MicroRNA-374b induces Endothelial-to-Mesenchymal Transition and neointima formation through the inhibition of MAPK7 Signaling

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Under review
ENDOTHELIAL MESENCHYMOAL TRANSITION OCCURS DURING INTIMAL HYPERPLASIA AND NEOINTIMA FORMATION VIA MECHANISMS THAT ARE INCOMPLETELY UNDERSTOOD. ENDOTHELIAL MAPK7 SIGNALING IS A KEY MECHANOSENSITIVE FACTOR THAT PROTECTS AGAINST ENDOTHELIAL-MESENCHYMAL TRANSITION, BUT ITS SIGNALING ACTIVITY LOST IN VESSEL AREAS THAT UNDERGOING PATHOLOGICAL REMODELING.

At sites of vascular remodeling in mice and pigs, endothelial MAPK7 signaling was lost. The TGFβ-induced microRNA-374b targets MAPK7 and its downstream effectors in endothelial cells and its expression induces endothelial-mesenchymal transition. Gain-of-function experiments, where endothelial MAPK7 signaling was restored, precluded endothelial-mesenchymal transition. In human coronary artery disease, disease severity associates with decreased MAPK7 expression levels and increased miR-374b expression levels.

Endothelial-mesenchymal transition occurs in intimal hyperplasia and neointima formation and is governed in part by microRNA-374b-induced silencing of MAPK7 signaling. Restoration of MAPK7 signaling abrogated these pathological effects in endothelial cells expressing miR-374b. Thus, our data suggest that the TGFβ-miR-374b-MAPK7 axis plays a key role in the induction of endothelial-mesenchymal transition during intimal hyperplasia and neointima formation and might pose an interesting target for anti-atherosclerosis therapy.
INTRODUCTION

The development of atherosclerosis is preceded by intimal hyperplasia and neointima formation [1]. Although the commonly recognized risk factors for the development of neointimal lesions and atherosclerosis are present at the systemic level [2], neointima formation and atherosclerosis manifest focally in so-called atheroprone regions [3]. These atheroprone regions are characterized by low levels of non-uniform shear stress, typically encountered at the outer walls of vascular bifurcations and at the inner wall of vascular curvatures, whereas atheroprotected regions are characterized by high levels of uniform laminar shear stress [4].

We and others have recently described a major contribution of endothelial cells to intimal hyperplasia and atherosclerosis development [5-7]. Upon exposure to inflammatory and pro-fibrotic growth factors (i.e. TGFβ) and cytokines, endothelial cells lose their endothelial cell markers and functionality, start to express markers of the mesenchymal lineage and gain contractile behavior [8-10]. During neointimal lesion formation, endothelial cells that undergo this Endothelial-Mesenchymal Transition (EndMT) acquire a fibroproliferative mesenchymal phenotype and migrate from the endothelial monolayer to the underlying neointima [5,6].

TGFβ is a major inducer of EndMT and highly expressed in neointimal lesions [11], which may cause EndMT. We uncovered that high levels of uniform laminar shear stress - observed at atheroprotected regions of the arteries - activates a specific mitogen-activated kinase (MAPK), namely MAPK7 (also known as Erk5 or Big MAPK (BMK1)), which inhibits the induction of EndMT by TGFβ1 [5]. Corroboratively, knockdown of MAPK7 in endothelial cells causes EndMT, even in the absence of exogenous TGFβ1 [5], suggesting a pivotal balance between TGFβ and MAPK7 signaling in the induction of EndMT and the formation of neointimal lesions. Indeed, TGFβ1 represses endothelial MAPK7 expression through unidentified mechanisms (unpublished data) and the loss of endothelial MAPK7 signaling aggravates atherosclerosis development [12].

MicroRNAs (miRNAs) are small non-coding RNAs that cause post-translational repression of their target genes. MicroRNAs elicit translational repression by imperfect base-pairing to the 3'UTR of their gene targets [13], which allows any specific microRNA to target multiple genes simultaneously. Moreover, multiplicity of microRNA targets might also allow one microRNA to specifically target multiple genes within one signal transduction cascade [14], efficiently abolishing its activity.

TGFβ induces a shift in endothelial miRNA expression levels [15-18] that may reduce MAPK7 signaling and thus facilitate EndMT induction. Here, we hypothesized that TGFβ1 induces the expression of a specific miRNA that targets MAPK7 and its signaling intermediates, resulting in the induction of EndMT.
CHAPTER 3

METHODS

CLINICAL SAMPLES

Human coronary arteries were obtained from autopsy specimens from 10 patients that died from an acute coronary episode at the Heart Institute (InCor), Sao Paulo, Brazil. During necropsy each dissected coronary artery was fixed in neutral-buffered formalin with constant perfusion at a quasi-normal perfusion pressure. The mean age of subjects contributing pathology specimens was 65 years. Hypertension was resent in 9 subjects, and diabetes in 6. Five individuals were active smokers. Next-of-kin gave informed consent and the investigation was performed according to institutional guidelines (InCor, Sao Paulo #SDC 3723/11/141 and #CAPPesq 482/11) and the Declaration of Helsinki.

ANIMALS AND SURGICAL PROCEDURES

Porcine abdominal trifurcations were obtained from healthy male slaughterhouse Yorkshire pigs (12-13 weeks of age; body weight 30-35 kg, n =3, V.O.F. van Beek, Lelystad, The Netherlands). Animals were fed a normal diet and were euthanized under anesthesia (ketamine (Nimatek) and midazolam with a bolus of pentobarbital and heparin (Actrapid)). Male C57Bl/6j wild-type mice (8–12 weeks of age, n = 8, Harlan, Horst, The Netherlands) were subjected to transverse aortic constriction under anesthesia (2% Isoflurane (Forene/Abbott, The Netherlands) and oxygen) and analgesia (Carprofen, 5 mg/kg). Briefly, an incision was made in the second intercostal space and a small incision was made in the parietal pleura to expose the ascending loop of the aorta. The aorta was supported with a 27G needle and a suture was placed around the aorta, drawn tight after which the needle was removed. Next, the pleura, muscle layers, and skin were closed by sutures. Animals received post-operative analgesia (Carprofen, 5 mg/kg/day for 48 h). Animals were kept on a 12 h light:dark cycle with ad libitum access to standard laboratory chow and water. Eight weeks after aortic constriction, animals were sacrificed under deep anesthesia [3% Isoflurane (Forene/Abbott, The Netherlands)] by exsanguination, after which the thoracic aorta was explanted. Experiments on mice were approved by the local Institutional Animal Care and Use Committee (University of Groningen, #DEC-5910).

HUMAN UMBILICAL VEIN ENDOTHELIAL CELL CULTURE

Human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD) were cultured in endothelial cell medium up to passage 5 as described previously [8]. EndMT was initiated by re-plating the HUVEC in RPMI1640, supplemented with 20% FCS, 1% Penicillin-Streptomycin, 2 mM L-glutamine, 5 U·mL$^{-1}$ heparin, and 10 ng·mL$^{-1}$ TGFβ1 (Peprotech, NJ, USA). For shear stress experiments, HUVEC were plated on 1% gelatin-coated µ-Slides (Ibidi, Martinsried, Germany) and grown to confluence prior to exposure to 20 dynes·cm$^{-2}$ of unidirectional uniform laminar shear stress (LSS). LSS was generated using the Ibidi Pump System (Ibidi, Martinsried, Germany).

