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Rationale: **Endothelial cells in situ are largely quiescent, and their isolation and culture are associated with the switch to a proliferative phenotype.**

Objective: **To identify antiangiogenic microRNAs expressed by native endothelial cells that are altered after isolation and culture, as well as the protein targets that regulate responses to growth factors.**

Methods and Results: **Profiling studies revealed that miR-223 was highly expressed in freshly isolated human, murine, and porcine endothelial cells, but those levels decreased in culture. In primary cultures of endothelial cells, vascular endothelial cell growth factor and basic fibroblast growth factor further decreased miR-223 expression. The overexpression of precursor-miR-223 did not affect basal endothelial cell proliferation but abrogated vascular endothelial cell growth factor–induced and basic fibroblast growth factor–induced proliferation, as well as migration and sprouting. Inhibition of miR-223 in vivo using specific antagomirs potentiated postnatal retinal angiogenesis in wild-type mice, whereas recovery of perfusion after femoral artery ligation and endothelial sprouting from aortic rings from adult miR-223$^{-/y}$ animals were enhanced. MiR-223 overexpression had no effect on the growth factor–induced activation of ERK1/2 but inhibited the vascular endothelial cell growth factor–induced and basic fibroblast growth factor–induced phosphorylation of their receptors and activation of Akt. β1 integrin was identified as a target of miR-223 and its downregulation reproduced the defects in growth factor receptor phosphorylation and Akt signaling seen after miR-223 overexpression. Reintroduction of β1 integrin into miR-223–ovexpressing cells was sufficient to rescue growth factor signaling and angiogenesis.**

Conclusions: **These results indicate that miR-223 is an antiangiogenic microRNA that prevents endothelial cell proliferation at least partly by targeting β1 integrin. (*Circ Res*. 2013;113:1320-1330.)**

Key Words: β1 integrin ■ angiogenesis inhibitors ■ hindlimb ■ microRNA-223 mouse ■ receptors, vascular endothelial growth factor

Under physiological conditions the vascular endothelium is quiescent and maintained in that state by several mechanisms, including contact inhibition, physical stimulation, and a balance of pro- and antiangiogenic factors. In fact it has been estimated that only 0.01% of the endothelial cells in the adult human vasculature are actively proliferating at any 1 time.[1,2] However, this situation is altered after vascular injury, inflammation, and growth factor stimulation, which result in endothelial cell activation. This induces a phenotypic switch from quiescent to proliferative during which endothelial cells remodel cell–cell junctions, begin to migrate and proliferate, eventually resulting in re-endothelization and angiogenesis.

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Editorial, see p 1270

MicroRNAs are small, noncoding RNAs of ≈20 to 26 nucleotides in length. They regulate gene expression post-transcriptionally by interacting with the 3′ untranslated region (UTR) of specific target mRNAs in a sequence-dependent manner.[3] Several microRNAs have been reported to regulate different steps in the process of angiogenesis, and both pro- and antiangiogenic microRNAs have been identified (for recent review see Dang et al[4]). The complexity of regulation becomes apparent even when focusing only on vascular endothelial cell growth factor (VEGF) signaling as VEGFA

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From the Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University and DZHK (German Centre for Cardiovascular Research) partner site Rhine-Main, Frankfurt, Germany (L.S., B.F., N.Z., T.F., J.H., A.E., R.P., I.F.); and Functional Proteomics, SFB 815 Core Unit, Faculty of Medicine, Goethe University, Frankfurt am Main, Germany (H.H.).

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Correspondence to Ingrid Fleming, PhD, Institute for Vascular Signaling, Centre for Molecular Medicine, Goethe University, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany. E-mail fleming@em.uni-frankfurt.de

Nonstandard Abbreviations and Acronyms

bFGF	basic fibroblast growth factor
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal regulated kinase
FGFR1	FGF receptor 1
UTR	untranslated region
VEGF	vascular endothelial cell growth factor
VEGFR2	VEGF receptor 2

expression is targeted by miR-93,[5] miR-200b,[6] miR-15a,[7] and miR-20b[8]; VEGF receptor 2 (VEGFR2) is regulated partially by miR-296,[9] and miR-126 targets negative regulators of the VEGF signaling cascade (ie, the Sprouty-related protein SPRED1[10–13] and the phosphatidylinositol 3-kinase regulatory subunit 2 [PIK3R2/p85-b]).[10,11] VEGF signaling also affects endothelial cell microRNA expression and can induce the expression of miR-16 and miR-424, which in turn downregulate the expression of the VEGFR2.[14]

Many of the initial screens that identified potentially angiogenic microRNAs were performed using cultured endothelial cells under basal and growth factor–stimulated conditions,[12,15,16] which is certainly more practicable than directly comparing microRNA expression in quiescent and proliferating endothelial cells in situ. However, focusing on cells in culture means that microRNAs involved in switching endothelial cells from a quiescent to proliferative phenotype may have been overlooked. The aim of this study was to compare the microRNA profile of endothelial cells directly after isolation and after short-term culture to identify microRNAs that may play a key role in endothelial cell homeostasis in vivo.

Methods

Reagents and other detailed methods are described in the Online Data Supplement.

Results

Rapid Downregulation of miR-223 in Cultured Endothelial Cells

To identify microRNAs that are altered during the quiescent to proliferative phenotypic switch and which may have antiangiogenic properties, we compared microRNA expression levels in freshly isolated and primary cultures of human umbilical endothelial cells. The most highly expressed microRNAs in freshly isolated CD144+ endothelial cells were miR-126, miR-26a, miR-24, miR-125a, miR-125b, miR-223, let-7, miR-16, let-7c, and miR-222 (Figure 1A). Although subsequent culture of the same cells to confluence had no effect on the majority of the microRNAs detected, we observed an increase in miR-222, whereas levels of miR-125a, miR-125b, and miR-223 were reduced (by 71.28±7.79%, 58.80±10.01%, and 95.24±6.59%, respectively).

MiR-223 was reported initially to be a myeloid-specific microRNA[17] and can be detected in high levels in neutrophils[17] and platelets.[18] To determine whether the high signal in the freshly isolated cells could be explained by contamination of the CD144+ endothelial cell populations isolated, we controlled the purity of the cells isolated using fluorescence activated cell sorting analysis. The cell suspension

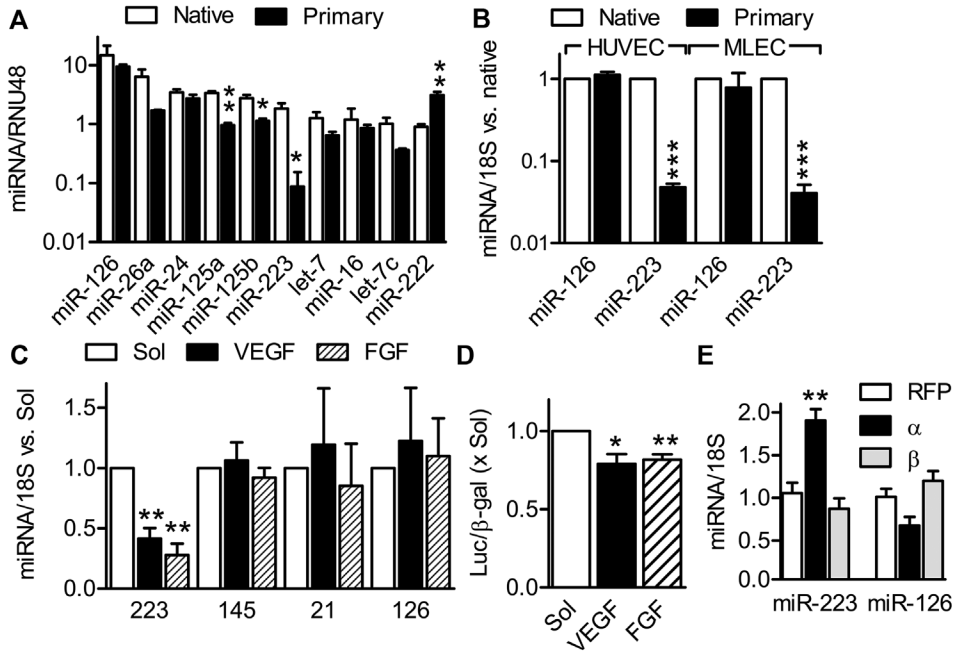

Figure 1. MicroRNA-223 expression in native and cultured endothelial cells. **A**, Comparison of the miR profile in freshly isolated (native) and confluent primary cultures of human umbilical vein endothelial cells (HUVEC). **B**, Effect of culture on miR-223 and miR-126 levels in HUVEC and mouse lung endothelial cells (MLEC). **C**, Effect of solvent (Sol), VEGF (30 mg/mL), and basic fibroblast growth factor (FGF; 30 mg/mL) on miR-223, miR-145, miR-21, and miR-126 expression in confluent primary cultures of HUVEC. **D**, Effect of growth factors on miR-223 core promoter activity in primary HUVEC cultures. **E**, Consequence of red fluorescent protein (RFP), CCAAT/enhancer binding protein-α (C/EBPα) or C/EBPβ overexpression on miR-223 expression in HUVEC (first passage). The graphs summarize data from 3 to 4 different cell batches; **P*<0.05, ***P*<0.01, and ****P*<0.001 vs native or solvent.

contained 80% to 85% CD31+ CD45− cells with 10% to 15% contamination with CD45+ cells. We then went on to deplete CD45+ cells after CD144+ selection. This resulted in cell suspensions that contained 95% CD31+ cells, with only 0.1% granulocyte (Ly6G+) and 0.05% monocyte (Ly6C+) contamination (Online Figure IA). Expression of miR-223 was not significantly different in human umbilical vein endothelial cells before and after sorting using CD144-coupled magnetic beads (Online Figure IB). In freshly isolated CD45-depleted endothelial cells, miR-223 could also be easily detected and again expression was markedly reduced by passaging (Online Figure IC). Both precursor (pre)-miR-223 and mature miR-223 levels were affected (Online Figure ID) and were paralleled by a marked decrease in miR-223 promoter activity (Online Figure IE). Comparable results were obtained when the expression of miR-223 was studied in CD144+ mouse lung endothelial cells (Figure 1B), and miR-223-like was studied in porcine aortic endothelial cells (Online Figure IF).

