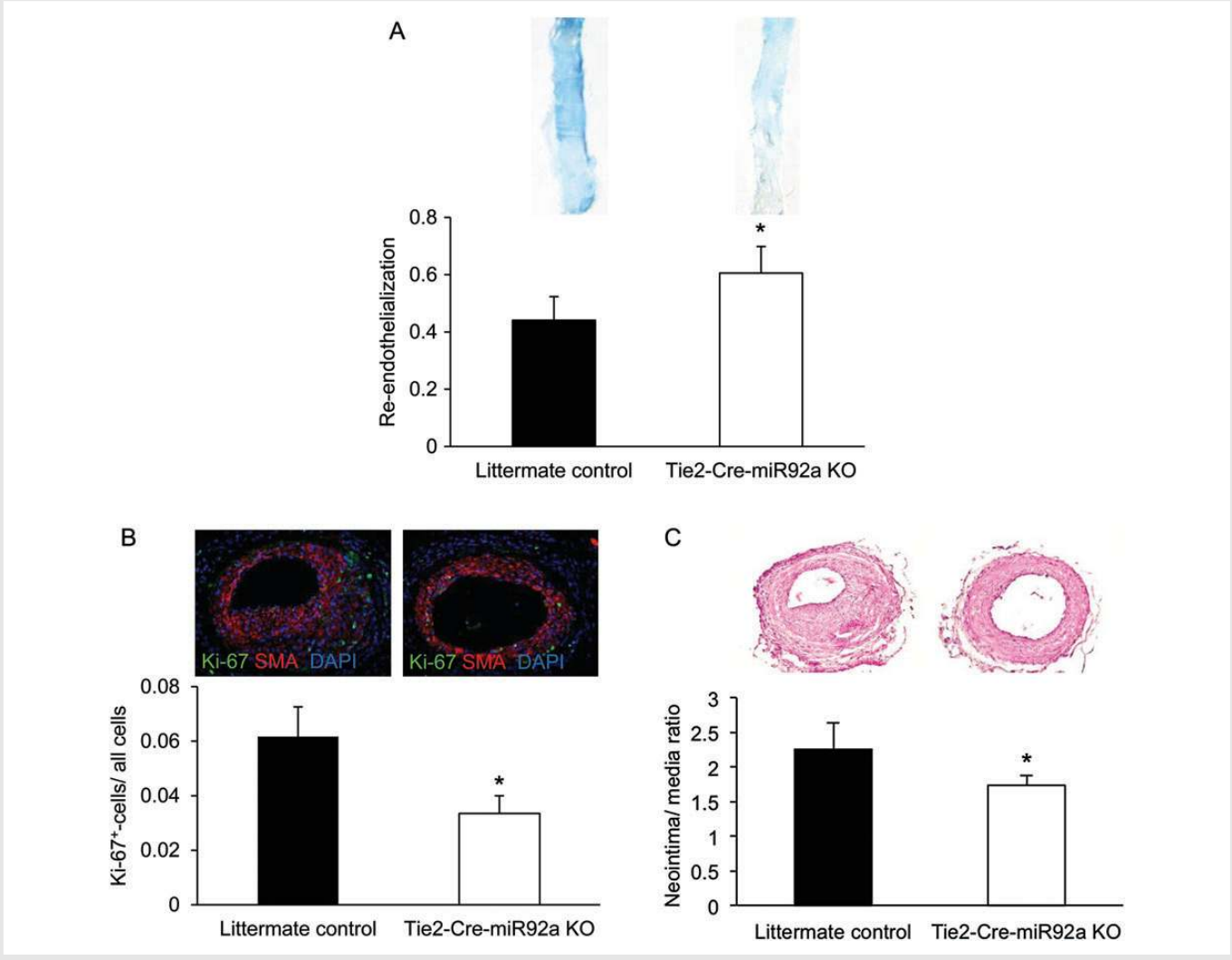

Figure 6 Conditional knockout of miR-92a in EC promotes re-endothelialization and inhibits neointimal formation. (*A*) Evans blue staining shows enhanced re-endothelialization in conditional miR-92a knockout mice compared to controls at 10 days after endothelial injury ($n = 6$, $^*P < 0.05$) (*B*) Quantification of Ki67 staining (green) showed a significantly reduced number of proliferating cells within the neointimal and medial layer of conditional miR-92a knockout mice compared with controls 21 days after injury ($n = 6$, $^*P < 0.05$). (*C*) Neointimal lesion development was significantly reduced in conditional miR-92a knockout.