3’UTR REPORTER ANALYSIS
Gene specific 3’ UTR fragments were isolated from a cDNA pool derived from various human tissues using oligonucleotides extended with SgfI (GGCAGGCG) and NotI (GCGGCCGC) restriction sequences at the sense and antisense primer (Table S1), respectively. Amplification was performed using the DyNAzyme EXT PCR kit (Finnzymes, Vantaa, Finland) according to the manufacturer’s instructions. Amplicon size was validated by gel electrophoresis on 1% agarose gels. 3’UTR fragments were cloned into the SgfI/NotI-sites of the psiCHECK-2 dual luciferase reporter vector (Promega, Madison, WI). COS-7 cells were transfected with 100 ng/ml 3’UTR reporter plasmid and 50 nM miR-374b mimic or scrambled control (Life Technologies, Carlsbad, CA) using Lipofectamine2000 (ThermoFisher, Waltham, MA). 48 h post-transfection, luciferase activity was assayed using the DualGlo Luciferase assay system (Promega, Madison, WI) and recorded for 1 s on a Luminoskan ASCENT (Thermo Scientific, Waltham, MA).

PLASMIDS AND LENTIVIRAL EXPRESSION OF MIR-374B, SHRNA AND MAPK7 SIGNALING MEMBERS

For lentiviral expression of miR-374b and small hairpin RNA (shRNA) against MAPK7 signaling members, DNA oligonucleotides containing the endogenous miR-374b hairpin or specific 21-mer targeting sequences for human MAP3K3, MAPK7, MEF2D or KLF4 (all Biolegio, Leiden, The Netherlands, Table S2) were cloned into the BamHI/EcoRI sites of the pGreenPuro shRNA expression vector (Systems Bioscience, CA, USA). Scrambled sequences were used as control.

Gene-CDS fragments were isolated from a cDNA pool derived from various human tissues using oligonucleotides extended with EcoRI (GAATTC) and BamHI (GGATCC) restriction sequences at the sense and antisense primer (Table S3), respectively. Amplification was performed using the DyNAzyme EXT PCR kit (Finnzymes, Vantaa, Finland) according to the manufacturer’s instructions. Amplicon size was validated by gel electrophoresis on 1% agarose gels. Gene-CDS fragments were cloned into the EcoRI/BamHI sites of the pCDH-CMV-MCS-EF1-Puro lentiviral expression vector (Systems Biosciences, Palo Alto, CA). Empty vectors served as control.

For lentiviral transduction, HEK293 cells were transfected with pGreenPuro or pCDH-CMV-MCS-EF1-Puro shuttle vectors and second-generation lentiviral helper plasmids using Endofectin (GeneCopoeia, MD, USA). Viral supernatants were collected every 24 hours, supplemented with 6 µg·mL⁻¹ polybrene and directly transferred to HUVEC cultures for two consecutive rounds. Transduced cells were selected for puromycin resistance 72 h post-transduction (4 µg·mL⁻¹ puromycin) for 24 hours and reseeded into puromycin-free medium for the experiments.

MICRORNA AND GENE TRANSCRIPT ANALYSIS

Total RNA was isolated using TRIlzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. For microRNA transcript analyses, 20 ng of total RNA was reversely transcribed using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems) using microRNA-specific stemloop primers (Table S4). For gene transcript analysis, 1 1 µg of total RNA was reversely transcribed using the RevertAid first Strand cDNA synthesis kit (Applied Biosystems, Carlsbad, CA) according to manufacturer’s protocol. Quantitive PCR expression analysis performed on a reaction mixture containing
10 ng cDNA equivalent, 0.5 μM sense primers and 0.5 μM antisense primers (for microRNA primers see Table S4 and gene transcript primers see Table S5, all Biolegio, Leiden, The Netherlands). Analyses were run on Viia7 real-time PCR system (Applied Biosystems, Carlsbad, CA).

IMMUNOFLUORESCENCE

Heat-induced antigen retrieval was performed with 0.1 M Tris-HCl (pH 9.0, 80°C, 20 mins) on the formalin fixed, paraffin-embedded sections prior to immunohistochemistry. Sections were incubated with primary antibodies at room temperature for 2 hours, followed by incubation with secondary antibodies at room temperature for 1 hour (Table S6). Detailed description of the imaging procedures is provided in the Supplementary Methods.

IMMUNOBLOTTING

Whole cell lysates were prepared in RIPA buffer (Thermo Scientific, IL, USA) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, Germany) and 1% HALT-phosphatase inhibitor cocktail (ThermoFisher Scientific). Protein concentrations were determined using the detergent compatible protein assay (Bio-Rad, VA), according to manufacturer's protocol. Equal amounts of protein were loaded on a 10% denaturing SDS–polyacrylamide gel and separated by gel electrophoresis (110V). Next proteins were blotted onto nitrocellulose membranes using the Trans-Blot Turbo System (Bio-Rad, AV) according to manufacturer's instructions. Blots were blocked in Odyssey Blocking Buffer (Li-COR Biosciences, NE, USA) at room temperature for 30 mins and incubated at 4°C with primary antibodies (Table S7) in Odyssey Blocking Buffer overnight, after which membranes were incubated with secondary antibodies (Table S7) in Odyssey Blocking Buffer at room temperature for 1 hour. Protein was detected using the Odyssey Infrared Imaging System (Li-COR Biosciences). Densitometric analysis was performed using Totallab 120 (Nonlinear Dynamics, Newcastle upon Tyne, England).

ANGIOGENIC SPROUTING CAPACITY

10 µl of MatriGel (BD Biosciences, San Jose, CA) was solidified in μ-Slide Angiogenesis (Ibidi, Martinsried, Germany). 10,000 cells per well were cultured on the solidified MatriGel in endothelial growth medium and the formation of sprouts was analyzed by conventional light microscopic analysis after overnight incubation.

COLLAGEN CONTRACTION ASSAY

Cells were suspended in a solution of rat tail Collagen type I (Corning, Corning, NY) containing 3 mg·mL⁻¹ NaHCO₃ and 0.1M Na₂HPO₄. Aliquots of 50 µL (containing 100,000 cells and 0.5 mg Collagen type I) were solidified at 37°C in a humidified incubator with 5% CO₂ for 30 mins. The collagen gels were released from the culture dishes by the addition of 2 ml endothelial growth medium and were imaged using a common Flatbed-scanner and allowed to contract for an additional 24 h.
The degree of gel contraction was determined by measuring the total gel area and dividing the areas of the contracted gels by the areas of the gels before contraction.

**STATISTICAL ANALYSIS**

Data are presented as means ± s.e.m. N-values relate to independent experiments/samples. P-values were calculated using one-way analysis of variance followed by Bonferroni’s post-hoc comparisons tests using Prism Graphpad (Graphpad Software, La Jolla, CA, USA). P < 0.05 was considered statistically significant.

**RESULTS**

**MICRORNA-374B TARGETS MAPK7 SIGNALING**

Database analysis (miRanda [19,20]) identified 12 microRNAs that putatively target MAPK7 (Suppl.fig.1). Next, we cross-checked these microRNAs against other genes in the MAPK7 signaling cascade (Suppl.fig.1) and found that the miR-374 family (miR-374a and miR-374b) target not only MAPK7, but also its upstream activators Rac1, MAP3K3 and MAP3K7. Moreover, miR-374 also putatively targets the downstream transcription factors of the myocyte enhancer factor (MEF)-2 family as well as Krüppel-like factor (KLF)-4 (Suppl.fig.2). TGFβ1 induced the expression of miR-374a, miR-374b, miR-143, miR-24 and miR-410 in endothelial cells (p<0.05 vs unstimulated control cells), whereas it decreased the expression of miR-488 (p<0.05 vs unstimulated control cells). The expression of miR-429, miR-200b, miR-200c, miR-183, miR-124 and miR-506 were unchanged (Suppl.fig.3) upon TGFβ1 treatment.

MicroRNA-374a and miR-374b collectively have 1305 putative gene targets, of which 527 and 434 are unique to miR-374a and miR-374b, respectively (Suppl.fig.2). 344 putative gene targets are shared between miR-374a and miR-374b. Analysis of genes within the MAPK7 signaling cascade indicates that MAP3K7 (TAK1), MAPK7 (ERK5), MEF2A and MEF2C are putative targets of miR-374a and miR-374b, whereas Rac1, MAP3K3 (MEKK3), MEF2D and KLF4 are targets of miR-374b only. None of the genes with in the MAPK7 signaling cascade where unique targets to miR-374a (Suppl.fig.2).