For miR-223 to act as a proliferative/angiogenic switch, we hypothesized that growth factors should decrease miR-223 expression. Indeed, in primary cultures of human endothelial cells (which still expressed the microRNA), both VEGF and basic fibroblast growth factor (bFGF) further decreased miR-223 without affecting the expression of miR-126 or the endothelial cell–enriched microRNAs: miR-145 and miR-21 (Figure 1C). The effect of the growth factors was related to altered transcription as VEGF and bFGF both decreased the activity of a miR-223 promoter construct in human umbilical endothelial cells (Figure 1D). CCAAT/enhancer binding protein-α (C/EBP) was reported previously to increase the activity of the miR-223 promoter,[19] and its overexpression (Online Figure II) in first-passage human endothelial cells increased miR-223 levels (Figure 1E). C/EBPβ was ineffective.

MiR-223 Attenuates Endothelial Cell Migration and Angiogenesis

To assess the role of miR-223 in angiogenesis, we overexpressed pre-miR-223 in cultured (passage 2) human endothelial cells, which expressed little endogenous miR-223. In these cells, pre-miR-223 overexpression had no effect on 5-bromo-2′-deoxyuridine (BrdU) incorporation in either the absence or the presence of serum (Figure 2A) but increased the basal rate of apoptosis (Online Figure III) and prevented the increase in proliferation induced by VEGF and bFGF (Figure 2B). Pre-miR-223 treatment also decreased endothelial cell migration on fibronectin-coated plates in a scratch-wound assay (Figure 2B), as well as VEGF- and bFGF-stimulated migration through a filter (Figure 2C). To assess the consequences of miR-223 inhibition on endothelial cell proliferation, endothelial cells were isolated from mice treated with a control antagomir or antagomir-223. Endothelial cells isolated from the latter animals demonstrated a significantly increased BrdU incorporation than cells from animals receiving the control antagomir (Online Figure IV).

The altered migration in pre-miR-223–expressing cells was also associated with a decrease in tube-forming ability

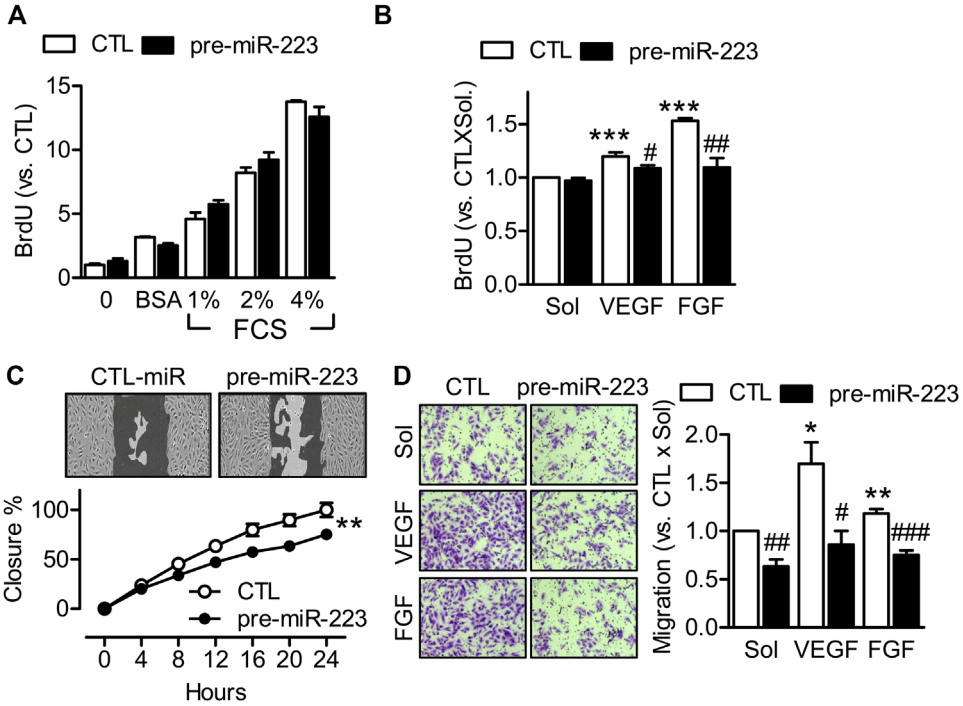

Figure 2. Effect of pre-miR-223 overexpression on the proliferation and migration of human endothelial cells. 5-Bromo-2′-deoxyuridine (BrdU) incorporation in pre-miR-223 or control miR (CTL) overexpressing human endothelial cells cultured (**A**) in the presence of different fetal calf serum (FCS) concentrations or (**B**) with solvent (Sol), vascular endothelial cell growth factor (VEGF; 30 ng/mL) or basic fibroblast growth factor (FGF; 30 ng/mL). **C**, Effect of pre-miR-223 on the migration of human endothelial cells in a scratch-wound assay; the black region indicates the area migrated after 24 hours. **D**, Effect of pre-miR-223 on endothelial cell migration through a Transwell filter after stimulation with VEGF or bFGF for 24 hours. Images show the crystal violet staining of migrated cells. The graphs summarize data from 3 to 9 different cell batches; **P*<0.05, ***P*<0.01, and ****P*<0.001 vs solvent. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs CTL with the same stimulation.

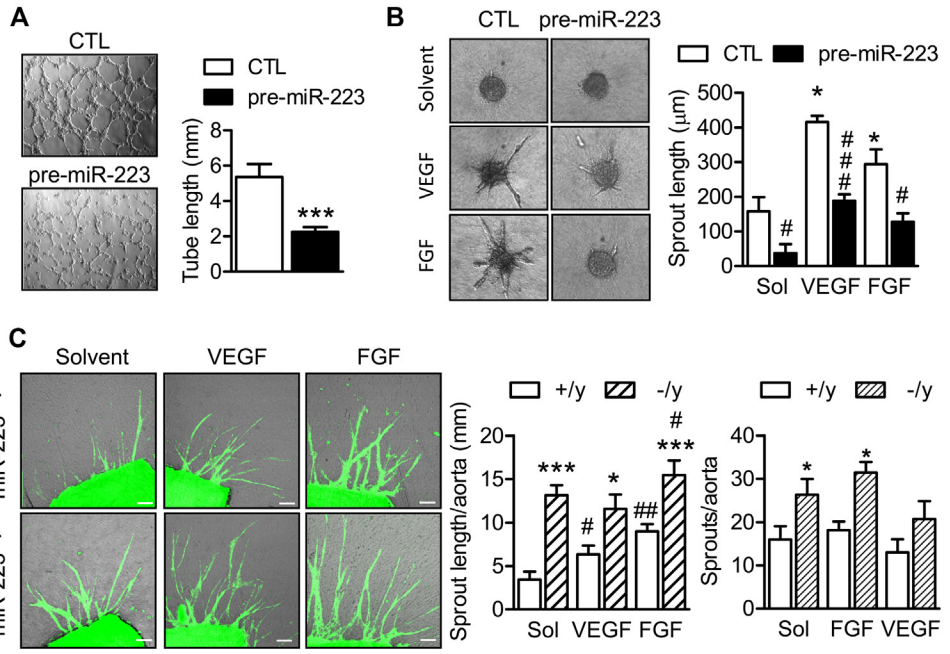

Figure 3. Effect of miR-223 on angiogenesis. Effect of pre-miR-223 on (**A**) tube formation on Matrigel and (**B**) sprouting from modified human endothelial cell spheroids. Experiments were performed in the absence and presence of vascular endothelial cell growth factor (VEGF; 30 ng/mL) or basic fibroblast growth factor (FGF; 30 ng/mL). **C**, Endothelial sprouting from aortic rings from wild-type (+/y) and miR-223$^{-/y}$ littermates, treated with solvent (Sol), VEGF, or FGF (30 ng/mL each); CD31, green; bar, 100 μm. The graphs summarized data from 4 to 5 independent experiments or animals; **P*<0.05, ****P*<0.001 vs CTL or +/y, #*P*<0.05 vs solvent.

(Figure 3A). Moreover, although VEGF and bFGF induced endothelial cell sprouting in a modified spheroid assay, this response was significantly impaired in pre-miR-223–expressing endothelial cells (Figure 3B). The miR-223 locus is located on the X chromosome[17] and to confirm our results in native endothelial cells, we studied endothelial sprouting by aortic rings from male wild-type (miR-223$^{+/y}$) and miR-223 knockout mice (miR-223$^{-/y}$). Although in the presence of mouse serum (2.5%) bFGF-induced endothelial cell sprouting from the isolated aortic rings (identified as CD31+ cells), both the sprout number and the sprout length were increased in samples from miR-223$^{-/y}$ mice (Figure 3C). A similar but slightly less pronounced effect was observed in response to VEGF.

miR-223 and Growth Factor Signaling

The next step was to determine at which level miR-223 interfered with growth factor signaling. Although VEGF and bFGF stimulated the phosphorylation of Akt (on Ser473) in human endothelial cells treated with a control pre-microRNA, this response was markedly attenuated in pre-miR-223–treated cells. In the same cells there was, however, no effect of miR-223 overexpression on the growth factor–induced phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2; Figure 4A and 4B). The inhibition of Akt phosphorylation by pre-miR-223 overexpression could not be explained by a decrease in the expression of VEGFR2 or FGF receptor 1 (FGFR1; Figure 4C) but by a significant attenuation in the agonist-induced tyrosine phosphorylation of both receptors: VEGFR2 by 68±13%; FGFR1 by 66±14% (both n=3; $P<0.05$; Figure 4D and 4E).