3.4 Conditional miR-92a knockout in EC but not HC accelerates re-endothelialization and attenuates neointimal formation

To further evaluate the specific contribution of endothelial miR-92a expression, we deleted miR-92a preferentially in EC by crossing miR-92a(fl/fl)-mice with Tie2-Cre deleter lines (Supplementary material online, *Figure S4*). These mice were viable and showed the expected mendelian ratio. In total heart tissue, miR-92a expression was slightly reduced, whereas miR-92a expression was abolished in sorted VE-cadherin positive lung EC (Supplementary material online, *Figure S5*). The expression of the other cluster members was not significantly changed in sorted EC (Supplementary material online, *Figure S5*). As expected, in freshly isolated EC from uninjured arteries, the expression of the miR-92a target Itga5 was significantly increased and a trend towards an enhanced expression of Sirt1 was observed (*Figure 5A*). As determined by immunohistochemistry, higher expression levels of Itga5 and Sirt1 were also detected in the recovering endothelial layer of the Tie2-Cre;miR-92a(fl/fl)-mice following vascular injury (*Figure 5B*). The conditional knockout of miR-92a in EC resulted in accelerated re-endothelialization 10 days after endovascular injury compared with the littermate controls (60 ± 4 vs. $44 \pm 8\%$; $^*P < 0.05$; $n = 5–6$) (*Figure 6A*). Conclusively, proliferation of vascular SMC as determined by Ki67 immunoreactivity (6.1 ± 1.1 vs. $3.4 \pm 0.6\%$; $^*P < 0.05$; $n = 5$) and the development of neointimal lesions were significantly reduced in Tie2-Cre;miR-92a(fl/fl)-mice 21 days after injury (neointima/media ratio: 1.73 ± 0.14 vs. 2.26 ± 0.39; $n = 6$; $^*P < 0.05$) (*Figure 6B* and *C*).

Since HC and inflammatory cells are critically involved in endothelial regeneration, we generated mice deleting miR-92a by Vav1-promoter-driven Cre recombinase, which is selectively expressed in HC.[11] However, re-endothelialization in Vav-Cre;miR-92a(fl/fl)-mice was not significantly altered when compared with littermate controls 10 days after wire-induced injury (59 ± 7 vs. $53 \pm 4\%$; $^*P =$ n.s.; $n = 5–6$) (*Figure 7*),

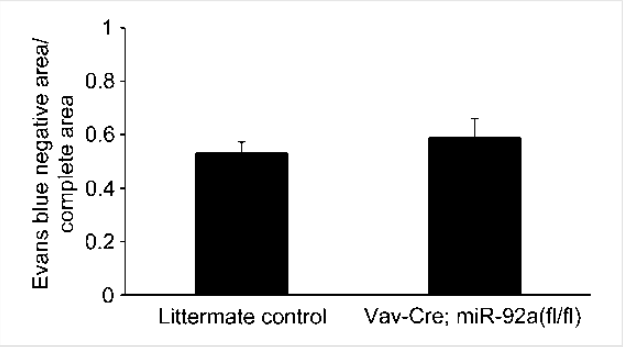

Figure 7 Conditional knockout of miR-92a in HC does not significantly promote re-endothelialization. Analysis of Evans blue staining in conditional miR-92a knockout mice under the control of a Vav1 promoter did not show enhanced re-endothelialization after knockout of miR-92a in HC compared with controls 10 days after endothelial injury ($n = 5–6$, $^*P$ = n.s.).

indicating that the effect of miR-92a deletion on re-endothelialization is mainly due to the reduced miR-92a-expression in local EC following injury.

4. Discussion

The data of the present study demonstrate that inhibition of miR-92a enhances re-endothelialization and prevents neointimal lesion formation after endovascular injury. The protective effects of inhibiting miR-92a on endothelial recovery in conjunction with the previously described enhancement of angiogenesis after ischaemia suggest that miR-92a inhibitors represent a very promising therapeutic option for the treatment of coronary artery disease.[5] Whereas previous studies in ischaemia models were using antagomirs, which are cholesterol-modified anti-sense RNAs, the present study used LNA-based antimiRs. After systemic injection, antagomirs and LNA-based antimiRs both efficiently suppressed miR-92a expression in the heart; however, LNA-92a suppressed miR-92a at much lower concentrations compared with antagomir treatment in previous studies.[16] Importantly, LNA-92a treated mice did not show signs of toxicity and all blood parameters were within the normal range (Supplementary material online, *Table S1*).