**MICRORNA-374B AND MAPK7 ARE DIFFERENTIALLY EXPRESSED AT AThEROPRONE REGIONS**

EndMT contributes to the formation of intimal hyperplasia [5], which is aggravated by the loss of protective MAPK7 signaling [12]. Cells co-expressing endothelial specific molecule (ESM)-1 and the mesenchymal protein SM22α were abundantly present in the hyperplastic intima of the porcine trifurcation compared to the non-hyperplastic intima (11.5 vs 1.0%; p<0.001, fig.1a). In the atheroprone hyperplastic regions, endothelial MAPK7 activity was decreased 2-fold (p=0.01) compared endothelial cells in the atheroprotected regions within the same trifurcation (fig.1b).
Figure 1. MicroRNA-374b and MAPK7 are differentially expressed in neointimal lesions. Porcine intimal hyperplastic lesions contain myo-endothelial cells (myoEC) that co-express the endothelial cell marker ESM-1 (red) and mesenchymal cell marker SM22a (green). Nuclei are stained with DAPI (blue) (A). The activity of MAPK7 (pMAPK7, green) was reduced in endothelial cells (ESM-1, red) in the atheroprone regions of the porcine trifurcation (B, n=5), whereas the expression of miR-374b was increased in the atheroprone areas compared to atheroprotected areas of the same porcine trifurcation (C, n=5). Mice were subjected to transverse aortic constriction (TAC). Eight weeks after TAC, myoEC that co-express the endothelial cell marker CD31 (red) and the mesenchymal cell marker SM22a (green) were detected at the areas exposed to disturbed flow (D, n=5, *=ligation). The activity of MAPK7 (pMAPK7, green) was reduced in endothelial cells (CD31, red) in the areas exposed to disturbed flow compared to areas exposed to laminar flow (E, n=5), which coincides with an increase in the expression of miR-374b (F, n=5). In vitro, endothelial cells treated with TGFβ1 were hyperplastic, which was inhibited by the ALK5-inhibitor SB431542 (G) and increased their expression of miR-374b (H, n=6). The expression of MAPK7 was reduced in TGFβ1-stimulated endothelial cells, compared to untreated control cells or endothelial cells treated with TGFβ1 and the ALK5-inhibitor SB431542 (I, n=6). The expression levels of miR-374b inversely associated with MAPK7 expression levels (J, n=6). Laminar fluid shear stress (20 dyne·cm⁻²) antagonized the cellular hypertrophy induced by TGFβ1 (K) in endothelial cells and inhibited the increase in miR-374b expression (L, n=5). Concurrently, when exposed to fluid shear stress, MAPK7 expression was unaltered in TGFβ1-treated endothelial cells compared to unstimulated control cells (M, n=5). T-test for comparison between two groups; One-way ANOVA for analyses between multiple groups. *p<0.05, **p<0.01, ***p<0.001.
We dissected the atheroprone and atheroprotected areas of the porcine trifurcation and found increased expression levels of miR-374b in the atheroprone regions (~3-fold, p=0.04, fig.1c). Concurrently, in mice with transverse aortic banding, atheroprone regions characterized by disturbed fluid shear stress were characterized by the accumulation of cells expressing both the endothelial marker CD31 and the mesenchymal protein SM22α (12.3%, fig.1d), indicative of EndMT. At these atheroprone sites, endothelial MAPK7 activity was decreased (1.6-fold, p=0.04, fig.1e), whereas the expression of miR-374b was elevated (4-fold, p=0.01, fig.1f).

In vitro, endothelial cells treated with TGFβ1 displayed apoptosis and hypertrophy (fig.1g) and increased their expression of miR-374b (~9-fold compared to non-stimulated cells, p<0.001), which was completely abolished by the addition of a small molecule inhibitor of ALK5 (SB431542)(fig.1h). The increase in miR-374b expression associated with decreased expression of MAPK7 (r²=0.797, p<0.001, fig.1i,j) and intermediates of MAPK7 signaling, i.e. Rac1 (r²=0.595, p<0.01), MAP3K7 (r²=0.688, p<0.01), MAPK7 (r²=0.797, p<0.01), MEF2D (r²=0.682, p<0.001) and KLF4 (r²=0.555, p<0.01, Suppl. fig.4a), as well as a decreased expression of endothelial markers VE-Cadherin (r²=0.678, p<0.001) and eNOS (r²=0.546, p<0.01, Suppl.fig.4b) and increased expression of mesenchymal markers SM22α (r²=0.872, p<0.001) and CNN1 (r²=0.814, p<0.001, Suppl.fig.4c).

Under LSS, endothelial cells aligned in the direction of flow, indicating mechanosensory behavior (fig.1k). In contrast to the static cell cultures, endothelial cells exposed to LSS did not become hypertrophic and did not increase their expression of miR-374b, even when exogenous TGFβ1 was applied (fig.1l). Corroboratively, MAPK7 expression, which is diminished by TGFβ1 in static cultures, remained high under LSS even in the presence of TGFβ1 (fig.1m).

MIR-374B TARGETS MULTIPLE MEMBERS OF THE MAPK7 SIGNALING CASCADE

Computational analysis of putative miR-374b targets identified multiple members of the MAPK7 signaling cascade (fig.2a). Reporter assays, wherein the 3’UTR regions of miR-374b gene targets are coupled to the Luciferase gene, revealed that the MAPK7 Signaling members Rac1, MAP3K3, MAP3K7, MAPK7, MEF2d and KLF4 are genuine miR-374b target genes (fig.2b) as co-transfection of these reporter plasmids with miR-374b mimics reduced luciferase activity (all p<0.01). In contrast, co-transfection of reporter plasmids with a scrambled miR-374b sequence did not alter luciferase activity (fig.2b). MEF2a and MEF2c were identified as putative gene targets of miR-374b by in silico analyses, however, co-transfection of their respective reporter constructs with miR-374b mimics, did not result in decreased luciferase activity (fig.2b) implicating that MEF2a and MEF2c are not genuine targets of miR-374b. To establish if MAPK7 signaling members are factual endogenous miR-374b targets in mature endothelial cells, we lentivirally overexpressed miR-374b or its scrambled sequence. Unstimulated control cells readily expressed MAPK7 and its signaling members (fig.2c). Stimulation of mature endothelial cells with TGFβ1 drastically reduced expression of these molecules (fig.2c) to approx. 50% of control endothelial cells (all p<0.01). Endothelial cells that expressed miR-374b had reduced expression of MAP3K3 (~33%, p<0.01), MAPK7 (~47%, p<0.01), MEF2d (~37%, p<0.01) and KLF4 (~42%, p<0.001) compared to endothelial cells treated with scrambled controls (fig.2c) indicating that miR-374b decreases endogenous MAPK7 signaling.
Figure 2. MicroRNA-374b interferes with MAPK7 signaling at multiple levels. In silico analysis (Tarbase [39]) reveals that miR-374b putatively targets multiple genes within the MAPK7 signaling cascade with different efficacies (miRSVR-scores indicate the predicted target site efficacy [20]). Co-transfection of 3’UTR reporter constructs with miR-374b mimics or scrambled control sequences in COS7 cells suggests that Rac1, MAP3K3, MAP3K7, MAPK7, MEF2D and KLF4 are genuine miR-374b target genes (B, n=5). Transfection of miR-374b mimics in endothelial cells mimics the TGFβ1-induced decrease in expression of MAPK7 signaling members (C,D; n=3). One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

