MicroRNA-24 Regulates Vascularity After Myocardial Infarction

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Background—Myocardial infarction leads to cardiac remodeling and development of heart failure. Insufficient myocardial capillary density after myocardial infarction has been identified as a critical event in this process, although the underlying mechanisms of cardiac angiogenesis are mechanistically not well understood.

Methods and Results—Here, we show that the small noncoding RNA microRNA-24 (miR-24) is enriched in cardiac endothelial cells and considerably upregulated after cardiac ischemia. MiR-24 induces endothelial cell apoptosis, abolishes endothelial capillary network formation on Matrigel, and inhibits cell sprouting from endothelial spheroids. These effects are mediated through targeting of the endothelium-enriched transcription factor GATA2 and the p21-activated kinase PAK4, which were identified by bioinformatic predictions and validated by luciferase gene reporter assays. Respective downstream signaling cascades involving phosphorylated BAD (Bcl-XL/Bcl-2–associated death promoter) and Sirtuin1 were identified by transcriptome, protein arrays, and chromatin immunoprecipitation analyses. Overexpression of miR-24 or silencing of its targets significantly impaired angiogenesis in zebrafish embryos. Blocking of endothelial miR-24 limited myocardial infarct size of mice via prevention of endothelial apoptosis and enhancement of vascularity, which led to preserved cardiac function and survival.

Conclusions—Our findings indicate that miR-24 acts as a critical regulator of endothelial cell apoptosis and angiogenesis and is suitable for therapeutic intervention in the setting of ischemic heart disease. (Circulation. 2011;124:720-730.)

Key Words: myocardial infarction • microRNAs • angiogenesis • antagonim • gene expression • heart failure
of organs,\textsuperscript{19} it is enriched in endothelial cells,\textsuperscript{20} but its role in the cardiovascular system remains basically uncertain. Here, we show that miR-24 acts as a critical regulator of endothelial cell apoptosis and angiogenesis and is suitable for therapeutic intervention in the setting of ischemic heart disease.

**Methods**

**miRNA/RNA Isolation, miRNA Reverse Transcription–Polymerase Chain Reaction, and Global Transcriptome Analysis**

RNA isolation was performed with TRIzol reagent (Invitrogen) or the miRVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. For detection of miRNAs in samples, different TaqMan miRNA assays (Applied Biosystems) were applied (online-only Data Supplement Table I). The small RNA molecule U6 small nuclear (Rnu6−2) was amplified as a control. Reverse transcription–polymerase chain reaction analysis was performed in an iCycler (Bio-Rad). To assess RNA integrity for downstream analysis, total RNA was subjected to capillary chromatography in an Agilent bioanalyzer 2100. Gene array analysis was performed with the Affymetrix GeneChip system according to the manufacturer’s instructions and with Human Gene 1.0ST arrays (Affymetrix Systems). Further microarray analyses and data handling were performed as described previously.\textsuperscript{21}

**Transfection Assays**

Transient liposomal transfection of small inhibitory RNAs (siRNAs) or miRNAs was performed according to the manufacturer’s instructions. Briefly, cells were split 1 day before transfection to reach 60% to 70% confluence on the day of transfection. Specific siRNAs/ miRNAs and control siRNA/miRNA and Lipofectamine 2000 (Invitrogen) were mixed separately and incubated for 5 minutes with Opti-MEM I media (Invitrogen). Complexes were added together and incubated for 20 minutes. Media were changed to antibiotic-free media before the addition of liposomal siRNA complexes (final concentration 150 nmol/L for siRNA and 100 nmol/L for miRNAs). Cells were incubated for 4 hours before the media were changed to fresh media. Silencing of proteins or miRNA targets was monitored for 48 hours (siRNA) or 72 hours (miRNAs) after transfection by Western blot analysis. Specific details about the siRNAs and miRNAs used are given in Table II in the online-only Data Supplement.

**Apoptosis Detection**

Apoptosis was measured with the Annexin-V-Fluos kit from Roche Diagnostics (Penzberg, Germany) according to the manufacturer’s instructions and as described previously.\textsuperscript{6} Detection of apoptosis was either done by fluorescent-activated cell sorter analysis on a FACScalibur (BD Biosciences) or by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays.

**Western Blotting and ELISA**

Western blot analysis was performed with 10 to 40 μg of total protein. Protein was blotted onto polyvinylidene fluoride membrane in Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Afterward, different antigens were detected by appropriate antibodies (online-only Data Supplement Table III). For phosphorylated Bcl-XL/Bcl-2–associated death promoter (phospho-BAD; Ser112) detection in cell culture samples, we applied a PathScan phospho-BAD (Ser112) sandwich ELISA kit (No. 7182; Cell Signaling) according to the manufacturer’s instructions. Phospho-BAD levels were related to total BAD expression levels as obtained by Western blotting.

**Analysis of Capillary and Arteriolar Density**

Analyses of capillary and arteriolar density were performed in transverse sections of the perinfarct zone and the remote zone from left ventricles 14 days after MI. Capillary and arteriolar densities were evaluated after fluorescent immunohistochemical staining for platelet and endothelial cell adhesion molecule-1 (PECAM-1; endothelial marker) or α-smooth muscle actin (Acta2; smooth muscle cells) with antibodies listed in online-only Data Supplement Table III. Arterioles were recognized as vessels with 1 or more continuous layers of Acta2\textsuperscript{+} vascular smooth muscle cells. The number of capillaries and arterioles per square millimeter was counted in a blinded fashion.