Effect of miR-223 Antagonism on Angiogenesis In Vivo

To confirm our findings in vivo, we assessed the vascularization of Matrigel plugs impregnated with either a control antagomir or an antagomir directed against miR-223, 7 days after implantation into wild-type mice. This model was chosen during a more general antagomir application to avoid potential systemic effects on the mobilization of progenitor/inflammatory cells. We found that the plugs containing antagomir-223 were better perfused and contained more Isolectin B4–positive cells than plugs impregnated with a control antagomir (Figure 5A).

In the postnatal retina, it was possible to demonstrate the incorporation of a fluorescently labeled antagomir in endothelial cells at the angiogenic front and in central areas of the developing retina when the probe was given to 2-day-old pups and incorporation assessed 3 days later (Figure 5B). When similar experiments were performed comparing a control antagomir with antagomir-223, we found that miR-223 inhibition increased BrdU incorporation into retinal endothelial cells (Online Figure V) and retinal angiogenesis (Figure 5C). The antagonism of miR-223 also increased tip cell numbers (Figure 5D).

We next assessed the vascularization of Matrigel plugs impregnated with either solvent or VEGF and bFGF, 7 days after implantation into wild-type or miR-223$^{-/y}$ mice. Although capillarization (Isolectin B4–positive cells) was similar in the solvent-impregnated plugs from both genotypes, the response to VEGF and bFGF was potentiated in the miR-223$^{-/y}$ animals (Figure 6A).

To investigate the impact of miR-223 on vascular repair in vivo in a more physiological model, we compared recovery after hindlimb ischemia in wild-type and miR-223$^{-/y}$ littermates. We found that as early as 7 days after surgery, the recovery of perfusion was accelerated clearly in miR-223$^{-/y}$ mice (Figure 6B). This difference became more pronounced at later time points and was associated with increased arteriogenesis (lectin and α-smooth muscle actin staining) in the ischemic adductor muscle and an increase in capillary density (angiogenesis) in the gastrocnemius muscle (Figure 6C).

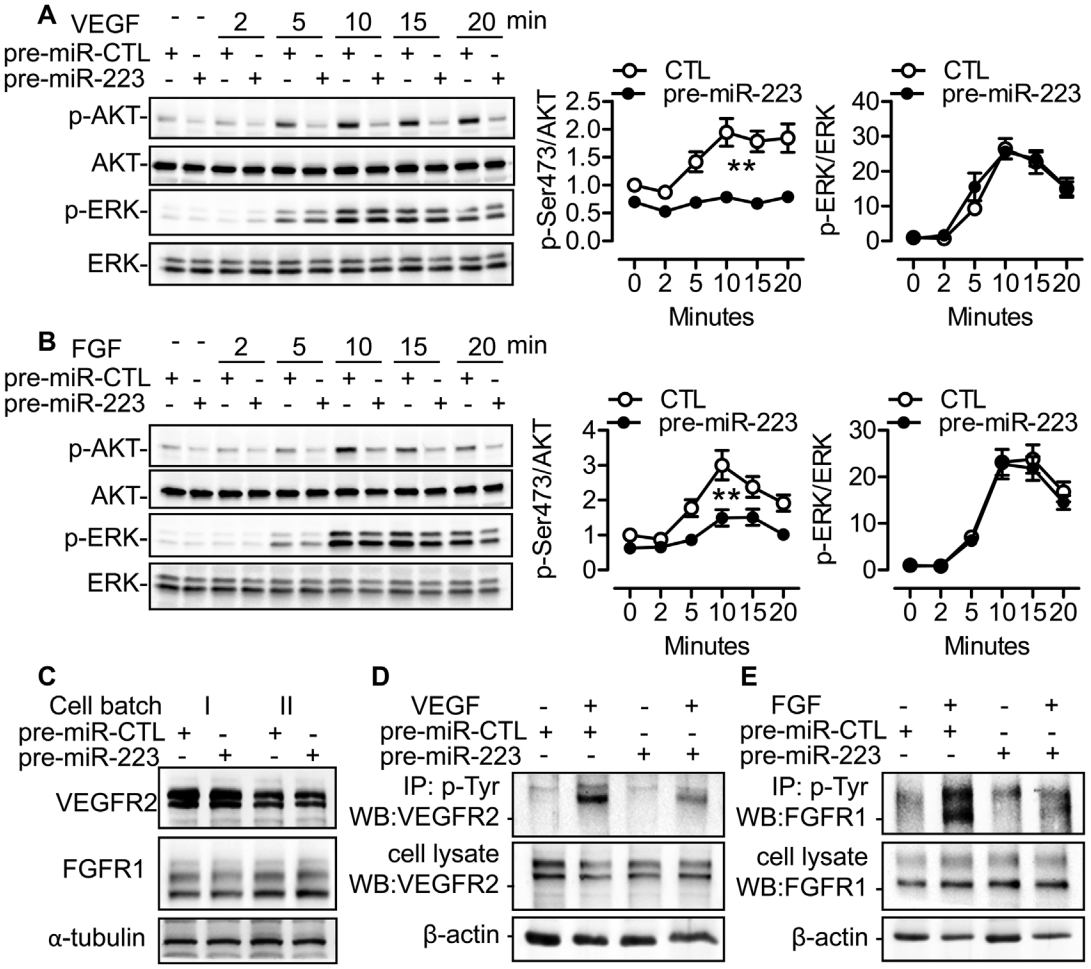

Figure 4. Consequences of miR-223 on vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) signaling in human endothelial cells. Endothelial cells were treated with either a control pre-miR (CTL) or pre-miR-223 48 hours before stimulation with (**A**) VEGF (30 ng/mL) or (**B**) FGF (30 ng/mL). **C**, Consequences of pre-miR-223 on the expression of VEGF receptor 2 (VEGFR2) and FGF receptor 1 (FGFR1). Consequences of pre-miR-223 overexpression on the agonist (30 ng/mL, 5 minutes)-induced phosphorylation of (**D**) VEGFR2 and (**E**) FGFR1. The graphs summarized data from 3 to 4 different cell batches; ***P*<0.01 vs CTL pre-miR. IP indicates immunoprecipitate; and WB, Western blot.

Identification of miR-223 Target Proteins in Endothelial Cells

To determine how miR-223 could regulate angiogenesis and identify miR-223 regulated endothelial cell proteins, we expressed pre-miR-223 in cultured human endothelial cells and used an isotope-coded affinity tag labeling strategy in combination with 2-dimensional chromatography and mass spectrometry.[20] Using this approach, we detected 64 differentially regulated proteins, 32 of which were upregulated and 32 of which were downregulated by ≥50% and 30%, respectively (Online Tables I and II).

We decided to study 3 of these proteins, endothelial nitric oxide synthase (eNOS), β1 integrin, and RhoB, in more detail as they have been linked previously with angiogenesis. The overexpression of pre-miR-223 in human endothelial cells that endogenously expressed only low levels of the microRNAs decreased the expression of eNOS, β1 integrin, and RhoB mRNA (Figure 7A) and protein (Figure 7B). However, no obvious seeding sequence for miR-223 could be detected within the eNOS 3′UTR, indicating that eNOS is likely to be an indirect rather than a direct target. Given that RhoB has already been identified as a miR-223 target[21] and has been implicated in angiogenesis,[22–24] we concentrated on β1 integrin.

A clear negative correlation between the expression of miR-223 and β1 integrin mRNA and protein was detected in human endothelial cells (Online Figure VI). Also, the expression of β1 integrin was markedly increased in endothelial cells in aortae and skeletal muscles from miR-223$^{-/y}$ mice versus their wild-type littermates (Figure 7C). A seeding sequence (7 base pairs of complementarity) for miR-223 was identified in 3′UTR region of β1 integrin. Cotransfecting human embryonic kidney-293 cells with increasing concentrations of pre-miR-223 and a luciferase construct containing the putative miR-223 binding sequence within the 3′UTR of β1 integrin, resulted in a concentration-dependent decrease in luciferase activity (Figure 7D). No effect of pre-miR-223 was detected when the binding sequence was mutated.