TGFβ-1 SUPPRESSES MAPK7 EXPRESSION THROUGH INDUCTION OF MIR-374B AND CAUSES ENDOTHELIAL-MESENCHYMAL TRANSITION

We next questioned if ectopic expression of miR-374b in endothelial cells would facilitate EndMT. Lentiviral expression caused a ~4-fold increase of miR-374b in endothelial cells (fig.3a). Consequently, endothelial cells lost their typical cobblestone morphology and started to show signs of hypertrophy (fig.3b). Consistent with EndMT, the expression of typical endothelial cell markers, i.e. VE-Cadherin and eNOS, was lost (fig. 3b,c) whereas expression of mesenchymal cell markers, i.e. SM22α and Calponin 1 (CNN1), was induced (fig. 3b,c). Additionally, miR-374b gain-of-function in endothelial cells, reduced the ability to form capillary-like sprouts on Matrigel (fig.3d) and fostered the gain of contractile behavior (fig.3e), all consistent with mesenchymal transition.
Figure 3. MicroRNA-374b gain-of-function induces EndMT. Transformation of endothelial cells with a lentivirus encoding the stemloop sequence of miR-374b increased its expression ~4-fold (A). MiR-374b expressing endothelial cells where hypertrophic and decreased their expression of the endothelial cell marker VE-cadherin and increased expression of the mesenchymal cell marker SM22α, compared to endothelial cells that expressed a scrambled control sequence (B). VE-Cadherin and eNOS expression were decreased in endothelial cells expressing miR-374b, whereas the expression of SM22α and CNN1 was increased (C). Endothelial cells expressing miR-374b had a reduced angiogenic sprouting capacity (D) and increased contractile capacity (E) compared to endothelial cells that expressed a scrambled control sequence. All n=5, T-test for comparison between two groups, *p<0.05, **p<0.01, ***p<0.001.

LOSS OF SPECIFIC MIR-374B TARGETS INDUCES ENDOTHELIAL-MESENCHYMAL TRANSITION

We questioned if the loss-of-function of specific miR-374b targets would suffice for EndMT induction and used a shRNA approach to specifically decrease MAP3K3, MAPK7, MEF2d or KLF4 expression in endothelial cells (Supl.fig.5). Decreased expression of MAPK7 signaling members caused the dissociation of endothelial cell-cell contacts and decreased the expression of VE-cadherin. Concurrently, the expression of SM22α was increased upon loss of MAPK7 signaling (fig.4a). The expression of endothelial markers VE-Cadherin and eNOS was abrogated and the expression of mesenchymal markers SM22α and Calponin was induced (fig.4b,c). Moreover, angiogenic sprouting ability decreased (fig.4d) and in contrast to the EndMT induction by the loss of downstream MAPK7 signaling members,
MAP3K3 deficiency did not alter the expression of endothelial, nor mesenchymal marker proteins (fig.4b,c). Yet, angiogenic sprouting ability was lost (fig.4d) and contractile behavior acquired (fig.4e) by endothelial cells deficient in MAP3K3.

Figure 4. Knockdown of specific microRNA-374b gene targets induces EndMT. Transformation of endothelial cells with lentiviruses encoding shRNA sequences to MAP3K3, MAPK7, MEF2D and KLF4 induced cellular hypertrophy, decreased the expression of the endothelial cell marker VE-cadherin, and increased expression of the mesenchymal cell marker SM22α, compared to endothelial cells that expressed scrambled control sequences (A). VE-Cadherin and eNOS expression were decreased in endothelial cells expressing shMAPK7, shMEF2D and shKLF4, whereas their expression was unaltered in endothelial cells expressing shMAP3K3. The expression of SM22α and CNN1 was increased in endothelial cells expressing shMAPK7, shMEF2D and shKLF4 (B,C). Endothelial cells expressing shRNA sequences to MAP3K3, MAPK7, MEF2D and KLF4 had a reduced angiogenic sprouting capacity (D) and increased contractile capacity (E) compared to endothelial cells that expressed a scrambled control sequence. All n=6, One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

MAINTENANCE OF ENDOTHELIAL MAPK7 SIGNALING ABROGATES MIR-374B-INDUCED ENDOTHELIAL-MESENCHYMAL TRANSITION

To investigate if restoration of MAPK7 signaling members could block miR-374b-induced EndMT, we lentivirally expressed miR-374b together with the protein-coding sequences of MAP3K3, MAPK7, MEF2D and KLF4 (fig.5a). VE-cadherin expression was reduced in endothelial cells that express miR-374b, whereas the expression of SM22α was induced.
In contrast, cells that co-expressed MAP3K3 or MAPK7 together with miR-374b maintained their expression of VE-cadherin and eNOS and were refractory to the induction of SM22a and Calponin by miR-374b (fig.5b-d), whereas cells co-expressing KLF4 and miR-374b maintained only the expression of eNOS (fig.5c,d). Restoration of MEF2D in endothelial cells expressing miR-374b, inhibited the expression of the mesenchymal proteins SM22a and Calponin, but failed to maintain the expression of endothelial marker proteins VE-Cadherin and eNOS (fig.5b-d). Restoration of all MAPK7 signaling members in endothelial cells expressing miR-374b maintained the angiogenic ability (fig.5e) and precluded the contractile behavior induced by miR-374b expression (fig.5f).

**Figure 5. Re-expression of microRNA-374b target genes inhibits EndMT.** The expression of miR-374b target genes was restored by lentiviral transformation using plasmids encoding the coding sequences (CDS) of MAP3K3, MAPK7, MEF2D and KLF4 in endothelial cells that overexpress miR-374b. The (re-)expression of these genes was confirmed by qPCR (A). Endothelial cells that express miR-374b decreased their expression of VE-Cadherin and increased their expression of SM22α. These effects were counteracted by the expression of MAPK7 signaling members (B-D). Endothelial cells that expressed MAP3K3, MAPK7, MEF2D or KLF4 have an enhanced angiogenic sprouting capacity (E) and decreased contractile capacity (F) compared to endothelial cell that express miR-374b. The levels of angiogenic sprouting and contractile behavior were similar to that of endothelial cells transformed using scrambled control sequences. All n=5, One-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.
Lastly, we assessed the expression levels of miR-374b and MAPK7 in samples from progressive human coronary artery stenosis. Increasing intima-media thickness (IMT) associates with the severity of coronary stenosis (fig.6a,b) and increased miR-374b expression (fig.6c). Moreover, miR-374b levels associated with IMT ($r^2=0.5874$, $p<0.01$, fig.6d). Conversely, MAPK7 expression levels progressively decrease with increasing stenosis (fig.6e). In human coronary artery stenosis, the levels of miR-374b are associated with the expression levels of MAPK7 ($r^2=0.3341$, $p<0.01$, fig.6f), suggesting that the miR-374b-induced loss of MAPK7 signaling might contribute to stenosis development and progression.

Figure 6. MicroRNA-374b is increased in human coronary artery stenosis. Verhoef stain of progressive coronary artery stenosis characterized by an increasing intima/media ratio (A). MiR-374b expression is elevated in human coronary artery stenosis and correlated to the degree of stenosis (B). Conversely, the expression levels of MAPK7 decrease with progressive stenosis and are inversely associated to the expression level of miR-374b (C). One-way ANOVA for comparison between groups, Pearson correlations, two-tailed. **$p<0.01$, ***$p<0.001$. 
Here, we show that EndMT occurs in intimal hyperplasia and neointima formation and is governed in part by microRNA-374b. We have previously identified the inhibitory effects of MAPK7 signaling on EndMT [5] and questioned if MAPK7 signaling is silenced at atheroprone areas in the vasculature. We uncovered that the TGFβ-induced microRNA-374b silences MAPK7 signaling and induces EndMT in the absence of exogenous TGFβ. Moreover, restoration of MAPK7 signaling abrogated these pathological effects in endothelial cells expressing miR-374b. Interestingly, we uncovered that miR-374b levels are elevated in human coronary artery disease and inversely related with MAPK7 expression. These data suggest that the TGFβ-miR-374b-MAPK7 axis plays a detrimental role in the induction of EndMT during intimal hyperplasia and neointima formation and might pose an interesting target for antiatherosclerosis therapy.