**Tube Formation and Spheroid Formation Assay**

Transfection or transduction of cultured cells was performed as mentioned previously. Transfection. Then, cells were harvested and 15 000 cells were seeded on top of Matrigel-coated chamber slides (BD). After 6 to 8 hours and 24 hours, pictures (MetaXpress Cell imaging system) were taken on a Zeiss Axioskop microscope (Jena, Germany). In selected experiments, an AP-1 assay was used. For spheroid formation, miRNA-transfected human umbilical vein endothelial cells were trypsinized and collected in EBM-2 medium containing FCS and 20% Methocel (Sigma-Aldrich). 500 cells in 100 μl of medium were plated per well in a 96-well plate (U-shaped bottom) and cultured overnight at 37°C, 5% CO2. The next day, spheroid formation was visualized by microscopy on a Nikon ECLIPSE Ti at 10× or 20× magnification. Spheroids were harvested at 200g and resuspended in a mixture of 80% Methocel and 20% FCS. Next, 50 spheroids per well were distributed in a 24-well plate by taking 500 μL of spheroid suspension together with 500 μL of collagen-containing matrix (DMEM, collagen solution, and NaOH). After 30 minutes incubation at 37°C, 100 μL of EBM-2 medium supplemented with 40% FCS and basic fibroblast growth factor (30 ng/mL) was given on top of casted gels. Twenty-four hours after initial seeding, endothelial sprouts were fixed with 10% paraformaldehyde before spraying capacity was evaluated by microscopy.

**Scratch Wound (Migration) Assay**

Transfected human umbilical vein endothelial cells were cultivated in EBM-2 medium at 37°C, 5% CO2. The scratches in the cell monolayer were generated with a 100-μL tip, and the cells were photographed at 0, 8, and 24 hours with a Zeiss Axiovert 135 microscope. Subsequently, the distance between cell fronts was measured with an AxioVision documentation system (Zeiss).

**Proliferation Assays**

To measure proliferative capacity in miRNA-modulated cells, a WST-1 (Roche) or standard bromodeoxyuridine proliferation assay (Calbiochem) was applied. miRNA transfection was performed as mentioned under Transfection Assays. Next, medium was changed and replaced by WST-1 or bromodeoxyuridine reagent as instructed by the manufacturer. WST-1 and bromodeoxyuridine absorbances were measured at 450 and 340 nm, respectively.

**Luciferase Reporter Assays**

A luciferase reporter assay system was applied to validate potential miRNA targets as described previously.\textsuperscript{6} A putative 3′UTR miRNA binding sequence was cloned into the SpeI and HindIII cloning site of pMIR-REPORT vector (Ambion). Mutations in the respective miR-24 binding sites were introduced by site-directed mutagenesis (Quick Change II–Site-Directed Mutagenesis Kit; Stratagene). The resulting construct was cotransfected with the miRNAs of interest in HEK293 reporter cells in 48-well plates by use of Lipofectamine
MiR-24 Is Upregulated in Endothelial Cells by Cardiac Ischemia

MiR-24 Triggers Endothelial Apoptosis and Impairs Angiogenesis

GATA2 and PAK4 Are Targets of miR-24

Myocardial Infarction

In Vivo Studies

Zebrafish Assays

Chromatin Immunoprecipitation

Viral Transduction

miRNA was applied. Cells were incubated for 24 hours before luciferase and β-galactosidase activity was measured (Promega).

2000 (Invitrogen). A total of 0.2 μg of plasmid DNA and 100 nmol/L miRNA was applied. Cells were incubated for 24 hours before luciferase and β-galactosidase activity was measured (Promega).

2-AU (MO1-pak4) and the fifth exon/intron was a gift from Marjo Simonen (Novartis, Basel, Switzerland).

The original green fluorescent protein (GFP)–murine-GATA2 plasmid was a gift from Marjo Simonen (Novartis, Basel, Switzerland). N-terminal GFP-tagged GATA2 was subcloned in an appropriate adenoviral entry vector. Adenoviruses were generated with the Gateway system (Invitrogen) by polymerase chain reaction amplification of the human cDNA sequence and recombination into the pAd/CMV/V5 destination vector (Invitrogen). Subsequently, 15 μg of purified recombinant adenoviral DNA was digested with PacI and precipitated with sodium acetate. One microgram of linearized vector was transfected to HEK 293 cells (Invitrogen) with Effectene precipitation with sodium acetate. One microgram of linearized pcDNA3 (Invitrogen). A total of 0.2 mol/L MO1-gata2a26 or 0.4 mol/L MO2-gata2a. In the case of pak4, morpholinos were designed against the ATG (MO1-pak4) and the fifth exon/intron boundary (MO2-pak4). One- to 2-cell stage embryos were injected with 2 to 4 ng of MO1-pak4 or 4 to 8 ng of MO2-pak4, respectively. Morpholino knockdown efficiency for start-site–targeting morpholinos was tested as recommended before by an in vivo translation-blocking assay. For the splice-site–targeting morpholino, MO2-pak4 knockdown efficiency was tested by RNA isolation from morpholino-injected zebrafish with TRizol (Invitrogen) and subsequent cDNA from DNase-treated total RNA with M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega). Primers used for reverse transcriptase polymerase chain reactions are available on request.

For confocal analyses, zebrafish embryos were fixed at 48 hpf in 4% paraformaldehyde solution overnight and embedded in 1.5% low melting point agarose. Confocal images were obtained with a Leica TCS SP2 confocal laser scanning microscope. The maximum projection algorithm of the Leica software was then used to calculate information sectored in different ranks to a 2-dimensional projection.

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dothelium (Table 30–42). Of major interest were the transcription factor \( \text{GATA2} \) and the \( \text{p21-activated kinase} \ \text{PAK4} \), both of which have established roles in vascular biology.\(^{30,43,44}\) To validate those targets, primary endothelial cells were first transfected with miR-24 precursors. This resulted in repression of GATA2 and PAK4 protein expression. PAK4 was additionally repressed at the mRNA level (Figure 3A and data not shown). Inhibition of miR-24 in endothelial cells by specific antagonists increased GATA2 and PAK4 expression (data not shown).