To determine the physiological relevance of β1 integrin in mediating the increased angiogenesis associated with miR-223

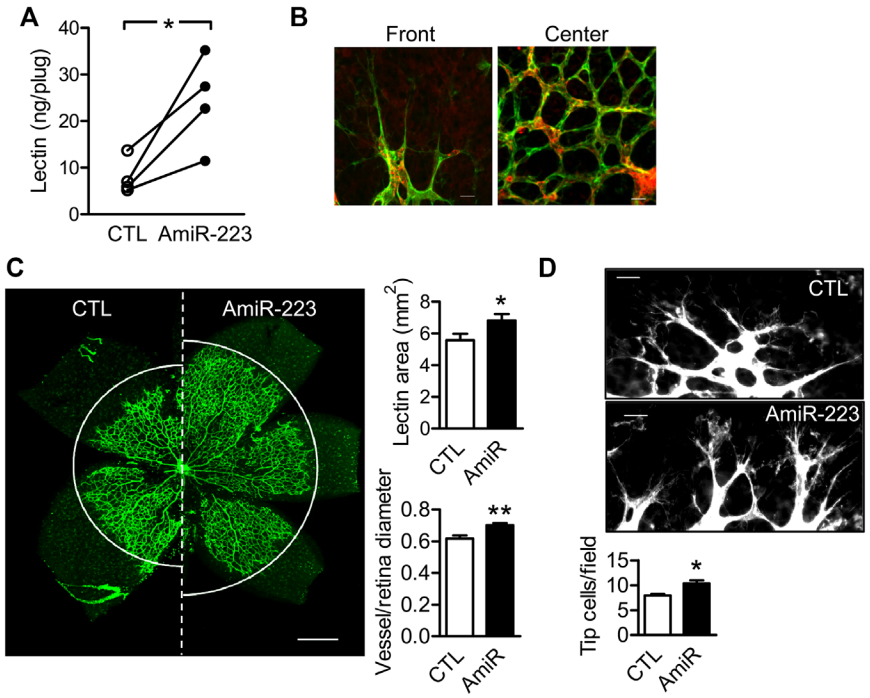

Figure 5. Effect of antagomirs directed against miR-223 on angiogenesis. **A**, Lectin content of Matrigel plugs impregnated with control (CTL) antagomir or antagomir-223 (AmiR) isolated 5 days after implantation into wild-type mice (n=4). **B**, Localization of Cy3-labeled AmiR-223 (red) in endothelial cells (green) in the front and central areas of the developing retinal vasculature in 5-day-old mice; bar, 20 μm. **C**, Consequences of CTL antagomir and AmiR-223 on retinal vascularization determined by Isolectin B4 staining; bar, 500 μm. **D**, High-magnification images and quantification of tip cells and filopodia in retinas from mice treated with control antagomir or antagomir-223. Isolectin B4 is shown in white; bar, 20 μm. The graphs summarize data from 4 to 10 different animals; **P*<0.05, ***P*<0.01 vs CTL antagomir treatment.

downregulation, a β1 blocking antibody was introduced into the vitreous of control antagomir- or antagomir-223–treated pups. Although the antibody did not alter retinal angiogenesis in animals treated with the control antagomir, it prevented the increase in angiogenesis in the antagomir-223–treated group (Figure 7E).

Rescue of the miR-223 Phenotype by β1 Integrin Overexpression

The downregulation of β1 integrin (small interfering RNA) in human umbilical vein endothelial cells reproduced the defects in VEGF- and bFGF-induced proliferation, migration, and tube formation seen previously in cells

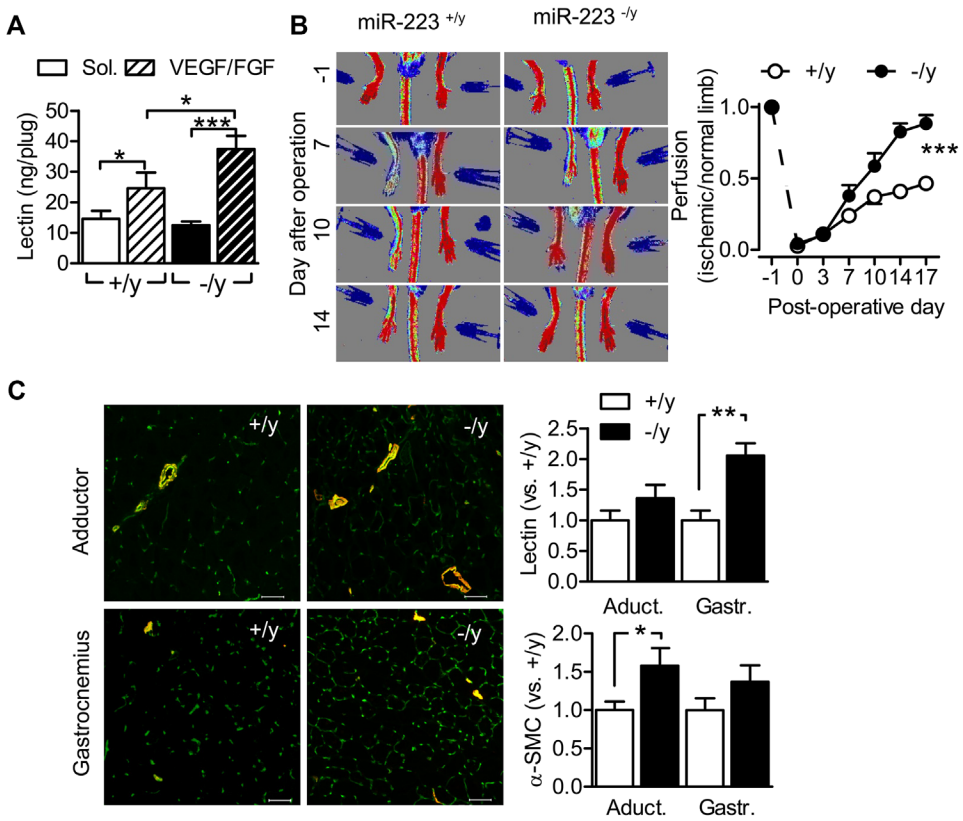

Figure 6. Increased angiogenesis and vasculogenesis in miR-223$^{-/y}$ mice. A, Fluorescein isothiocyanate-lectin content in Matrigel plugs impregnated with solvent (Sol) or vascular endothelial cell growth factor (VEGF) plus basic fibroblast growth factor (FGF; each 200 ng/mL) isolated 7 days after implantation into miR-223$^{+/y}$ or miR-223$^{-/y}$ littermates. **B**, Recovery of blood flow after hindlimb ischemia in miR-223$^{+/y}$ and miR-223$^{-/y}$ littermates. **C**, Isolectin B4 (green) and smooth muscle actin (red) staining 17 days after hindlimb ischemia in the adductor and gastrocnemius muscles; bar, 50 μm. The graphs summarize data from 6 to 7 different animals per group; **P*<0.05, ***P*<0.01 vs miR-223$^{+/y}$ or solvent.

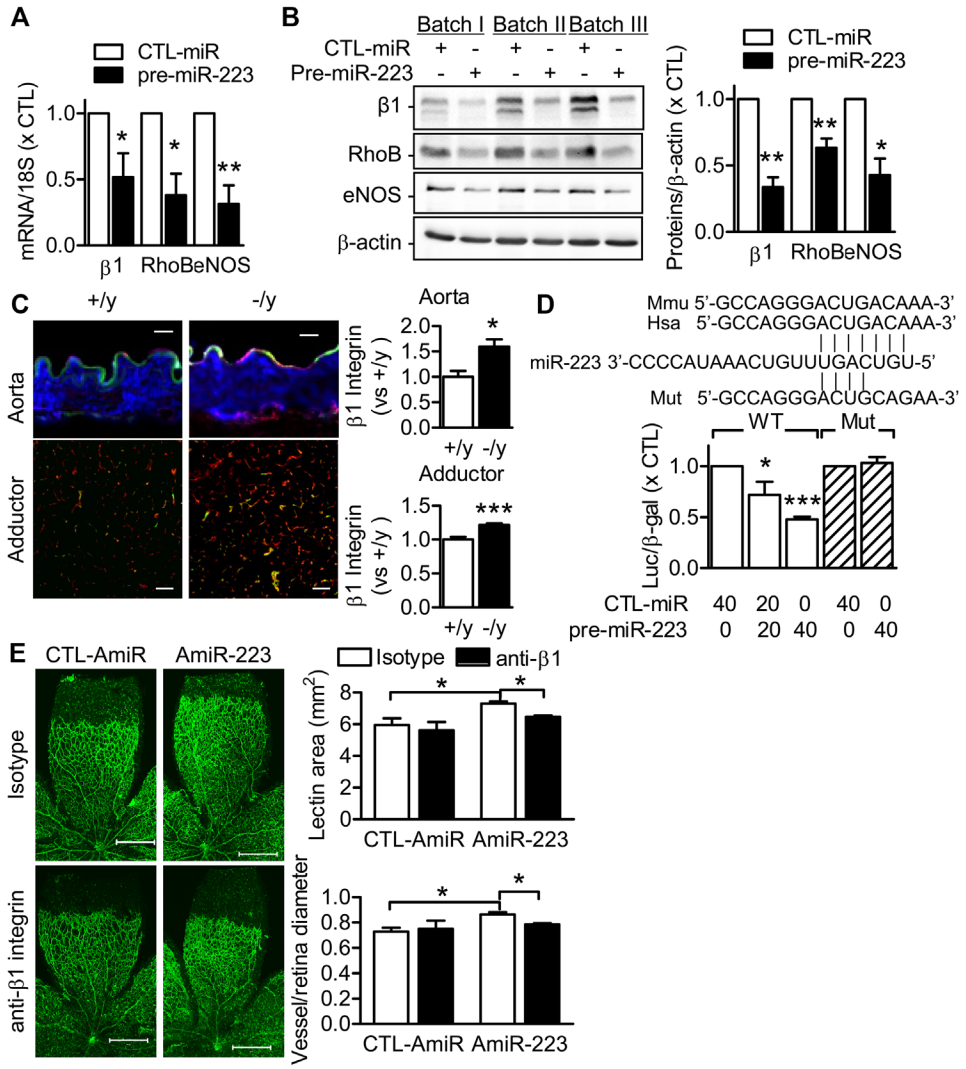

Figure 7. Identification of β1 integrin as a direct target of miR-223. The proteomics results were confirmed by (**A**) reverse transcription quantitative polymerase chain reaction and (**B**) Western blotting in 3 different batches of human endothelial cells. **C**, β1 integrin expression in aortae and adductor muscles from wild-type (+/y) and miR-223$^{-/y}$ mice; red, β1 integrin; green, isolectin B4; blue, α-smooth muscle actin; bar, 20 μm. **D**, Effect of control (CTL) miR and pre-miR-223 on the activity of the wild-type or mutated β1 integrin 3′UTR. **E**, Effect of a β1 integrin blocking antibody or its isotype CTL on retinal angiogenesis (isolectin B4 staining) in animals treated with CTL antagomir or antagomir-223; bar, 500 μm. The graphs summarize the data from 3 independent cell batches/independent experiments or 3 to 6 different animals per group; *P<0.05, **P<0.01, and ***P<0.001 vs CTL or +/y.