Atherosclerosis is characterized by systemic risk factors, and anti-atherosclerosis therapies are focused on maintaining these systemic contributors within clinically acceptable ranges (e.g. anti-hypertensives, anti-inflammatory agents and lipid-lowering drugs) [21-23]. Yet, it is becoming increasingly clear that focal risk factors, such as fluid shear stress levels, play a major role in the pathogenesis of atherosclerosis. Indeed, endothelial MAPK7 signaling has been identified as a major contributor to the initiation and severity of atherosclerosis [5,12]. The atheroprotective effects of MAPK7 include anti-inflammatory effects, the reduction of oxidative stress and the increased biosynthesis of Nitric Oxide [24-26], which decrease smooth muscle cell proliferation and inflammatory cell infiltration into the atherosclerotic neointima. We recently uncovered that endothelial MAPK7 signaling additionally confers atheroprotective effects through the inhibition of EndMT [5], a process increasingly recognized in the initial phases of intimal hyperplasia and neointima formation [5,7,27,28]. Indeed, the inhibition of MAPK7 activity by SUMOylation increases atherosclerosis [29,30] and endothelial specific deletion of MAPK7 aggravates atherosclerosis development and progression [12].

MicroRNAs are involved in atherosclerosis development and progression [31], and microRNA-based therapies that target endothelial dysfunction reduce atherosclerosis development in mice [32-34]. MicroRNAs regulate gene expression by imperfect base-pairing with the 3’ UTR region of their gene target, causing translational repression [35]. This imperfect base-pairing allows for gene target multiplicity, wherein one microRNA targets multiple genes with a specific signaling cascade [36]. Hence, we questioned if MAPK7 signaling would be regulated by a specific microRNAs and if such microRNA would be differentially expressed at atheroprone and atheroprotected sites. We found that miR-374b expression is elevated at atheroprone areas in the vessel wall and is associated with decreased MAPK7 activity.

A role for miR-374b in atherosclerosis has not been described before, yet elevated levels of miR-374b have been reported in the plasma of acute coronary syndrome patients [37] and in stenosis of the arteriovenous fistulae of dialysis patients [38], however its relevance in these pathologies remains elusive. Here, we show that miR-374b is a shear stress-sensitive microRNA that targets MAPK7 signaling at multiple levels ranging from its upstream activating kinase (i.e. MAP3K3) to its downstream transcription factor (i.e. KLF4). The loss of MAPK7 signaling culminates in the induction of EndMT in the absence of exogenous triggers, which contributes to intimal hyperplasia and neointima formation.
Moreover, we show that restoration of MAPK7 signaling in endothelial cell that express miR-374b abolishes EndMT. From a clinical perspective, our data imply that targeting miR-374b in atherosclerosis might restore endothelial MAPK7 activity and limit neointima formation through the inhibition of EndMT.

**Figure 7. Schematic representation of miR-374/MAPK7 signaling in atheroprone and atheroprotected areas.** High laminar fluid shear stress induces the expression and activation of atheroprotective MAPK7 signaling, which maintains endothelial homeostasis and inhibits the proatherogenic TGFβ signaling (A). At areas of low, or non-uniform disturbed shear stress, MAPK7 activity is low and its expression is decreased by the TGFβ1-induced miR-374b, resulting in enhanced TGFβ signaling in endothelial cells, culminating in EndMT (B).

In conclusion, here we show that miR-374b expression is elevated in coronary artery stenosis and neointima formation and abolishes endothelial MAPK7 activity, culminating in EndMT. The restoration of endothelial MAPK7 activity surmounts the induction of EndMT by miR-374b.
EXTENDED METHODS

CLINICAL SAMPLES

Human coronary arteries were obtained from autopsy specimens from 10 patients that died from an acute coronary episode at the Heart Institute (InCor), Sao Paulo, Brazil. Coronary arteries were fixed in neutral buffered formalin prior to paraffin embedding. During necropsy each dissected coronary artery was fixed in neutral-buffered formalin with constant perfusion at a quasi-normal perfusion pressure. The mean age of subjects contributing pathology specimens was 65 years. Hypertension was recent in 9 subjects, and diabetes in 6. Five individuals were active smokers. Next-of-kin gave informed consent and the investigation was performed according to institutional guidelines (InCor, Sao Paulo #SDC 3723/11/141 and #CAPPesq 482/11) and the Declaration of Helsinki.

Human coronary artery samples were deparaffinized using 100% Xylol for 10 mins and rehydrated using a series of EtOH solution of decreasing concentration (100%; 75%; 50%; 0%, all 10 mins). Samples were stained in Verhoef’s solution (92 mM hematoxylin, 137 mM FeCl₃, 27 mM KI, 4 mM I₂ in 55% EtOH) at room temperature for 1 hour. Samples were rinsed in tap water and differentiated in FeCl₃ (123 mM in dH₂O) for 1 minute and treated with Sodium Thiosulphate (316 mM in dH₂O) at room temperature for 1 min. Samples were dehydrated using increasing concentrations of EtOH (50%; 75%; 100%, all 1 min) and cleared in 100% xylene. Samples were mounted in Permount resinous mounting medium.

ANIMALS AND SURGICAL PROCEDURES

Porcine abdominal trifurcations were obtained from healthy male slaughterhouse Yorkshire pigs (12-13 weeks of age; body weight 30-35 kg, n =3, V.O.F. van Beek, Lelystad, The Netherlands). Animals were fed a normal diet and were euthanized under anesthesia (ketamine (Nimatek) and midazolam with a bolus of pentobarbital and heparin (Actrapid)). Male C57Bl/6j wild-type mice (8-12 weeks of age, n=8, Harlan, Horst, The Netherlands) were subjected to transverse aortic constriction under anesthesia (2% Isoflurane (Forene/Abbott, The Netherlands) and oxygen) and analgesia (Carprofen, 5 mg·kg⁻¹). Briefly, an incision was made in the second intercostal space and a small incision was made in the parietal pleura to expose the ascending loop of the aorta. The aorta was supported with a 27G needle and a suture was placed around the aorta, drawn tight after which the needle was removed. Next, the pleura, muscle layers, and skin were closed by sutures. Animals received post-operative analgesia (Carprofen, 5 mg·kg⁻¹·day⁻¹ for 48 h). Animals were kept on a 12 h light:dark cycle with ad libitum access to standard laboratory chow and water. Eight weeks after aortic constriction, animals were sacrificed under deep anesthesia [3% Isoflurane (Forene/Abbott, The Netherlands) by exsanguination, after which the thoracic aorta was explanted. Experiments on mice were approved by the local Institutional Animal Care and Use Committee (University of Groningen, #DEC-5910).
CHAPTER 3

HUMAN UMBILICAL VEIN ENDOTHELIAL CELL CULTURE

Human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD) were cultured on 1% gelatin (#G9391 Sigma-Aldrich, St. Louis, MO) coated tissue culture plastics in endothelial cell medium up to passage 5 in RPMI1640 (#BE12-702F, Lonza, Verviers, Belgium) supplemented with 20% fetal bovine serum (#SH30071, HyClone Laboratories, Logan, UT), 50 µg·ml⁻¹ ECGF (own isolation according to Burgess et al 1985)[39], 2 mM L-glutamine (#G7513, Sigma-Aldrich, St. Louis, MO), 1% Penicillin/Streptomycin (#P4333, Sigma-Aldrich, St. Louis, MO) and 5 U·ml⁻¹ heparin (Leo Pharma, Ballerup, Denmark). EndMT was initiated by re-plating the HUVEC in RPMI1640, supplemented with 20% FCS, 1% Penicillin–Streptomycin, 2 mM L-glutamine, 5 U·mL⁻¹ heparin, and 10 ng·mL⁻¹ TGFβ1 (Peprotech, NJ, USA) for 96 hours. For shear stress experiments, HUVEC were plated on 1% gelatin-coated µ-Slides (Ibidi, Martinsried, Germany) and grown to confluence prior to exposure to 20 dynes·cm⁻² of unidirectional uniform laminar shear stress (LSS). LSS was generated using the Ibidi Pump System (Ibidi, Martinsried, Germany) according to manufacturer’s instructions.