When we fused the respective 3’UTR regions to a luciferase reporter gene and determined luciferase activity in cells transfected with synthetic miR-24 precursors, miR-24 significantly repressed luciferase activity, whereas unrelated miRNAs showed no effect (Figure 3B). We thus identified GATA2 and PAK4 as direct targets of miR-24. Bioinformatic miR-24 target prediction identified further potential targets (Table). We also validated the RAS p21 protein activator \( \text{RASA1} \) and the histone coding gene \( \text{H2AFX} \), because of its described role in tumor vascularization.\(^{35}\) Overexpression of miR-24 in endothelial cells reduced RASA1 and H2AFX protein expression, and luciferase gene reporter assays identified them as direct miR-24 targets (online-only Data Supplement Figures Ila and IIb). Functionally, RASA1 silencing induced modest endothelial apoptosis but did not alter tube formation, whereas no effects were seen after H2AFX down-regulation (online-only Data Supplement Figures Iic through IIe). In strong contrast, silencing of both GATA2 and PAK4 in endothelial cells by siRNA abrogated tube formation.

Figure 1. Selective miR-24 upregulation in endothelial cells after myocardial infarction (MI). A, Expression of miR-24 relative to Rnu6–2 in fractionated endothelial cells (magnetic affinity cell-sorted CD146\(^+\) cells), cardiac fibroblasts, and cardiomyocytes of the perifarct (MI) or remote region 0, 1, 3, or 14 days after MI in mice. In situ hybridization of miR-24 with fluorescent probes and costaining against (B) endothelial (platelet and endothelial cell adhesion molecule-1 [Pecam1]) and (C) smooth muscle cell (Acta2) markers in mice after MI. D, Expression of \( \text{miR-24/RNU6–2} \) in human umbilical vein endothelial cells 24 hours after normoxic (21% O\(_2\)) or hypoxic conditions (1% O\(_2\)), \( n = 3 \) to 7 per experiments or animals per group. Data are mean and SEM; \( * P < 0.05, ** P < 0.005 \). FC indicates fold change.

Figure 2. Activation of endothelial apoptotic programs and impairment of angiogenic properties by miR-24. A, Relative changes of apoptotic cells 72 hours after transfection of human umbilical vein endothelial cells with scrambled-miR (scr), synthetic miR-24 precursors (pre-24), or miR-24 antagonists (anti-24). B, Changes of apoptotic endothelial cells after transfection with scr-miR, pre-miR-24, or anti-miR-24 for 72 hours and subsequent exposure to hypoxia (1% O\(_2\), 24 hours) or normoxia (21% O\(_2\), 24 hours). C, Tube formation (top), spheroid formation (middle), and sprouting (bottom) capacity of human umbilical vein endothelial cells 72 hours after transfection with scr-miR, pre-miR-24, or anti-miR-24. D and E, Migratory (scratch wound assay; D) and proliferation (bromodeoxyuridine assay; E) capacity of human umbilical vein endothelial cells 72 hours after transfection with miR-24 or scrambled controls. \( n = 3 \) to 4 experiments per group. Data are mean and SEM; \( * P < 0.05, ** P < 0.01, *** P < 0.005 \).
capacity and significantly induced apoptosis (Figures 3C and 3D). Immunohistochemical and Western blotting analyses revealed enriched cardiac endothelial expression of GATA2, PAK4, and RASA1 (online-only Data Supplement Figures IIIa and IIIb), which suggests cell-type specific coexpression of miR-24 with those targets within the heart.

### Table. Predicted microRNA-24 Target Messenger RNAs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Endothelial Expression (Reference)</th>
<th>Evolutionary Conserved No. of Species (miRBase)</th>
<th>Predicted Target (miRBase)</th>
<th>Predicted Target (PicTar)</th>
<th>Seed Match for miR-24 (TargetScan)</th>
</tr>
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<tbody>
<tr>
<td>GATA2</td>
<td>Endothelial transcription factor GATA2</td>
<td>30</td>
<td>4</td>
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<td>8mer</td>
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<tr>
<td>PAK4</td>
<td>Serine/threonine-protein kinase PAK4</td>
<td>31</td>
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<td>No</td>
<td>Yes</td>
<td>8mer</td>
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<tr>
<td>RASA1</td>
<td>Ras GTPase-activating protein 1</td>
<td>32</td>
<td>10</td>
<td>No</td>
<td>Yes</td>
<td>8mer</td>
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<tr>
<td>CDKN1B</td>
<td>Cyclin-dependent kinase inhibitor 1B (p27kip1)</td>
<td>33</td>
<td>4</td>
<td>No</td>
<td>Yes</td>
<td>8mer</td>
</tr>
<tr>
<td>AMOTL2</td>
<td>Angiomotin like 2</td>
<td>34</td>
<td>3</td>
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<td>Yes</td>
<td>8mer</td>
</tr>
<tr>
<td>H2AFX</td>
<td>Histone family, member X</td>
<td>35</td>
<td>5</td>
<td>Yes</td>
<td>Yes</td>
<td>7mer</td>
</tr>
<tr>
<td>RAP1B</td>
<td>RAP1B, member of RAS oncogene family</td>
<td>36</td>
<td>5</td>
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<td>Yes</td>
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</tr>
<tr>
<td>AXL</td>
<td>AXL receptor tyrosine kinase</td>
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<td>8</td>
<td>No</td>
<td>Yes</td>
<td>7mer</td>
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<td>S1PR1</td>
<td>Sphingosine-1-phosphate receptor 1</td>
<td>38</td>
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<tr>
<td>MAGI1</td>
<td>Membrane associated guanylate kinase, WW and PDZ domain containing 1</td>
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<td>No</td>
<td>NA</td>
<td>8mer</td>
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<tr>
<td>TFF1</td>
<td>Tissue factor pathway inhibitor</td>
<td>40</td>
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<td>No</td>
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<tr>
<td>ANGPT4</td>
<td>Angiopoietin 4</td>
<td>41</td>
<td>5</td>
<td>No</td>
<td>NA</td>
<td>8mer</td>
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<tr>
<td>BMPR2</td>
<td>Protein receptor, type II</td>
<td>42</td>
<td>6</td>
<td>No</td>
<td>NA</td>
<td>7mer</td>
</tr>
</tbody>
</table>

The microRNA databases and target prediction tools miRBase (http://microrna.sanger.ac.uk/), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/index.html) were used to identify potential microRNA-24 targets. NA indicates not available.