overexpressing pre-miR-223 (Online Figure VIIA–VIIC). Moreover, the loss of β1 integrin attenuated the ability of VEGF and bFGF to stimulate the phosphorylation of Akt, without affecting ERK phosphorylation (Online Figure VIID). Again this effect was related to the attenuated growth factor–induced phosphorylation of the VEGFR2 and FGFR1 receptors (Online Figure VIIE and VIIF). To determine how crucial β1 integrin was in mediating the antiangiogenic effects of pre-miR-223, we performed rescue experiments in which β1 integrin and pre-miR-223 were coexpressed. Although pre-miR-223 overexpression decreased the surface expression of β1 integrin, it was normalized by the approach used (Figure 8A). Similarly, although pre-miR-223 markedly disturbed endothelial cell tube formation, the overexpression of β1 integrin (40% transfection efficiency by electroporation; Online Figure VIII) partially restored tube-forming capacity (Figure 8B). This functional recovery was associated with a restored VEGF and bFGF signaling as the overexpression of β1 integrin in pre-miR-223–expressing human endothelial cells partially restored the growth factor–induced phosphorylation of Akt (Figure 8C and 8E), as well as the phosphorylation of the VEGF and bFGF receptors (Figure 8D and 8F).

Discussion

The results of the present investigation revealed that although miR-223 is expressed in native endothelial cells, it is rapidly downregulated by cell isolation and culture. Extrapolation of studies using pre-miR-223 suggested that the downregulation of miR-223 is required for VEGF- and bFGF-stimulated endothelial cell migration and tube formation, at least partly by targeting β1 integrin. Decreased miR-223 levels in vivo were also associated with a marked increase in angiogenesis in the murine retina and hindlimb; therefore, it seems that miR-223 may be required for the maintenance of endothelial cell quiescence.

The decision on which microRNA to study in a given situation is usually based on the analysis of differential microRNA expression but how reliable and physiologically relevant this information is, really depends on the experimental material studied (ie, whether tissue is fresh or studies are performed in primary or multi-passaged cell cultures). Such considerations are particularly relevant to endothelial cells. These

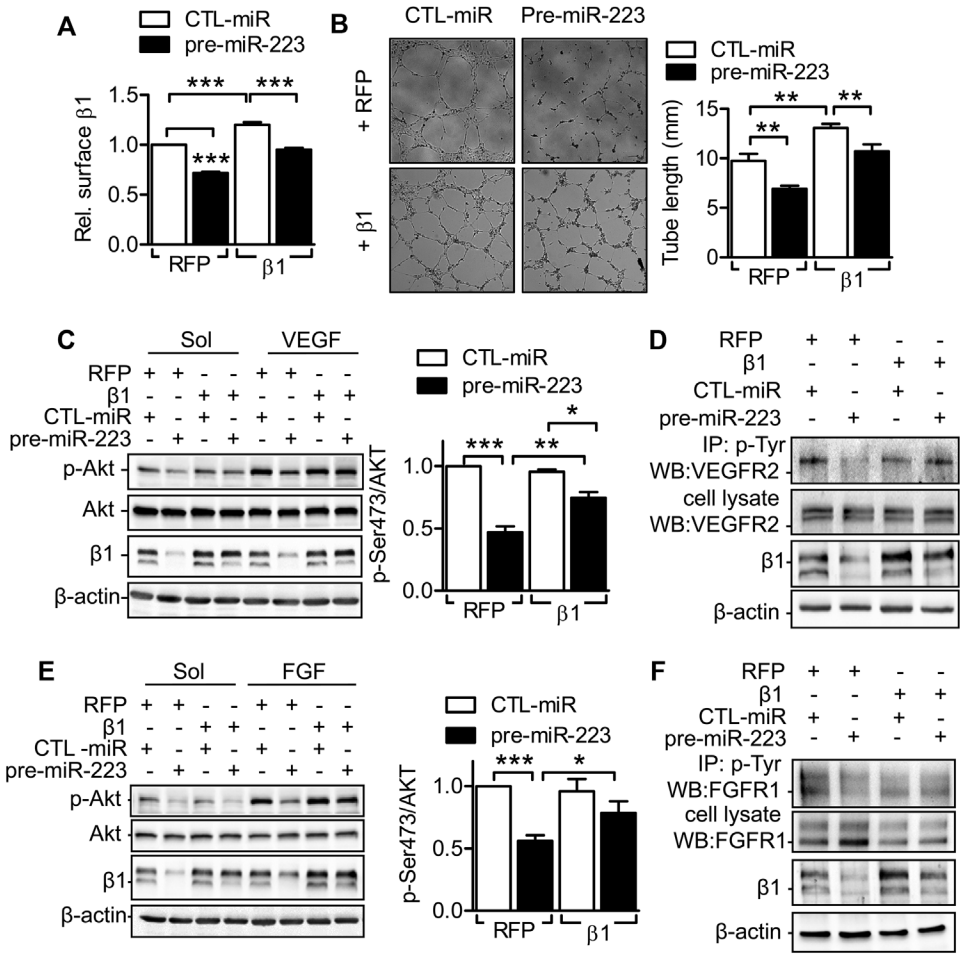

Figure 8. Overexpression of β1 integrin rescues impaired angiogenesis and signaling in miR-223–expressing cells. Human endothelial cells were transiently transfected with red fluorescent protein (RFP) or β1 integrin and either a control (CTL) microRNA or pre-miR-223. **A**, Cell surface expression of β1 integrin after transfection. **B**, Endothelial cell tube formation on Matrigel 48 hours after transfection. **C**, β1 integrin expression and vascular endothelial cell growth factor (VEGF; 30 ng/mL, 15 minutes)–induced Akt phosphorylation 48 hours after transfection. **D**, Consequences of β1 integrin overexpression on the tyrosine phosphorylation of VEGF receptor 2 (VEGFR2). **E**, β1 integrin expression and basic fibroblast growth factor (FGF; 30 ng/mL, 15 minutes)–induced Akt phosphorylation 48 hours after transfection. **F**, Consequences of β1 integrin overexpression on the tyrosine phosphorylation of FGF receptor 1 (FGFR1). The graphs summarize data obtained using 3 to 6 different cell batches; **P*<0.05, ***P*<0.01, and ****P*<0.001. IP indicates immunoprecipitate; and WB, Western blot.

cells proliferate at a low rate in situ but once isolated and placed in culture, endothelial cells switch to a proliferative phenotype, a process which must go hand-in-hand with de-differentiation and marked changes in gene expression. We performed microRNA expression profiling using freshly isolated human umbilical vein endothelial cells and the same cells after culture and found that although miR-223 could be detected in freshly isolated cells, levels decreased markedly (by ≈95%) by the time the cells reached confluence. This was not a phenomenon specific to venous endothelial cells as similar effects were recorded in mouse lung and in porcine aortic endothelial cells.

MiR-223 is reportedly enriched in neutrophils and monocytes,[17] as well as in platelets,[18,25] whereas it has been largely undetectable in cultured endothelial cells. As a result, it was important to demonstrate that the high levels of miR-223 detected in freshly isolated human endothelial cells did not reflect contamination with other cell types. We went about this in several different ways, first we controlled the purity of the CD144+ cells from human umbilical veins using fluorescence activated cell sorting analysis and found that the cell suspension after CD45 depletion resulted in a cell population that contained 0.1% granulocyte and 0.05% monocyte contamination. We detected miR-223 levels in cells isolated before and after sorting using CD144-coupled magnetic beads and found that expression was similar. Comparable results were obtained when miR-223-like expression was studied in porcine aortic endothelial cells and in CD144+ mouse lung endothelial cells. Although miR-223 levels decreased significantly after isolation, its expression was still clearly detectable in cells in primary culture. In these cells VEGF and bFGF both further attenuated miR-223 levels, as would be expected for an antiangiogenic microRNA, by decreasing miR-223 promoter activity. The overexpression of C/EBPα to increase the activity of the miR-223 promoter,[19] however, increased miR-223 levels. Why the expression of miR-223 decreases so markedly and so quickly in endothelial cells is unclear but may be related to epigenetic silencing mechanisms. Another alternative that cannot be excluded is that circulating cells contribute to the maintenance of high miR-223 levels in endothelial cells in vivo. Indeed, microvesicle-mediated microRNA transfer was demonstrated previously between platelets and endothelial cells,[26] as well as monocytes/macrophages and endothelial cells.[27,28] Such intercellular microRNA transfer is difficult to assess in vivo, but activated platelets have been reported to deliver Ago2-microRNA complexes to endothelial cells.[26] However, we found that native endothelial cells express both precursor miR-223 (absent from Ago2-miR complexes) and mature miR-223, and that the levels of both are decreased but still measurable in confluent primary endothelial cell cultures. Thus, although microRNA transfer may take place, part of

the decrease in mature miR-223 levels seems to be because of altered transcriptional regulation, which fits well with the observed decrease in miR-223 promoter activity.