3’UTR REPORTER ANALYSIS

Gene specific 3’UTR fragments were isolated from a cDNA pool derived from various human tissues using oligonucleotides extended with SgfI (GCGATCGC) and NotI (GCGGCCGC) restriction sequences at the sense and antisense primer (Table S1), respectively. Amplification was performed using the DyNAzyme EXT PCR kit (Finnzymes, Vantaa, Finland) according to the manufacturer’s instructions. 10 ng of cDNA was amplified in a 50 µl reaction volume containing 50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 15 mM (NH₄)₂SO₄, 0.1% Triton X-100, 200 µM dNTPs, 0.6 µM 3’UTR primers and 2U DyNAzyme EXT DNA Polymerase (Biometra, Göttingen, Germany) using an initial denaturation step of 94°C for 5 mins and 30 cycles of 94°C for 30 sec, 58-72°C for 2 mins, and 72°C for 2 mins, followed by a final extension step of 72°C for 10 mins. The reaction was halted at 82°C for 5 mins.

Amplicon sizes were validated by gel electrophoresis on 1% agarose gels and amplicons were dissected from the gels. 3’UTR fragments were purified using the QIAquick PCR Purification Kit (#28104, Qiagen, Venlo, The Netherlands) according to manufacturer’s protocol. Purity and concentration of 3’UTR fragments was determined spectrophotometrically on a Nanodrop 1000 (ThermoFisher, Waltham, MA). 3’UTR fragments were inserted into the SgfI/NotI (both Fermentas, Vilnius, Lithuania)-linearized psiCHECK-2 dual luciferase reporter vector (Promega, Madison, WI) using T4 DNA Ligase (Sigma-Aldrich, St. Louis, MO). 3’UTR-containing plasmids were amplified in competent TOP10 E.coli (Invitrogen, Carlsbad, CA) and plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands) according to manufacturer’s instructions. COS-7 cells were transfected with 100 ng/ml 3’UTR reporter plasmid and 50 nM miR-374b mimic or scrambled control (Life Technologies, Carlsbad, CA) using Lipofectamine2000 (ThermoFisher, Waltham, MA). 48 h post-transfection, luciferase activity was assayed using the DualGlo Luciferase assay system (Promega, Madison, WI) and recorded for 1 s on a Luminoskan ASCENT (Thermo Scientific, Waltham, MA).
MICRORN-374B INDUCES ENDOTHELIAL-TO-MESENCHYMAL TRANSITION

PLASMIDS AND LENTIVIRAL EXPRESSION OF MIR-374B, shRNA AND MAPK7 SIGNALING MEMBERS

For lentiviral expression of miR-374b and small hairpin RNA (shRNA) against MAPK7 signaling members, equimolar amounts of DNA oligonucleotides containing the endogenous miR-374b hairpin or specific 21-mer targeting sequences for human MAP3K3, MAPK7, MEF2D or KLF4 (all Biolegio, Leiden, The Netherlands, Table S2) were annealed in a 100 µl reaction volume, containing 100 µM sense and antisense oligonucleotides, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl and 1 mM EDTA. Annealing was performed by heating the reaction mixture to 98°C for 5 mins and cooling the reaction mixture at 1.5°C·min⁻¹ to 20°C in a Biometra TPprofessional thermocycler. Duplexed oligonucleotides were cloned into the BamHI/EcoRI (both Fermentas, Vilnius, Lithuania)-linearized pGreenPuro shRNA expression vector (Systems Bioscience, CA, USA) using T4 DNA Ligase (Sigma-Aldrich, St. Louis, MO). Scrambled sequences were used as control.

Gene-CDS fragments were isolated from a cDNA pool derived from various human tissues using oligonucleotides extended with EcoRI (GAATTC) and BamHI (GGATCC) restriction sequences at the sense and antisense primer (Table S3), respectively. Amplification was performed using the DyNAzyme EXT PCR kit (Finnzymes, Vantaa, Finland) according to the manufacturer’s instructions. 10 ng of cDNA was amplified in a 50 µl reaction volume containing 50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 15 mM (NH₄)₂SO₄, 0.1% Triton X-100, 200 µM dNTPs, 0.6 µM 3'UTR primers and 2U DyNAzyme EXT DNA Polymerase (all Finnzymes, Vantaa, Finland) in a Biometra TPprofessional thermocycler using an initial denaturation step of 94°C for 5 mins and 30 cycles of 94°C for 30 sec, 58-72°C for 2 mins, and 72°C for 2 mins, followed by a final extension step of 72°C for 10 mins. The reaction was halted at 82°C for 5 mins. Amplicon size was validated by gel electrophoresis on 1% agarose gels and amplicons were dissected from the gels. 3’UTR fragments were purified using the QIAquick PCR Purification Kit (#28104, Qiagen, Venlo, The Netherlands) according to manufacturer’s protocol. Purity and concentration of 3’UTR fragments was determined spectrophotometrically on a Nanodrop 1000 (ThermoFisher, Waltham, MA). Gene-CDS fragments were cloned into the EcoRI/BamHI sites of the pCDH-CMV-MCS-EF1-Puro lentiviral expression vector (Systems Biosciences, Palo Alto, CA) using T4 DNA Ligase (Sigma-Aldrich, St. Louis, MO). Empty vectors served as control.

ShRNA and Gene-CDS-containing plasmids were amplified in competent TOP10 E.coli (Invitrogen, Carlsbad, CA) and plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands) according to manufacturer’s instructions. For lentiviral transduction, 1.0·10⁶ HEK293 cells were transfected with 1 µg pGreenPuro or pCDH-CMV-MCS-EF1-Puro shuttle vectors and 1 µg second-generation lentiviral helper plasmids using Endofectin (GeneCoopoia, MD, USA). Viral supernatants were collected every 24 hours, supplemented with 6 µg·mL⁻¹ polybrene and directly transferred to HUVEC cultures for two consecutive rounds. Transduced cells were selected for puromycin resistance 72 h post-transduction (4 µg·mL⁻¹ puromycin) for 24 hours and reseeded into puromycin-free medium for the experiments.

MICRORNA AND GENE TRANSCRIPT ANALYSIS

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. In brief, 1·10⁶ cells or 10 mg of tissue was homogenized in 1
ml TRIzol reagent and supplemented 0.2 ml Chloroform. After centrifugation (12.000xg for 15 min) the aqueous phase was supplemented with 0.5 ml 2-propanol and the RNA pelleted by centrifugation (12.000xg for 10 min). RNA was further purified using two consecutive washes with 75% EtOH. RNA integrity was validated on 2% agarose gels and RNA purity and concentration were determined spectrophotometrically on a NanoDrop 1000 (ThermoFisher, Waltham, MA). For microRNA transcript analyses, 20 ng of total RNA was reversely transcribed using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems) in a 10 µl reaction volume containing 1 µl 10x RT reaction buffer, 1.6 µM microRNA-specific stemloop primers (Table S4), 1 mM dNTPs, and 3U RT enzyme (all Taqman, ThermoFisher, Waltham, MA) in a Biometra TPProfessional thermocycler at 16°C for 30 mins, 42°C for 60 mins, and the reaction was stopped at 85°C for 5 mins. For gene transcript analysis, 1 µg of total RNA was reversely transcribed using the RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA) in a in a 20 µl reaction volume containing 4 µl 5x RT reaction buffer, 5 µM random hexamer primers, 20U RiboLock RNAse Inhibitor, 1 mM dNTP, and 200U RT enzyme (all Fermentas/ThermoFisher, Waltham, MA) in a Biometra TPProfessional thermocycler at 25°C for 5 mins, 42°C for 60 mins, and the reaction was stopped at 70°C for 5 mins. Quantitative PCR expression analysis was performed on a reaction mixture containing 1-10 ng cDNA equivalent, 0.5 µM sense primers and 0.5 µM antisense primers (for microRNA primers see Table S4 and gene transcript primers see Table S5, all Biolegio, Leiden, The Netherlands) and FastStart SYBR Green (Roche, Almere, The Netherlands). Analyses were run on a Viia7 real-time PCR system (Applied Biosystems, Carlsbad, CA) using an initial denaturation step of 94°C for 5 mins and 40 cycles of 94°C for 30 sec, 60°C for 1 min. Cycle thresholds (Ct) values were determined at the middle of the linear phase. MicroRNA expression was normalized against the expression of RNU6B and mRNA expression levels were normalized against the expression of GAPDH prior to normalization against control samples.