### Downstream GATA2 and PAK4 Target Regulation

To understand the observed cellular changes in miR-24 target regulation, we further investigated the downstream signaling cascades of the direct miR-24 targets GATA2 and PAK4. First, we used a global transcriptome analysis after viral overexpression or silencing of GATA2 in endothelial cells.

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**Figure 3.** MiR-24 regulates GATA2 and PAK4 in endothelial cells. **A**, Protein expression of GATA2, PAK4, and GAPDH 72 hours after transfection with scrambled miRNAs (scr-miR) or synthetic miR-24 precursors (pre-miR-24) to human umbilical vein endothelial cells and statistical summary. **B**, Activities of luciferase reporter constructs comprising the 3’UTR regions of GATA2 and PAK4 mRNA relative to β-Gal control plasmids after transfection of synthetic miRNAs. mut indicates mutant. **C**, Tube formation of HUVECs after transfection of scrambled small inhibitory RNA (scr-siRNA) or siRNA against GATA2 or PAK4 24 hours after seeding on top of Matrigels and statistical analysis. **D**, Apoptosis of endothelial cells after transfection of scrambled siRNA (scr-siRNA) or siRNAs specific for GATA2 (siRNA-GATA2) or PAK4 (siRNA-PAK4). Data are mean and SEM; *P*<0.05, **P*<0.01,** ***P*<0.005.
and identified 3 reciprocally regulated mRNAs after GATA2 modulation (BMP and activin membrane-bound inhibitor [BAMBI], endothelial cell–specific molecule-1 [ESM1], and netrin-4 [NTN4]). Bioinformatics studies identified BAMBI, ESM1, NTN4, heme-oxygenase-1 (HMOX1), and sirtuin-1 (SIRT1) as harboring GATA2 binding sites within their promoter sequence (data not shown). To further validate GATA2-mediated regulation, we used GATA2-ChIP and found those 5 mRNAs to be highly enriched after ChIP (Figure 4A). GATA2 overexpression strongly induced protein expression of SIRT1 and HMOX1 (both of which play important roles in angiogenesis45,46), whereas siRNA-mediated GATA2 silencing reduced expression (Figure 4B; online-only Data Supplement Figures IVc and IVd). HMOX1 was regulated by miR-24 via modulation of GATA2 (Figure 4C; online-only Data Supplement Figures Va and Vb). HMOX1 exerts vasoprotective and antiapoptotic actions in endothelial cells,46 and consequently, we found miR-24–mediated repression of HMOX1 resulted in enhanced reactive oxygen species formation in endothelial cells (online-only Data Supplement Figure Vc), which may also contribute to recent findings that miR-24 upregulation predisposes cells to DNA damage.47

To identify proteins that further mediated the proapoptotic action of endothelial miR-24, we hybridized endothelial protein extracts after transfection of scrambled miRs or synthetic miR-24 precursors to a protein microarray spotted with antibodies for proteins involved in apoptosis (Figure 4D). A number of proapoptotic proteins were upregulated, eg, HIF-1α (HIF-1α) and FAS, although we found a strong reduction of BAD and HMOX1 (Figures 4C through 4E). The direct miR-24 target PAK4 promotes BAD phosphorylation, thus inactivating the proapoptotic protein BAD.48 MiR-24 overexpression led to a reduction of phosphorylated BAD (pBAD), whereas miR-24 antagonism increased the pBAD/BAD ratio (Figure 4E). Repression of PAK4 resulted in reduced BAD phosphorylation (Figure 4F; online-only Data Supplement Figure IVa), which contributed to increased apoptosis in endothelial cells (Figure 3D). We also blocked miR-24 expression to derepress PAK4, which resulted in enhanced pBAD levels (Figure 4E). Knockdown of PAK4 led to reduced pBAD levels in the absence or presence of miR-24 blockade, which suggests that PAK4 is a main modulator of miR-24–mediated BAD phosphorylation in endothelial cells (online-only Data Supplement Figure VD).

To test whether the miR-24 targets GATA2, PAK4, and HMOX1 are among the main mediators of the antiangiogenic and proapoptotic action of miR-24 in endothelial cells, we reconstructed those targets in miR-24–overexpressing endothelial cells. Reconstitution of miR-24–resistant GATA2, PAK4, and HMOX1 rescued both miR-24–mediated endothelial apoptosis and miR-24–impaired tube formation capacity (Figures 4G and 4H). We thus identified a network of
direct and indirect miR-24 targets that regulated apoptosis and angiogenic properties of endothelial cells. These important cellular characteristics may affect (neo)vascularization in vivo, especially after ischemic events.

MiR-24 Overexpression and Knockdown of miR-24 Targets Impair Zebrafish Vascular Development

To analyze miR-24 effects in vivo, we first injected miR-24 precursors into Tg(kdrl:eGFP) embryos that express GFP in the vasculature. Embryos at 48 hpf had increased miR-24 expression levels (Figure 5A), as well as abnormal vessel architecture and insufficient blood transport, which demonstrates that miR-24 activation results in a robust vascular phenotype, although we cannot exclude other cell-type–specific effects (Figures 5B through 5I). To test the involvement of the direct miR-24 targets Gata2 and Pak4 in this model, we used target knockdown experiments. Morpholino-based knockdown of the target gene gata2a (Figures 5L and 5N), as well as pak4 (Figures 5P and 5R; online-only Data Supplement Figure VI), led to blood accumulation and pericardial edema compared with controls (Figure 5J). Furthermore, confocal images of the trunk vasculature revealed impaired vascular formation. In MO1-gata2a (Figure 5M), MO2-gata2a (Figure 5O), MO1-pak4, and MO2-pak4 embryos, very similar to the vascularization defects seen in embryos with forced miR24 overexpression. Blood accumulation and hemorrhaging were more severe in MO-pak4 knockdown embryos than in MO-gata2a injected embryos (see also online-only Data Supplement Figure VI D). ctr indicates control.
Therapy With miR-24 Inhibitors Increases Vascularization and Improves Cardiac Function After MI