Although the overexpression of pre-miR-223 had little effect on endothelial cell proliferation in the presence of serum, it abrogated VEGF- and bFGF-induced cell proliferation, migration, and tube formation in vitro. The deletion of miR-223, however, increased the ability of native endothelial cells to form tubes ex vivo in an aortic ring assay. In vivo, antagomirs directed against miR-223 increased the endothelization of Matrigel plugs and increased retinal angiogenesis. To investigate the impact of miR-223 on vascular repair in vivo in a more physiological model, we compared recovery after hindlimb ischemia in wild-type and miR-223$^{-/y}$ littermates. We found that as early as 7 days after surgery the recovery of perfusion was clearly accelerated in miR-223$^{-/y}$ mice and was associated with increased arteriogenesis in the ischemic adductor muscle and an increase in capillary density in the gastrocnemius muscle. All of the evidence supported the concept that high levels of miR-223 attenuate angiogenesis, whereas angiogenesis is accelerated after miR-223 downregulation. Given the high expression of miR-223 in neutrophils and monocytes,[17] a potential additional consequence of miR-223 deletion could be the altered mobilization/function of circulating progenitor cells with angiogenic potential (previously referred to as endothelial progenitor cells). The potential contribution of such cells to the recovery of perfusion after hindlimb ischemia is difficult to rule out without generating an endothelial cell–specific miR-223 knockout mouse. However, the clear effects of miR-223 inhibition in Matrigel plugs and the retina as well as the increased endothelial cell sprouting from isolated miR-223$^{-/y}$ aortic rings, all models in which progenitor cell mobilization would not be expected to play a major role, strongly indicate that at least part of the effects of miR-223 deletion are endothelium dependent.

How does miR-223 attenuate the endothelial response to VEGF and bFGF? The overexpression of miR-223 in cells that expressed low levels of the microRNA had no effect on VEGFR2 or FGFR1 expression but markedly attenuated the growth factor–induced phosphorylation of both receptors. This phenomenon was associated with the impaired growth factor–induced phosphorylation of Akt, whereas the phosphorylation of ERK1/2 was not affected and indicated that a specific step in the growth factor signaling pathway was targeted by miR-223.

To identify proteins regulated by miR-223 in endothelial cells, we used a proteomic approach to study differential protein expression in human endothelial cells overexpressing a control pre-miR or pre-miR-223. Of the >60 differentially regulated proteins, we focused on eNOS, RhoB, and β1 integrin. eNOS is a protein known to play a key role in regulating vascular tone and endothelial cell proliferation, migration, and angiogenesis. A role for microRNAs in the regulation of eNOS has long been suspected because knocking down Dicer, which is necessary for microRNA maturation, increased eNOS expression.[29] Although eNOS levels were decreased in pre-miR-223–overexpressing cells, there was no identifiable miR-223 seeding sequence in the eNOS 3′UTR, and it seems that miR-223, like miR-221 and miR-222,[29] has only an indirect effect on eNOS expression. The 3′UTR of RhoB was reported previously to be targeted by miR-223 in different cell lines,[21] and the protein has been implicated in angiogenesis.[22–24] Therefore, we concentrated on determining a link between miR-223 and β1 integrin expression.

There is certainly no doubt about the importance of β1 integrin in angiogenesis as its deletion is associated with impaired cell adhesion, migration, survival, and angiogenesis,[30] as well as with the establishment of endothelial cell polarity and lumen formation.[31] Indeed, the endothelial cell–specific deletion of β1 integrin results in disorganized capillaries and is embryonically lethal.[30] β1 integrin was found to be a direct target of miR-223 as cotransfection with increasing concentrations of miR-223 significantly attenuated the expression of a reporter gene construct containing the putative miR-223 binding sequence within the 3′UTR of the β1 integrin mRNA. Mutation of the miR-223 binding sequence resulted in the loss of this effect. Moreover, β1 integrin levels were decreased in cultured endothelial cells that overexpressed pre-miR-223 but increased in native endothelial cells from miR-223$^{-/y}$ mice. MicroRNAs potentially target hundreds of different target proteins to elicit a biological effect. To determine how crucial β1 integrin was to the biological actions of miR-223 the consequences of its downregulation (small interfering RNA) were analyzed. We found that β1 integrin small interfering RNA decreased VEGF- and bFGF-induced endothelial cell proliferation, migration, and tube formation, as well as the growth factor–induced phosphorylation of the VEGFR2 and FGFR1 receptors and Akt phosphorylation. Thus, reduced β1 integrin expression was largely able to account for the antiangiogenic effects of miR-223. These observations fit well with other recent reports that β1 integrin is required for VEGFR2[32] and FGFR1[33] phosphorylation.

Performing rescue experiments in endothelial cells is hampered by the difficulties in transfecting these cells. However, although maximal transfection efficiency was estimated at 40%, it was possible to compensate partially for the miR-223–induced defects in endothelial angiogenesis. Although the rescue was not complete, the transient overexpression of β1 integrin in cells pretreated with pre-miR-223 also improved growth factor–induced receptor phosphorylation and Akt activation. Exactly why the loss of β1 integrin attenuated VEGF- and bFGF-induced Akt phosphorylation, but not that of ERK1/2, is unclear but may be related to β1 integrin binding proteins such as integrin cytoplasmic domain–associated protein-1, which differentially regulates ERK and Akt phosphorylation.[34]

MiR-223 is currently of interest as circulating miR-223 levels are correlated inversely with the risk of developing diabetes mellitus[35] and myocardial infarction.[36] Although changes in circulating miR-223 levels have been suggested to reflect largely changes in platelet microRNAs, it will be interesting to determine whether the endothelial dysfunction associated with cardiovascular disease is also associated with altered miR-223 levels in endothelial cells.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Endothelial cells proliferate at a low rate in situ but once isolated and placed in culture they switch to a proliferative phenotype.
- Endothelial cell proliferation must go hand-in-hand with dedifferentiation and marked changes in gene expression.
- Several microRNAs have been reported to regulate different steps in the process of angiogenesis and both pro- and antiangiogenic microRNAs have been identified.

What New Information Does This Article Contribute?

- miR-223 is expressed in native endothelial cells. Cell isolation and culture, as well as by treatment with vascular endothelial cell growth factor and basic fibroblast growth factor, rapidly downregulate miR-233 level.
- miR-223 targets β1 integrin. Expression of pre-miR-223 attenuates the vascular endothelial cell growth factor–induced and fibroblast growth factor–induced phosphorylation of their receptors and downstream Akt phosphorylation.
- Decreased miR-223 levels markedly increased angiogenesis in vivo in the murine retina and ischemic hindlimb.

The majority of the pro- and antiangiogenic microRNAs identified to date have been characterized using cultured endothelial cells. Therefore, microRNAs playing a potentially crucial role in switching endothelial cells from a quiescent to proliferative phenotype might have been largely overlooked. We report that a microRNA (miR-223) typically associated with neutrophils and platelets is expressed in native endothelial cells and is rapidly downregulated after isolation and culture of the endothelial cells. MicroRNA-223 attenuates endothelial cell proliferation by targeting the expression of β1 integrin and reducing the vascular endothelial cell growth factor–induced and basic fibroblast growth factor–induced phosphorylation of their receptors, as well as downstream Akt phosphorylation. Downregulation or deletion of miR-223 markedly increased β1 integrin expression, as well as angiogenesis and vascular repair. Whether the rapid decrease in miR-223 expression in culture reflects epigenetic silencing mechanisms and the lack of miR-223 transfer to the endothelium from other circulating cells remains unclear. Circulating miR-223 levels are inversely correlated with the risk of developing diabetes mellitus and myocardial infarction. It will, therefore, be interesting to determine whether the endothelial dysfunction associated with cardiovascular disease is also associated with altered miR-223 levels in endothelial cells.

SUPPLEMENTAL MATERIAL

MicroRNA-223 antagonises angiogenesis by targeting β1 integrin and preventing growth factor signaling in endothelial cells

Materials

Cell culture media were purchased from Gibco (Invitrogen, Karlsruhe, Germany), human VEGF165, murine VEGF, human bFGF and murine bFGF were from PeproTech (Hamburg, Germany) and Matrigel was from BD Biosciences (Heidelberg, Germany). The antibodies directed against eNOS, RhoB, VEGFR2, phospho-tyrosine (clone PY20) were from Santa Cruz (Heidelberg, Germany), the antibody directed against β1 integrin used for Western blotting was from BD Biosciences, the β1 integrin antibody used for immunohistochemistry was from R&D Systems (Wiesbaden, Germany) and the β1 integrin blocking antibody was from Biolegend (Fell, Germany). The antibodies against, FGFR1, phospho-Ser473 Akt, total Akt, phospho ERK1/2 and total ERK were from New England Biolabs (Frankfurt am Main, Germany). The antibody against β-actin and all other substances were from Sigma-Aldrich (Munich, Germany), Merck or Roth (Darmstadt, Germany).

Animals

C57BL/6 mice (6-8 weeks old) were purchased from Charles River (Sulzfeld, Germany). miR-223-deficient mice, generated as described,[1] were kindly provided by Fernando D. Camargo, (Harvard University, Cambridge, MA 02138) and bred by the animal facility at the University of Frankfurt. As the miR-223 locus is located on the X chromosome, miR-223$^{-/Y}$ mice and their miR-223$^{+/Y}$ littermates were studied. Mice were housed in conditions that conform to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23). Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany; # F28/33) approved the study protocol.