The mechanisms underlying the therapeutic benefit of LNA-92a very likely involve a direct pro-regenerative effect on local ECs adjacent to the injury, which secondarily influences leucocyte accumulation and SMC proliferation. Both, systemic inhibition as well as genetic deletion of miR-92a in Tie2-expressing cells, which comprise preferentially EC, provides a therapeutic benefit with increased endothelial regeneration and an inhibition of neointimal lesion formation. On the other hand, our data clearly indicate that modulating miR-92a levels did not directly influence proliferation and migration of SMC *in vitro*. Moreover, selective deletion of miR-92a in HC, which contribute to endothelial regeneration following vascular injury, resulted only in a mild but non-significant enhancement in re-endothelialization. Therefore, we speculate that the enhancement of local EC-regenerative capacity and the resulting acceleration of re-endothelialization, which represents an established mechanism for decreasing the inflammatory response and suppressing SMC proliferation following vascular injury *in vivo*,[17] is the key mechanism that explains the observed benefit of LNA-92a treatment on neointimal lesion formation (Supplemental material online, *Figure S6*). This hypothesis is further supported by a recent report demonstrating that systemic inhibition of miR-92a expression prevents ox-LDL-dependent EC activation and subsequent atherosclerotic plaque progression.[18] The reason why miR-92a inhibition preferentially targets EC function may also be due to the fact that miR-92a is predominantly expressed in EC in intact vessels *in vivo* when compared with SMC and other vascular cells (*Figure 1C* and *D*).

Among the verified targets of miR-92a, Itga5 and Sirt1 are known to critically influence EC proliferation and migration.[19] Whereas in our previous work only a trend but no significant down-regulation of Sirt1 by miR-92a was observed in human umbilical vein ECs as assessed by microarray, we now find a significant down-regulation of Sirt1 at 24 h in human coronary artery ECs by qPCR.[5] Overexpression of Sirt1 has been shown to reduce atherosclerotic lesion formation and to protect against neointima formation following vascular injury in mice.[20,21] The expression of Itga5 in EC is essential for the regenerative capacity of the cells following vascular injury, mainly due to its interaction with fibronectin, which represents an abundant component of the extracellular matrix (ECM) following vascular injury.[22] Indeed, the concerted interaction of integrins with ECM components is a prerequisite for the adhesion, proliferation, and migration of EC in the process of re-endothelialization. However, it seems likely that also other direct or indirect targets of miR-92a (e.g. such as Klf2 and Klf4 or eNOS) may affect endothelial repair and neointimal formation. Even though eNOS is not a direct target of miR-92a, we do find a down-regulation that has been shown in previous work to be mediated by Itga5[5] or Klf2.[23] Moreover, Sirt1 was shown to directly deacetylate and thereby activate eNOS.[24,25] Therefore, the indirect up-regulation and activation of eNOS may represent an additional effect contributing to enhanced endothelial regeneration following the knock-down of miR-92a. In addition, miR-92a might be involved in more complex epigenetic regulations of cell function since histone deacetylase-9, which was associated with coronary artery disease in GWAS studies,[26] has been shown to increase the expression of the miR-17–92 cluster in EC.[27] And the histone-deacetylase Sirt1, as a target of miR-92a, might in turn modulate chromatin remodelling and cellular reprogramming.

In summary, we show that inhibition of miR-92a does not only improve angiogenesis in ischaemic tissues, but also accelerates re-endothelialization after vascular injury and thus reduces neointimal lesion formation. Given the recent demonstration that LNA-based inhibitors directed against miR-92a improved neovascularization in a large animal model[28] and the safety and efficacy of LNA-based inhibitors directed against miR-122 in clinical phase II trials in hepatitis,[29] one may speculate that miRNA inhibitors may serve as an attractive strategy to improve outcomes of patients with acute or chronic vascular diseases.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: E.v.R. is a scientific co-founder and employer of miRagen Therapeutics, Inc. S.D. and A.B. have a patent regarding the use of miR-92 for neovascularization.

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