To further study the effects of miR-24 on vascularization in mammals, we injected chemically engineered cholesterol-conjugated single-strand RNA analogues (antagomirs) that targeted miR-24 or scrambled controls into mice. In initial experiments, we found Cy3-labeled antagomirs to be effectively taken up by endothelial cells in vitro (Figure 6A). Because we wanted to target mainly the endothelial cell fraction, we first performed titration experiments with Cy3-labeled antagomirs to achieve preferential delivery to endothelial cells. Injection of a Cy3-labeled antagomir at a low dose (5 mg/kg) mainly resulted in cellular uptake in cardiac endothelial cells, whereas injection of a high dose (80 mg/kg) led to a strong homogeneous uptake in all cardiac cells, including cardiomyocytes (Figure 6B). Consequently, we found injections of low doses of an antagomir (5 mg/kg, day 0 and day 2) against miR-24 to repress miR-24 but not unrelated miRNAs mainly in fractionated endothelial cells obtained from healthy or ischemic heart tissue (Figures 6B and 6C and data not shown).

We then tested the effects of endothelial miR-24 antagonism in a mouse model of MI. Immunohistochemical studies revealed a greater amount of apoptotic endothelial cells in the periinfarct zone (online-only Data Supplement Figure VII). In contrast, endothelial apoptosis measured by TUNEL+/PECAM1+ cells was reduced and both capillary and arteriolar density were increased in the periinfarct region after miR-24 antagonism, whereas no changes were observed in the remote myocardium (Figure 7A; online-only Data Supplement Figure VII). Improved capillary density correlated with significant smaller infarct size 14 days after MI (control 54±6% versus antagonim-24 38±3%, P<0.05).

MI led to an impairment of cardiac function 14 days after intervention (Figure 7B). Systolic and diastolic left ventricular diameter and lung wet weight increased after MI (Figures 7C and 7D). In contrast, immediate treatment after MI with an antagonim against miR-24 (days 0 and 2) improved cardiac function and attenuated pulmonary congestion and left ventricular dilatation (Figures 7B through 7E). Baseline data of fractional shortening and left ventricular diameters were not different between the investigated groups (data not shown). Survival of MI animals was significantly improved by miR-24 antagonim treatment (surviving animals at day 14: scrambled antagonim 43.5% versus antagonim-24 78.3%; P=0.02). To exclude significant off-target effects and to confirm specificity of antagonim-24, we injected an antagonim against a scrambled sequence, which did not affect miR-24 expression or infarct healing (Figures 6C and 7B through 7E). A schematic summary of our findings is presented as Figure 7F.

Discussion

In the present study, we identified miR-24 as a critical regulator of endothelial cell survival and angiogenesis. We found miR-24 to be enriched in cardiac endothelial cells and to be upregulated after ischemic injury. However, miR-24 is also expressed in other cells with different cell-specific functions, such as insulin production of pancreatic β-cells, in part via regulation of the transcriptional repressor,50 cell cycle regulation and proliferation of leukemia cells,51 and activation of smooth muscle cells via repression of Tribbles-like protein 1.52 In the latter study, long-term hypoxia led to miR-24 upregulation in lung tissue and to activation of lung smooth muscle cells. Thus, inhibition of miR-24 in certain pulmonary diseases may be of future therapeutic relevance.

With regard to function in other cardiac cell types, miR-24...
overexpression has been reported to increase cardiomyocyte hypertrophy in vitro, although a recent study revealed an additional role in cardiomyocyte apoptosis. In that report, the authors described a downregulation of miR-24 in the border zone after MI. In line with those results, we detected a downregulation of miR-24 in fractionated cardiomyocytes early after MI, whereas its expression was massively increased in cardiac endothelial cells. We used post-MI treatment with a low dose of an antagomir (2 mg/kg 1 d 1), which silenced miR-24 specifically in endothelial cells but not cardiomyocytes in the heart. Interestingly, high dosing of an antagonist (2×80 mg · kg 1 · d 1) against miR-24 had less favorable effects on cardiac function after MI (data not shown), which suggests potential toxicity of the high-dose group or negative effects on cardiomyocyte biology, as suggested recently. Future studies are therefore needed to develop improved cell-type–specific delivery systems of antagomirs. The role of miR-24 in other ischemic disease conditions and its potential therapeutic value remain to be investigated.

We used bioinformatics analyses to characterize the endothelial miR-24 targetome. So far, we have identified GATA2 (via SIRT1 and HMOX1) and PAK4 (via pBAD) as major miR-24 targets that control a complex network of apoptotic and angiogenic programs in endothelial cells (Figure 7F). However, many additional targets may be involved in the action of miR-24, especially in other disease conditions in which target availability may change. Although many other targets might be involved, target reconstitution experiments showed dominant roles of GATA2 and PAK4 for the proapoptotic and antiangiogenic effects of miR-24. The role of other potential targets important for miR-24 biological actions remains to be elucidated.

Importantly, application of low-dose antagomirs permitted silencing of miR-24 expression predominantly in endothelial cells in vivo, which resulted in reduced endothelial apoptosis, enhanced vascularization, decreased infarct size, and improved cardiac function after MI. Thus, miR-24 and its downstream targets may serve as valuable therapeutic entry points to interfere with endothelial genetic programs and
thereby improve vascularity and cardiac performance after ischemic injury.

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Disclosures
Dr. Fiedler and Thum filed a patent application about the use of miR-24 in ischemic diseases. Dr. Tuschl serves as a consultant/advisor board member for Regulus Therapeutics. The remaining authors report no conflicts of interest.