Matrigel Plug Assay

Male miR-223+/y (wild-type) and miR-223$^{-/y}$ littermates (8 weeks old) were anesthetized by inhalation of Isoflurane and 500 µL Matrigel (BD Biosciences, Heidelberg, Germany) impregnated with heparin (25 U/mL) containing solvent of growth factors (VEGF, basic FGF, each 200 ng/mL) were injected s.c. along the dorsal midline on each side of the spine. In some experiments the Matrigels were impregnated with control miR or antagomiR-223 (8 µg/mL, VBC-Biotech GmbH, Vienna, Austria).

Oligonucleotide	**Sequence**
Control antagomir-GFP	5'-AAGGCAAGCUGACCCUGAAGUU-3'
Antagomir-223	5'-UGGGGUAUUUGACAAACUGACA-3'

After seven days FITC-lectin (20 µg i.v.) was applied via the tail vein 10 minutes before sacrifice. To quantify the lectin content of the plugs they were dissected and dissolved in RIPA buffer (Tris 50 mmol/L, NaCl 150 mmol/L, 0.1%SDS, 0.5% deoxycholate, 1.0% Triton X-100) and the lectin concentration in the supernatant was measured using a plate reader (Envision 2014 Multilabel

reader, Perkin Elmer, Rodgau, Germany; excitation λ495 nm; emission λ528 nm). In order to evaluate the efficiency of antagomir uptake into endothelial cells, we implanted Matrigel plugs impregnated with Cy3-labelled antagmiR-223 (8µg/mL) into mice. Seven days after implantation, the Matrigel plugs were isolated, digested with collagenase II and the incorporation of the Cy3 into CD31+ cells was assessed by FACs analysis. Using this approach, we detected Cy3 fluorescence in approximately 93% of the CD31+ endothelial cells isolated.

Hindlimb Ischemia

Neovascularization capacity was investigated in a murine model of hindlimb ischemia using 6 to 8 week old wild-type and miR-223$^{y/-}$ mice. The deep femoral artery was ligated using an electric coagulator (ERBOTOM ICC50, ERBE), afterwards the superficial femoral artery and vein as well as the epigastric arteries were completely excised as described.[2] The overlying skin was closed with 3 surgical staples. Relative blood flow was determined by laser Doppler imaging (Laser Doppler Perfusion Imager System, Wilmington, Germany) at regular intervals for up to 24 days post-ligation. The perfusion of the ischemic and nonischemic limb was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature and to maintain a constant body temperature, mice were exposed to infrared light for 10 minutes before laser Doppler scans. The calculated perfusion was expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

Retina Preparation and Analyses. Retinas for whole-mount immunohistochemistry were fixed in 4% PFA for 2 hours at room temperature, or overnight at 4 °C. After fixation, retinas were blocked and permeabilized in 1% BSA and 0.5% Triton X-100 over night at 4°C. Then retinas were washed three times in Pblec buffer (0.5% Triton X-100, 1 mM $CaCl_2$, 1 mmol/L $MgCl_2$, and 1 mmol/L $MnCl_2$ in PBS [pH 6.8]), and incubated overnight in Pblec containing FITC-labelled Isolectin B4 (1:100, Sigma). After staining, retinas were post-fixed in 4% PFA for 15 minutes before flat-mounting in mounting medium (DAKO). All quantification was done with high resolution images taken using a Zeiss LSM 510 meta laser scanning confocal microscope as described.[3] In some experiments, control antagomir or antgomiR-223 (8 mg/kg) was administered (8 mg/kg i.p) to wild-type pups from postnatal day 2 to 4. In a further subset of animals BrdU (50 mg/kg i.p) was applied on day 5, 3 hours before sacrifice and retinas were isolated and analyzed.

To assess the role of β1 integrin in retinal angiogenesis, animals receiving control antagomir or antagomiR-223 were administered either a β1 integrin blocking antibody (1 µL, 1 mg/mL intravitreally) or an isotype control antibody on postnatal day 4. Retinas harvested on day 6 and analysed as outlined above.

Immunohistochemistry

Cryosections (10 µm) were cut after tissues (aortas and muscles) were embedded in Tissue-Tek® OCT Compound (Sakura, Staufen, Germany). Frozen sections were dried (37°C, 10 minutes) and fixed in PFA (4% in PBS, room temperature, 5 minutes). After washing in PBS, samples were incubated with blocking buffer (PBS containing 1% BSA and 0.5% Triton X-100) at room temperature for 2 hours, before being exposed to the primary antibody (overnight 4°C). After extensive washing and exposure to the appropriate secondary antibody (1 hour, room

temperature) samples were mounted in fluorescent mounting medium (Dako) and analyzed with a confocal microscope (Zeiss, LSM meta 501).

Cell Isolation and Culture

Human umbilical vein endothelial cells were isolated and purified using VE-cadherin (CD144) antibody-coated magnetic beads (Dynal Biotech, Hamburg, Germany) and cultured as described.[4] Mouse lung endothelial cells were prepared as described,[5] after purification by positive selection for CD144 expressing cells; and negative selection for the lymphatic endothelial cells using anti-podoplanin antibody-coupled beads (Biozol, Eching, Germany). Porcine aortic endothelial cells were isolated and cultured as described [6]. HEK293 cells were purchased from the American Tissue Culture Collection (Wesel, Germany) and cultured in minimal essential medium (PAA, Cölbe, and Germany) supplemented with 8% FCS, 0.1 mmol/L non essential amino acids, sodium pyruvate (1 mmol/L) and penicillin (50 U/mL) and streptomycin (50 µg/mL).

Transfection

Transfection of RNA oligonucleotides; pre-miR-223, control (scrambled) pre-miR, antisense miR-223 (Ambion, Darmstadt, Germany) or plasmids, were performed using RNA-Max or lipofectamine 2000 separately (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. Transfection of human endothelial cells was performed by electroporation using the NEON transfection kit (Invitrogen, Darmstadt, Germany). Trypsinised cells were resuspended ($6x10^5$ cells, 105 µL in buffer R provided with the kit) and incubated with 6 µg plasmid in a cuvette. Cells were electroporated with 1050 volts for 30 milliseconds and quickly resuspended in culture medium. Transfected cells were transferred to 3.5 cm plates and cultured in EBM medium with 8% FCS. The electroporation protocol generally resulted in a transfection efficiency of 40% determined by overexpression of GFP.

Plasmid Construction and Reporter Gene Assays

The 3' UTR of β1 integrin (2545- 3751 in NM 133376) was amplified from HUVEC genomic DNA using Thermo prime plus DNA polymerase (Abgene, Hamburg, Germany) and subcloned into a pMIR-reporter plasmid (Ambion, Darmstadt, Germany) into the Sac1 and Mlu1 restriction enzyme sites. The mutation of the β1 integrin miR-223 seeding sequence was generated using the QuickChange kit (Stratagene, Waldbronn, Germany) with miR-223 mutation primers. The primer sequences used for cloning and mutation were:

Cloning primer	**Sequence**
β1 integrin 3' UTR Forward primer(Sac1)	5'-TCTGAGCTCGCAAATCCCACAACACT -3'
β1 integrin 3' UTR Reverse primer(Mlu1)	5'-ATCACGCGT TTCATGCACACAACC -3'
β1 integrin 3' UTR mutation Forward primer	5'-GTAATTCATGCCAGGGACTGCAGAAAGACTTGAGACA GGATGG-3'
β1 integrin 3' UTR mutation Reverse primer	5'-CCATCCTGTCTCAAGTCTTTCTGCAGTCCCTGGCATGA ATTAC-3'

For the 3' UTR reporter gene assay, the appropriate plasmids were transfected into HEK293 cells in the presence and absence of pre-miR-223 and pcDNA3.1CMV-β-gal (Invitrogen, Darmstadt, Germany) for 48 hours.

For the miR-223 promoter activity assay, a plasmid containing luciferase under the control of the miR-223 core promoter (kind gift from by Masanobu Tanabe, Keio, Japan) together with pcDNA3.1CMV-β-gal were cotransfected into human endothelial cells and cultured in EBM medium with 2% FCS EBM medium with or without VEGF or bFGF stimulation (30 ng/mL each) for 48 hours. Luciferase (Promega, Mannheim, Germany) and β-galactosidase (Tropix, Bedford, MA) activities were measured according to the manufacturers' protocols. Luciferase activity was normalized to β-galactosidase activity and data are expressed as fold of the non-treated controls.

Cell Migration

Scratch wound assay. A wound maker (Essen Bioscience; Welwyn Garden City, United Kingdom) was used to generate a uniform wound in the endothelial cell monolayer, and cell migration was recorded and visualized by an automated microscope system (IncuCyte, Essen Bioscience) for up to 24 hours.

Transwell migration. Endothelial cells (5×10^5) were seeded on the upper chambers of a 24 well format cell culture insert with 8 µm pores (FALCON, Le Pont De Claix, France). The lower chamber contained EBM with 8% FCS and 30 ng/mL of either VEGF or bFGF. Twenty four hours later, the number of transmigrated cells was counted after trypsin treatment. Alternative, after methanol fixation, crystal violet staining (0.1%) was used to visualize transmigrated cells.

Cell Proliferation

Human endothelial cells were seeded in 96 well plates (0.5×10^3 cells per well) cultured with EBM medium containing 0.1% BSA, 1%, 2%, 4% FCS or VEGF or bFGF (30 ng/mL) in 2% FCS for 2 days. BrdU incorporation was assessed on the second day after seeding using a specific ELISA (Roche, Mannheim, Germany) according to the manufacturers' instructions.

***In vitro* angiogenesis assays**

Tube formation: HUVEC ($2X10^4$ in 50 µL EBM with 0.1% BSA) were seeded on Ibitreat angiogenesis slides (Ibidi, Martinsried, Germany) pre-coated with 10 µL Matrigel, and the formation of tubular like structure was recorded by Axio-Observer Z1, Zeiss) evaluated at 6 hours or 24 hours later and tube length was measured with Axiovision software (Zeiss, Germany).