IMMUNOFLUORESCENCE

Heat-induced antigen retrieval was performed with 0.1 M Tris-HCl (pH 9.0, 80°C, 20 mins) on the formalin fixated, paraffin-embedded sections prior to immunohistochemistry. Sections were allowed to cool to room temperature for 45 mins, and incubated with 2%BSA/2% normal donkey serum in PBS for 20 mins. Sections were incubated with primary antibodies (Table S6) at room temperature for 2 hours, followed by extensive washing in TBS-Tween (0.05%). Subsequently, samples were incubated with secondary antibodies in DAPI/PBS at room temperature for 1 hour (Table S6) and mounted in CitiFluor AP1 (Electron Microscopy Sciences, Hatfield, PA). Overview images of porcine aortic trifurcation tissue and murine TAC tissue were obtained through ×10 (Plan-Neofluar 0.4 NA, dry, Ph2) and ×20 (Plan-Neofluar, 1.30 NA, dry, DIC) objectives of an upright epifluorescence microscope (Zeiss AxioObserver Z1), controlled by TissueFAXS (TissueGnostics GmbH, Vienna, Austria) and analyzed using TissueQuest software (TissueGnostics GmbH, Vienna, Austria). Confocal images were obtained using a Leica SP8 spectral confocal microscope through a ×63 (HCX PL APO 1.40 NA, oil) objective. 555 and 647 channels were scanned sequentially. Images were analyzed using Imaris 7.2.1 (Bitplane AG, Zürich, Switzerland), z-stacks were created using ImageJ version 1.43u (NIH, USA).
IMMUNOBLOTTING

Whole cell lysates were prepared in RIPA buffer (Thermo Scientific, IL, USA) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, Germaby) and 1% HALT-phosphatase inhibitor cocktail (ThermoFisher Scientific). Protein concentrations were determined using the detergent compatible protein assay (Bio-Rad, VA), according to manufacturer’s protocol. Equal amounts of protein were loaded on a 10% denaturing SDS–polyacrylamide gel and separated by gel electrophoresis (110V). Next proteins were blotted onto nitrocellulose membranes using the Trans-Blot Turbo System (Bio-Rad, AV) at 2.5A for 7 mins, according to manufacturer’s instructions. Blots were incubated in Odyssey Blocking Buffer (Li-COR Biosciences, NE, USA) in TBS at room temperature for 30 mins and incubated at 4°C with primary antibodies (Table S7) in Odyssey Blocking Buffer overnight. After extensive washes with TBST, membranes were incubated with secondary antibodies (Table S7) in Odyssey Blocking Buffer at room temperature for 1 hour. Protein was detected using the Odyssey Infrared Imaging System (Li-COR Biosciences). Densitometric analysis was performed using Totallab 120 (Nonlinear Dynamics, Newcastle upon Tyne, England) set for automatic lane detection, Rolling ball background subtraction (20 pixel radius) and automatic lane detection (minimum slope=100; Noise reduction = 5%, % Max peak=1). Lane intensities were corrected for protein-loading using the intensities of GAPDH and normalized to the average intensity of control samples.

ANGIOGENIC SPROUTING CAPACITY

10 µl of MatriGel (BD Biosciences, San Jose, CA) was solidified in µ-Slide Angiogenesis (Ibidi, Martinsried, Germany). 15,000 cells per well were cultured on the solidified MatriGel in endothelial growth medium and the formation of sprouts was analyzed by conventional light microscopic analysis after overnight incubation. The number of hexagonal shapes per well was quantified manually.

COLLAGEN CONTRACTION ASSAY

Cells were suspended in a solution of rat tail Collagen type I (Corning, Corning, NY) containing 3 mg·mL⁻¹ NaHCO₃ and 0.1M Na₂HPO₄. Aliquots of 50 µL (containing 100.000 cells and 0.5 mg Collagen type I) were solidified at 37°C in a humidified incubator with 5% CO₂ for 30 mins. The collagen gels were released from the culture dishes by the addition of 2 ml endothelial growth medium and were imaged using a common Flatbed-scanner and allowed to contract for an additional 24 h. The degree of gel contraction was determined by measuring the total gel area and dividing the areas of the contracted gels by the areas of the gels before contraction.

STATISTICAL ANALYSIS

Data are presented as means ± s.e.m. N-values relate to independent experiments/samples. P-values were calculated using one-way analysis of variance followed by Bonferroni’s post-hoc comparisons tests using Prism Graphpad (Graphpad Software, La Jolla, CA, USA). P < 0.05 was considered statistically significant.
### Supplementary Figures

**Supplementary figure 1. In silico analysis identifies miR-374 as putative regulator of MAPK7 signaling.**

(A) Overview of the MAPK7 signaling pathway containing the upstream activators Rac1, MAP3K7 (TAK1), MAP3K3 (MEKK3), MAP2K5 (MEK5), MAPK7 (ERK5), and the downstream transcription factors MEF2A-D and KLF4. (B) In silico analysis (miRanda(40, 41)) identifies 12 microRNAs that target MAPK7. SVR-scores indicate the predicted target site efficacy. (C) In silico analysis of genes within MAPK7 signaling targeted by microRNA-374, -429, -200b/c, -143, -24, -488, -410, -183, -124 and miR-506. The miR-374 family targets 8/9 genes in the MAPK7 signaling pathway.

**Supplemental figure 2. MicroRNA-374b targets MAPK7 signaling.** In silico analysis (TarBase(42)) reveals that miR-374a and miR-374b collectively have 1305 putative gene targets, of which 527 and 434 are unique to miR-374a and miR-374b, respectively. 344 putative gene targets are shared between miR-374a and miR-374b. Analysis of genes within the MAPK7 signaling cascade indicates that MAP3K7 (TAK1), MAPK7 (ERK5), MEF2A and MEF2C are putative targets of miR-374a and miR-374b, whereas Rac1, MAP3K3 (MEKK3), MEF2D and KLF4 are targets of miR-374b only.
Supplemental figure 3. TGFβ1-induced microRNA expression. TGFβ1 induced the expression of miR-374a, miR-374b, miR-143, miR-24 and miR-410 in endothelial cells (p<0.05 vs unstimulated control cells), whereas it decreased the expression of miR-488 (p<0.05 vs unstimulated control cells). The expression of miR-429, miR-200b, miR-200c, miR-183, miR-124 and miR-506 were unchanged (Suppl.fig.2) upon TGFβ1 treatment. *** p<0.001, ** p<0.01, * p<0.05, n=5, one-sample t-test.
Supplemental figure 4. miR-374b expression levels associate with decreased expression of MAPK7 signaling members and EndMT. The increased expression of miR-374b in endothelial cells treated with TGFβ1 (black) compared to TGFβ1 and SB431542-treated endothelial cells (dark grey) or non-stimulated endothelial cells (light grey) were associated with decreased expression of MAPK7 signaling members RAC1, MAP3K3, MAPK7, MEF2D, and KLF4 (A), and associated with decreased expression of endothelial cell markers (VE-Cadherin and eNOS; B) and increased expression of mesenchymal cell markers (SM22α and CNN1; C). n=4, Pearson correlation, two-tailed.
Supplemental figure 5. RNAi-mediated repression of MAPK7 Signaling members. Lentiviral expression of shRNA oligonucleotides against MAPK7 signaling members reduced their expression >2-fold compared to endothelial cells that were transformed using scrambled-constructs. *** p<0.001, ** p<0.01, n=3, one-sample t-test.
### Table S1. Primers used for 3’-UTR-reporter construct preparations.