References
Ischemic heart disease is the leading cause of death worldwide, often presenting as acute myocardial infarction. Myocardial infarction frequently progresses to the development of heart failure. Insufficient myocardial vascularization prevents optimal blood support for cardiomyocyte survival, although the underlying mechanisms of cardiac vascularization are not well understood. MicroRNAs are small RNA molecules regulating approximately half of the genome and recently have emerged as powerful therapeutic targets for various heart diseases. We identified miRNA-24 (miR-24) as being activated in cardiac endothelial cells after myocardial infarction, leading to endothelial cell apoptosis and abolishment of capillary network formation. These effects are mediated through targeting of the endothelium-enriched transcription factor GATA2, the p21-activated kinase PAK4, and the respective downstream signaling cascades. Blocking of endothelial miR-24 in a mouse model of myocardial infarction limits myocardial infarct size by preventing endothelial apoptosis and enhancing vascularity, which leads to preserved cardiac function and survival. Thus, miR-24 acts as a critical regulator of cardiac vascularization and is suitable for therapeutic intervention in the setting of ischemic heart disease. Our findings may lead to clinical applications, whereby miRNA modulators are used systemically or locally to improve vascularization in patients after myocardial infarction.

CLINICAL PERSPECTIVE
MicroRNA-24 Regulates Vascularity After Myocardial Infarction

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SUPPLEMENTAL MATERIAL

Expanded Methods and Results

**Cultivation of cardiovascular cells.** Human umbilical vein endothelial cells (HUVECs) were cultured in EGM2 media supplemented with 10% (v/v) fetal calf serum (FCS) and supplements (all reagents from Cambrex Lonza, UK). Cells were grown in a humidified atmosphere at 5% CO$_2$ and 37°C.

**Fractionation of cardiac cell types from heart tissue.** The thorax of mice was opened and the aorta was cannulated. After washing with 37°C PBS, the heart together with the cannula was removed and perfused with a collagenase solution for 5 min (Joklik MEM medium supplemented with 10 mM butanedione monoxime, 20 µM calcium chloride, 1mg/ml collagenase II) as described $^1$. Then the heart was placed in 37°C pre-warmed collagenase solution for further 25 min and was subsequently divided in infarct and remote areas, minced and filtered through a nylon mesh (200 µm pore size). Then, cardiomyocytes and cardiac fibroblasts were separated by a sedimentation step as described $^2$. Within the non-cardiomyocyte cell fraction retained in the supernatant an incubation step with CD146-antibodies coupled to microbeads was performed and subjected to magnetic affinity cell sorting according to the manufacturers’ recommendations (Mouse CD146 microbead endothelial isolation kit, Miltenyi Biotec, Germany).

**Apoptosis protein array.** Apoptosis array data were generated by applying a human apoptosis array kit (ARY009, R&D, USA). 200 µg protein from a pool of three samples were incubated with antibody-coated membranes following manufacturers’ instructions. Various regulated proteins were then validated by Western blotting.
Detection of reactive oxygen species (ROS). The redox-sensitive, cell-permeable fluorophore dihydroethidium (DHE) becomes oxidized in the presence of O$_2^-$ to yield fluorescent ethidium. Thus, dye oxidation is an indirect measure of the presence of reactive oxygen intermediates \(^3\). MiRNA-transfected HUVECs were incubated with DHE (2.5 \(\mu\)M) for 30 min. After washing, HUVECs were immediately analyzed with FACS (FACS Calibur, BD Bioscience).

**MircoRNA target prediction.** The microRNA databases and target prediction tools miRBase (http://microrna.sanger.ac.uk/), PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/index.html) were used to identify potential microRNA targets. Specifically, we searched for targets with known expression in cardiovascular tissue and endothelial cells. We focused on targets predicted by at least two prediction data bases and containing a miR-24-8mer seed match in the respective 3'UTR region.

**In situ hybridization.**

We combined in situ hybridization of miR-24 with endothelial and smooth muscle cell marker stainings. Mouse heart cryosections (10 \(\mu\)m) were dried at room temperature and fixed with 4\% PFA for 10 min. at room temperature and washed two times with DEPC-PBS for 3 min. To increase tissue permeability and reduce unspecific background, the sections were treated with proteinase K (end concentration 5 \(\mu\)g/ ml) for 15 min at 37\(^{\circ}\)C and then were washed two times for 3 minutes with DEPC-PBS. The sections were acetylated 15 minutes at room temperature using acetic anhydride dissolved in DEPC-water, 6N HCl and Triethanolamine and subsequently washed two times with DEPC-PBS for 3 min. Pre-hybridisation was performed 1 hour at 60\(^{\circ}\)C in pre-hybridization buffer containing 50\% formamide, 5x SSC, 0.5 mg/ml yeast tRNA, 1x Denhardt’s solution and 9.2 mM citric acid. Subsequently, sections were hybridized with 2.5 pmol of miR-24- specific probe labelled with fluorescein at 5’ end (#18121-04, Exiqon) or with fluorescein labelled scrambled miR- probe (#99004-04, Exiqon) diluted in pre-hybridization buffer, overnight at 60\(^{\circ}\)C. The described RNA melting
temperature of the miR-24 and scrambled control were approximately similar (±3 °C). Next day, the sections were washed three times with 0.1x SSC at 65°C and, finally, one time with 2x SSC for 5 min, permeabilized for immunostaining with 0.1% Triton X-100 and washed 2 times with PBS. Unspecific background was blocked with 5% donkey serum diluted in PBS 30 min. at room temperature. Endothelial cells were stained with rat anti-CD31 antibody (# MCA 2388, AbD Serotec) at 1:50 dilution for 2 hours at room temperature and sections were washed several times with PBS. Smooth muscle cells were stained with rabbit anti-alpha smooth muscle actin antibody (Acta2; ab5694, Abcam) at 1:50 dilution overnight at 4°C. Then, sections were incubated with donkey anti-rat-AlexaFluor594 antibody (#21209, Invitrogen; dilution 1:500; to detect Pecam1) or with donkey anti-rabbit-AlexaFluor594 antibody (#21207, Invitrogen dilution 1:500; to detect Acta2) together with DAPI (1: 1000), for 30 min at room temperature, washed several times with PBS and mounted the sections using VectaShield Hard Set (#H-1400, Vector laboratories) mounting medium.

**Immunofluorescence.** Frozen heart sections were acetone-fixed, washed and blocked with 5% (v/v) donkey sera or MOM Mouse IgGs (for RASA1 stain) before addition of appropriate Alexa-conjugated secondary antibodies (Invitrogen). Slides were mounted in VECTASHIELD/DAPI (Linaris, Germany). Details about used antibodies are shown in Supplemental Table 3.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was used to detect protein-DNA interactions. First, protein G sepharose beads were blocked o/n at 4°C. HUVECs from confluent T75 flasks were first cross-linked and harvested. The pellet was lysed and sonified to yield DNA fragments from 100-1000 bp in length. Afterwards, samples were centrifuged at maximum speed to yield cleared lysates. Aliquots were separately taken to measure sonification efficiency by agarose gel analysis. To reduce non-specific background, cleared lysates were pre-cleared on blocked Protein G Sepharose beads (GE Healthcare) twice. Samples were subjected to either immunoprecipitation with 5 µg GATA2
antibody (Santa Cruz Biotechnology, sc-267X) or control mouse IgGs (Santa Cruz Biotechnology, sc-2025) o/n at 4 °C. To block non-specific background BSA and herring sperm DNA were added. One sample with cell lysis and IP dilution buffer was used as mock control. The next day GATA2/DNA cross-links were collected by incubation with Protein G beads. Beads were washed twice with dialysis buffer and four times with IP wash buffer. Finally, beads were washed twice with TE-buffer. Antibody-GATA2/DNA complexes were eluted from the beads by adding 150 µl IP elution buffer and heating at 65°C. The elution step was repeated and combined eluates were reverse cross-linked. Samples were subjected to RNA and protein degradation. Afterwards, DNA was isolated and purified with Qiagen PCR purification kit (Qiagen).

For ChIP primer-design we first identified 2000-2500 bp upstream promoter region of candidate target genes by Ensembl Genome Browser (http://www.ensembl.org/index.html). Then we screened the promoter region for potential GATA2 binding sites by the use of ALLGEN-Promo and selected appropriate primer pairs that amplify potential GATA2 binding sites. Subsequent PCR analysis of chipped DNA fragments was done by mixing 2.5 µl sample, 2.5 µl 4 µM appropriate primer pairs, 10 µl HotStarTaq Mix (Qiagen) and applying the following protocol: 94°C 10 min, [94°C 1 min, 57°C 30 sec, 72°C 1 min]x33, 72°C 10 min, 4°C hold. Used oligonucleotide primer sequences are given in Supplemental Table 4.

**Antagomir injection.** Antagomirs were designed and provided by Regulus Therapeutics (USA) and as described ⁴. Sequences were: Antagomir-24: 5’-CTGTTCCTGCTGAACTGAGCCA-chol-3’ and scrambled Antagomir: 5’-ACAAACACCAUUGUCACACUCCA-chol-3’. Antagomirs were diluted in nuclease-free water and 100 µl at concentrations of 5 mg/kg and 80 mg/kg were applied to mice via retroorbital injection.

**Determination of apoptotic endothelial cells and cardiomyocytes in vivo.** Apoptosis was quantified at 14 days after MI by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP
nick-end labeling (TUNEL) technique (in situ cell death detection kit Fluorescein, Roche, Germany) and combined cell-type specific stainings of either endothelial cells (Pecam1) or cardiomyocytes (Tnni3). Following treatment of slides with proteinase K (20μg/ml, 30min at 37°C), TUNEL assay was performed as described by the manufacturer. Sections were additionally stained with DAPI to recognize nuclei. At least ten high power fields (400x) from the peri-infarct zone were analysed.

**Myocardial infarction.** Male Mice (C57BL/6, 8-10 weeks) underwent coronary artery ligation for the production of myocardial infarction (MI). Successful generation of MI after occlusion of the left ascending artery was monitored by parallel electrocardiogram (ECG; ST-elevation) measurements and impaired wall motion by echocardiography. Only mice with significant ST-elevation in the ECG analysis and impaired wall motion by echocardiography were included in the study. Briefly, mice were anesthetized, placed on a heating pad, intubated and ventilated with a mixture of oxygen and isoflurane. After left lateral thoracotomy and exposure of the heart by retractors, the left anterior descending coronary artery (LAD) was permanently ligated. Successful production of MI was checked by measurements of ST-elevation in electrocardiograms as well as impaired left ventricular wall motion by echocardiography. Animals that did not show ST-elevation and impaired left ventricular wall motion after myocardial infarction were excluded from further studies. Fourteen days after MI, additional echocardiography measurements were performed and finally hearts were excised and cut into transverse sections. From the middle ring, sections were cut and stained with appropriate antibodies (see above). Cardiac dimensions and function were analyzed by pulse–wave Doppler echocardiography.

**Determination of infarct size.** This was done essentially as described previously. From cardiac rings sections were stained with picrosirius red and infarct size was determined by planimetric measurement using a microscope and calculated by dividing the sum of
endocardial and epicardial circumferences of infarct areas by the sum of the total endocardial and epicardial circumferences.
Additional Figures

Figure legends

Supplemental Figure 1. MiR-24 inhibits endothelial tube formation independently from its pro-apoptotic activity but has no effects on number and function of endothelial progenitor cells. Endothelial tube formation 72 h after transfection of scrambled miRNAs (scr-miR, 100 nM) or miR-24 precursors (pre-miR-24, 100 nM) in the presence or absence of a pan-caspase inhibitor (Caspase 3 inhibitor I, 100 µM, 72 h). **P,0.01. n=3-5 per group.