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All primers were obtained from Sigma-Aldrich, MO.

### Table S2. Oligos used for lentiviral miR-374b and shRNA expression.

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<th>Construct</th>
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| miR-374b  | NR_030620          | ACTCGGATGGGATATAATACAACCTGCTAAGTGTCCTAGCACTTA GCAGGTGTATATCTGGGTGTTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT...
MICRORNA-374B INDUCES ENDOTHELIAL-TO-MESENCHYMAL TRANSITION

Table S4. Primers used for microRNA transcript analysis.

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<td>TGCGGTATCCACTGGTCAGCTATTC</td>
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<tr>
<td>hsa-miR-410</td>
<td>GTCGTATCCAGTGCCAGGTCGAGTTTGCCTGATGCACTGGGAC</td>
<td>TGCGGTATCCACTGGTCAGCTATTC</td>
</tr>
<tr>
<td>hsa-miR-429</td>
<td>GTCGTATCCAGTGCCAGGTCGAGTTTGCCTGATGCACTGGGAC</td>
<td>TGCGGTATCCACTGGTCAGCTATTC</td>
</tr>
<tr>
<td>hsa-miR-488</td>
<td>GTCGTATCCAGTGCCAGGTCGAGTTTGCCTGATGCACTGGGAC</td>
<td>TGCGGTATCCACTGGTCAGCTATTC</td>
</tr>
<tr>
<td>hsa-miR-506</td>
<td>GTCGTATCCAGTGCCAGGTCGAGTTTGCCTGATGCACTGGGAC</td>
<td>TGCGGTATCCACTGGTCAGCTATTC</td>
</tr>
<tr>
<td>RNU6B</td>
<td>GTCGTATCCAGTGCCAGGTCGAGTTTGCCTGATGCACTGGGAC</td>
<td>TGCGGTATCCACTGGTCAGCTATTC</td>
</tr>
</tbody>
</table>

Amplification reactions for mature microRNAs used the same antisense primer (5' - CCAGTGCCAGGTCGAGTTTGCCTGATGCACTGGGAC-3'). All primers were obtained from Sigma-Aldrich, MO.

Table S5. Primers used for mRNA transcript analysis.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Reference Sequence</th>
<th>Sense Primer (5' → 3')</th>
<th>Antisense Primer (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH5</td>
<td>NM_001795</td>
<td>GTTCCACCTTCTGGGAGG</td>
<td>GTGCTGTGCGGCTGTCCT</td>
</tr>
<tr>
<td>CNN1</td>
<td>NM_001308341</td>
<td>CCAACCTGCGAGCTGTCGCGAG</td>
<td>TACCTGGTGCTCGCTGCTGCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>AGCCACATCGCTGACACAG</td>
<td>GCCCAATGACACCAATC</td>
</tr>
<tr>
<td>KLF4</td>
<td>NM_004235</td>
<td>GGGGAGAGAGACAGCTGCTCA</td>
<td>GGGGGAGGCTCTGAG</td>
</tr>
<tr>
<td>MAP3K3</td>
<td>NM_203351</td>
<td>ACACCGCGATCTGACG</td>
<td>AAGGGGAAGCAGACGTGTTGA</td>
</tr>
<tr>
<td>MAP3K7</td>
<td>NM_003188</td>
<td>AACTCCATTACGCCCTCGTCA</td>
<td>TTGGGAGCGGCTGCTAGAG</td>
</tr>
<tr>
<td>MAPK7</td>
<td>NM_139032</td>
<td>CCTGATGCTCAACCTGTGCTG</td>
<td>CTTGTTGCTGCTGAGAAG</td>
</tr>
<tr>
<td>MEF2D</td>
<td>NM_005920</td>
<td>AACCGGCGCATCATCGAG</td>
<td>GGCCTGCTGTGAGGGGGAG</td>
</tr>
<tr>
<td>NOS3</td>
<td>NM_000603</td>
<td>CACATGGGCCTGGGACTGAG</td>
<td>CAGAGGCTGCTGCTGAG</td>
</tr>
<tr>
<td>RAC1</td>
<td>NM_018890</td>
<td>CTGATGCGCAACATCAGAG</td>
<td>CAGAGGAAGCTGCTGAGGGAG</td>
</tr>
<tr>
<td>TAGLN</td>
<td>NM_003186</td>
<td>CTGAGGACTATGCGGGACTC</td>
<td>TAGTGGGCATCATCCTGAG</td>
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</tbody>
</table>

All primers were obtained from Sigma-Aldrich, MO.
### Table S6. Antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Supplier</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td>Santa Cruz Biotechnology, CA</td>
<td>sc-1506</td>
<td>1:200</td>
</tr>
<tr>
<td>ESM-1 (Endocan)</td>
<td>Lunginnov, France</td>
<td>lia-0901</td>
<td>1:200</td>
</tr>
<tr>
<td>MAPK7 (Erk5/BMK1)</td>
<td>Biosis, MA</td>
<td>bs-5486r</td>
<td>1:100</td>
</tr>
<tr>
<td>SM22α</td>
<td>Abcam, UK</td>
<td>ab14106</td>
<td>1:250</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Cell Signaling, CA</td>
<td>2500</td>
<td>1:100</td>
</tr>
<tr>
<td><strong>Secondary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Mouse IgG Alexa 594</td>
<td>Life Technologies, The Netherlands</td>
<td>A21203</td>
<td>1:500</td>
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<tr>
<td>anti-Goat IgG Alexa 594</td>
<td>Life Technologies, The Netherlands</td>
<td>A11058</td>
<td>1:500</td>
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<tr>
<td>anti-Rabbit IgG Alexa 647</td>
<td>Life Technologies, The Netherlands</td>
<td>A31573</td>
<td>1:500</td>
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</tbody>
</table>

### Table S7. Antibodies used for immunoblotting.

<table>
<thead>
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<th>Antigen</th>
<th>Supplier</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calponin (CNN1)</td>
<td>Abcam, UK</td>
<td>ab46794</td>
<td>1:1000</td>
</tr>
<tr>
<td>eNOS (NOS3)</td>
<td>BD Pharmingen</td>
<td>610299</td>
<td>1:500</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Abcam, UK</td>
<td>ab9484</td>
<td>1:5000</td>
</tr>
<tr>
<td>MAP3K3 (MEKK3)</td>
<td>Abcam, UK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK7 (Erk5/BMK1)</td>
<td>MerckMillipore, MA</td>
<td>07-039</td>
<td>1:1000</td>
</tr>
<tr>
<td>SM22α (Transgelin)</td>
<td>Abcam, UK</td>
<td>ab14106</td>
<td>1:1000</td>
</tr>
<tr>
<td>MEF2D</td>
<td>Bethyl Laboratories, TX</td>
<td>A303-522A</td>
<td>1:100</td>
</tr>
<tr>
<td>KLF4</td>
<td>Abcam, UK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>Cell Signaling, CA</td>
<td>2500</td>
<td>1:1000</td>
</tr>
<tr>
<td><strong>Secondary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Ms IRDye 800</td>
<td>LI-COR Biosciences, Germany</td>
<td>926-32212</td>
<td>1:10.000</td>
</tr>
<tr>
<td>anti-Rb IRDye 680</td>
<td>LI-COR Biosciences, Germany</td>
<td>926-68073</td>
<td>1:10.000</td>
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</table>
REFERENCES