Supplemental Figure 2. Regulation and functional importance of further miR-24 targets RASA1 and H2AFX in endothelial cells. (a) Western Blots of RASA1 and H2AFX 72 h after transfection of scrambled (scr-miR) or miR-24 precursors (pre-24). (b) Activities of luciferase reporter constructs comprising the 3'UTR region of RASA1 and H2AFX mRNA relative to beta-Gal control plasmids after transfection of synthetic miRNAs. (c) Western blots of RASA1 and H2AFX 48 h after transfection of specific siRNAs against RASA1, H2AFX or appropriate control siRNAs (si-scr). TBP=TATA box binding protein (nuclear housekeeping protein). (d) Relative changes in apoptosis (Annexin V-assay) and changes in tube formation (e) 48h after transfection of specific siRNAs against RASA1, H2AFX or appropriate scrambled siRNAs. n=3-4 experiments per group. Data are mean and s.e.m.; *P,0.05, ***P,0.005.

Supplemental Figure 3. Cardiac endothelial expression of miR-24 targets. (a) Localization of endothelial protein Pecam1 and the miR-24 targets Gata2, Pak4 and Rasa1 in sections of mouse hearts. Nuclei were counterstained with 4',6-diamidino-2-phenylindol-dihydrochlorid (DAPI). White arrows indicate perinuclear region of PAK4 expression in cardiac endothelial cells. (b) Protein expression of miR-24 targets in fractionated cardiomyoctyes and cardiac endothelial cells. n=3-4 experiments per group.

Supplemental Figure 4. MiR-24 target modulation in endothelial cells. (a) Expression of GATA2 and PAK4 48h after transfection of specific siRNAs against GATA2 or PAK4 or scrambled controls (si-scr). Right, Statistical summary. (b) Gata2 expression three days after transfection of a murine GFP-Gata2 construct or a YFP-labeled control construct to human umbilical vein endothelial cells. Note, cytoplasmic localisation of the control construct and nuclear expression of the GFP-Gata2 construct. (c) Western blots of SIRT1 after up- or downregulation of GATA2 in HUVECs. (d) SIRT1 expression after transfection of increasing doses (m.o.i.) of the adenoiral GATA2 construct to endothelial cells. n=3-4 experiments per group. Data are mean and s.e.m.; *P,0.05; ***P,0.005.

Supplemental Figure 5. MiR-24 regulates HMOX1 expression via GATA2 and Bad phosphorylation via PAK4 in endothelial cells. (a) HMOX1 expression is regulated by miR-24 in endothelial cells.(b) GATA2 and HMOX1 expression in endothelial cells after miR-24 inhibition and/or GATA2 silencing. (c) FACS-based analysis of ROS formation 72 h after transfection of synthetic miR-24 precursors (pre-24) or scrambled controls (scr) to HUVECs. (d) BAD phosphorylation in endothelial cells after miR-24 inhibition and/or PAK4 silencing. n=3-6 experiments per group. Data are mean and s.e.m.; *P,0.05, **P,0.01, ***P,0.005.

Supplemental Figure 6. Knockdown strategy for pak4. (a,b) Lateral view of 24 hpf zebrafish embryos injected with (a, a') cRNA containing Pak4-morpholino target site in front of a cassette encoding GFP and 4 ng of control morpholino or (b, b') cRNA containing Pak4-morpholino target site in front of a cassette encoding GFP and 4 ng of MO1-pak4. (a') Whereas the control embryos displayed GFP expression, (b) GFP expression was suppressed by silencing of the Pak4-target site after injection of MO1-pak4 morpholino. (c) The splice site morpholino MO2-pak4 was directed against the exon 5, intron 5 boundary of
pak4 which consists of 10 exons. Arrows depict the location of the two primers which were utilized in the RT-PCR analysis. RT-PCR analysis of total RNA isolated of MO2-pak4 and control morpholino injected embryos at 24 hpf revealed a significant decrease of correctly spliced pak4 transcripts in MO2-pak4 morphants in contrast to the control. RT-PCR analysis of β-actin served as loading control. (d) A representative pak4-MO2 injected embryo at 48hpf displaying blood accumulation and hemorrhaging (arrows) in the trunk and head region. In this particular experiment, 110 embryos were scored and 74 displayed blood retention and compromised circulation. 11 embryos had hemorrhages.

**Supplemental Figure 7. Prevention of endothelial apoptosis in vivo by antagomir-24 treatment.** Apoptotic endothelial cells (TUNEL+/Pecam1+ cells) within the periinfarct region 14 d after myocardial infarction (MI) and treatment with scrambled antagomirs (Scr) or antagomir-24 (Ant24). **Bottom,** Statistical summary. n=6-8 experiments per group. Data are mean and s.e.m.; **P,0.01.
### Additional Tables

#### Supplemental Table 1: TaqMan miRNA detection assays

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#### Supplemental Table 2: Used siRNAs and miRNAs

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**miRNAs**

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#### Supplemental Table 3: Antibodies applied in this work for Western blotting and immunofluorescence

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### Supplemental Table 4: Primers used for ChIP-PCR

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### Supplemental Table 5: Morpholino sequences for target knockdown studies in zebrafish

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References (to Expanded Methods)


Supplemental Figures

Supplemental Figure 1

a

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![Graph showing tube length (fold change of scr)](graph.png)
Supplemental Figure 2

(a) Western blot analysis showing the expression levels of RASA1, H2AFX, and GAPDH under scr-miR and pre-24 conditions.

(b) Luciferase activity assay for RASA1 3'UTR and H2AFX 3'UTR under miR-24 conditions.

(c) siRNA transfection experiment showing the effect of si-RASA1 and si-H2AX on RASA1 and GAPDH expression.

(d) Western blot analysis of apoptosis-related proteins under scr, si-RASA1, and si-H2AX conditions.

(e) Tubule length assay showing the effect of si-RASA1 and si-H2AX on tubule formation.
Supplemental Figure 3

(a) Intact heart

(b) Cardiomyocytes

- Gata2
- Pak4
- Rasa1
- Gapdh

Endothelial cells

- miR-24 target
- Overlay