Endothelial cell spheroids: Spheroids containing 400 cells were generated as described.[7] After 24 hours in a collagen gel, angiogenesis was quantified by measuring the cumulative length of all capillary like sprouts originating from the central plain of an individual spheroid using a computer assisted microscope (Axio-Observer Z1, Zeiss, Jena, Germany). At least 5 randomly selected spheroids per experimental group and experiment were analyzed.

Aortic ring assay: Aortae from miR-$223^{-/y}$ and miR-$223^{+/y}$ mice were removed, cleaned and embedded in a collagen gel (collagen type I, BD Biosciences) in a 48 well plate containing MCDB 131 medium (Gibco/Invitrogen) supplemented with murine serum (2.5%; PAA, Pasching,

Austria) as described.[8] After 24 hours, murine VEGF (30 ng/mL) and basic FGF (30 ng/mL) were added and the tube-like structures allowed to develop over 7 days. Thereafter, the samples were fixed (4% paraformaldehyde) and endothelial cells were visualized using antibodies against CD31.

FACS Analysis

After trypsination human endothelial cells were suspended and fixed with PBS containing 1% PFA and 1% BSA (30 min). FITC conjugated primary antibodies (FITC-CD29, GeneTex, Offenbach, Germany; or PE/cy7-CD31, Biolegend, Fell, , Germany) were incubated with the cells (1:100 dilution) at 4°C for 1 hour. After centrifugation (1200g, 4 min,) cells were resuspended in PBS with 1% PFA and surface protein levels were determined by FACS (FACSCalibur, BD Biosciences, Heidelberg, Germany).

Microarray

Total RNA was isolated from freshly isolated or P0 passaged HUVEC. The qPCR array analysis (360 mouse microRNA genes) was performed by Febit (Heidelberg, Germany). Expression values were normalized to the values of small nucleolar RNAs (RNU48 and RNU44).

Reverse transcription and real time PCR (RT-qPCR)

Total RNA from lung or cultured primary cells was extracted using a miRNA easy kit (Qiagen, Hilden, Germany) and equal amounts (1 µg) of total RNA was reverse transcribed (Superscript III, Invitrogen, Heidelberg, Germany). mRNA levels were detected using SYBR Green (Absolute QPCR SYBR Green Mix; Thermo Scientific, Hamburg, Germany). To quantify miRNAs, specific stem-loop primers were used for reverse transcription then specific primers and FAM-labeled oligonucleotides (universal probe library probe #13, Roche Diagnostics, Mannheim, Germany) were used for the subsequent real-time PCR. The relative expression level of miRNA and genes were calculated using the $2^{-\Delta\Delta Ct}$ method with 18S RNA as a reference.[9]

Primer	**Sequence**
miR-223 RT primer	5'-GTCTCTGCCTGTGCAGGGTCCGAGGTATTCGCACAGGC AGAGACTGG GGT-3'
miR-126 RT primer	5'-GTCTCTGCCTGTGCAGGGTCCGAGGTATTCGCACAGG CAGAGACCGCATT-3'
miR-21 RT primer	5'-GTCTCTGCCTGTGCAGGGTCCGAGGTATTCGCACA GGCAGAGACTCAACA-3'
miR-145 RT primer	5'-GTCTCTGCCTGTGCAGGGTCCGAGGTATTCGCACAGGC AGAGACAGGGAT-3'
miRNA qPCR Reverse primer (conserved)	5'-GTGCAG GGTCCG AGGT-3'
miR-223 qPCR Forward	5'-GTGGCTGTCAGTTTGTCAAATA-3'
miR-126 qPCR Forward	5'-GTGGCTCGTACCGTGAGTAATA-3'
miR-21 qPCR Forward	5'- GTGGCTAGCTTATCAGACTGAT-3'
miR-145 qPCR Forward	5'-GTGGCGTCCAGTTTTCCCAGGAA -3'
18S qPCR Forward primer (human, mouse)	5'-CTTTGGTCGCTCGCTCCTC-3'
18S qPCR Reverse primer (human, mouse)	5'-CTGACCGGGTTGGTTTTGAT-3'
18S qPCR Forward	5'-GAGGTTCGAAGACGATCAGA-3'

primer (pig)	
18S qPCR Reverse primer (pig)	5'-TCGCTCCACCAACTAAGAAC-3'
β1 integrin qPCR Forward (human)	5'-AATGAATGCCAAATGGGACACGGG-3'
β1 integrin qPCR Reverse (human)	5'-TTCAGTGTTGTGGGATTTGCACGG-3'
eNOS qPCR Forward (human)	5'-TGG CTG TCT GCA TGG ACC T-3'
eNOS qPCR Reverse (human)	5'-TGG TSA CTT TGG CTA GCT GGT-3'
RhoB qPCR Forward (human)	5'-CCATCCGCAAGAAGCTGGA-3'
RhoB qPCR Reverse (human)	5'-TCTTGGCAGAGCACTCGAGGTA-3'

Immunoblotting and Immunoprecipitation

Cells or aortic homogenates (by Tissuelyser, Qiagene, Hilden, Germany) were lysed with SDS sample buffer on ice for 10 minutes. Samples were heated at 95 °C for 5 minutes and subjected for 10 minutes to centrifugation at 10000g. SDS-PAGE was performed and proteins were detected using their respective antibodies, as described.[10] For immunoprecipitation cells were lysed in Triton X-100 lysis buffer (10 mmol/L Tris-HCL pH7.5, 30 mmol/L NaCl, 2 mmol/L $Na_4P_2O_7$, 20 mmol/L NaF, 200 µmol/L ortho vanadate, 10 µmol/L okadeic acid, 230 µmol/L PMSF). Antibodies against phospho-tyrosine (PY20, Santa Cruz, Heidelberg, Germany) were used to immunoprecipitate tyrosine-phosphorylated proteins. Antibody/antigen complexes were captured by Protein A/G-sepharose (GE-healthcare, Uppsala, Sweden) and beads were washed three times with lysis buffer. Samples were heated in SDS-PAGE sample buffer proteins and separated by SDS-PAGE as described[11]. After transfer to nitrocellulose membranes individual proteins were detected by specific antibodies.

Proteomic analyses

The identification and quantification of differentially expressed endothelial cell proteins was achieved using an isotope coded affinity tag (ICAT) labeling strategy in combination with two dimensional chromatography and mass spectrometry[12] using a cleavable variant of the ICAT reagent.

Isotope coded affinity tag (ICAT) labeling: Human endothelial cells were transfected with a control pre-miR or pre-miR-223. Forty eight hours after transfection the control and pre-miR-223-expressing cells were labeled in parallel using the Cleavable ICAT Reagent Kit (Applied Biosystems, Darmstadt, Germany) according to the supplier's protocol. Briefly, cells containing 100 µg of total protein were treated with 10% trichloroacetic acid (TCA) and pelleted. TCA pellets were resolubilized in denaturing/reducing buffer containing 50 mM Tris, pH 8.5, 0.1 % SDS and 1 mM TCEP (Tris-(2-carboxyethyl)-phosphine hydrochloride) and heated for 10 min in boiling water. After reduction, proteins from control and treated cells were labeled with the light and heavy cleavable ICAT reagent respectively. Light and heavy labeled samples were combined, the proteins tryptically digested and the resulting peptides cleaned up by cation

exchange. ICAT-labeled peptides were further enriched by biotin-avidin interaction using supplied affinity columns and the biotin moiety was cleaved of from the ICAT tag according to the supplier's protocol.

Mass spectrometry analysis of ICAT labeled peptides: ICAT labeled peptides were identified by 2-dimensional HPLC combined with electrospray ionization (ESI)-MS/MS analysis. At first ICAT-labeled peptides were separated by strong cation exchange chromatography (SCX) in 12 fractions. SCX fractions were further separated by reversed phase chromatography on a *nano*-HPLC C18 column and eluted peptides online submitted to MS/MS analysis in a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Briefly, 3 µm C18 reversed phase silica (Hypersil^{TM} GOLD, Thermo Fisher Scientific) was filled into a 10.5 cm x 75 µm ID PicoTip emitter (New Objective, Woburn, USA). Sixty minute HPLC runs using 30 minute gradients of 5% to 50% acetonitrile in water with 0.1 % formic acid, followed by a 90% acetonitrile column wash and 5% acetonitrile re-equilibration steps for 15 minutes each were applied. Eluting peptides were analyzed using acquisition method consisting of alternating cycles of a full MS scan with a mass resolution of 30,000 at 400 m/z following by Data Dependent^{TM} acquisition of collision induced dissociation spectra in the linear ion trap of 10 most abundant precursor ions with charge states of 2 and greater at 35 % normalized collision energy.

Evaluation of MS/MS spectra and quantification: The collected MS/MS spectra were identified and quantified in Proteome Discoverer 1.2 environment (Thermo Fisher Scientific). Spectra were matched with the human reviewed database downloaded from Uniprot (July 2011) using the Mascot 2.2 server search engine with following parameters: full tryptic peptides with one missed cleavage, mass precision of 10 ppm on precursor and 0.8 Da on fragment ions, optional oxidation of methionine, labeling of cysteines with the light or heavy ICAT tag (227.127 and 236.157 Da mass shift respectively). Peptide identifications were filtered by significance threshold set to 0.05. Protein quantification was achieved using the precursor ions quantifier node with mass precision set to 3 ppm and ratio reporting of heavy to light on cysteine side chain using only unique peptides. Experimental quantitation bias was normalized on the protein median of all quantified proteins using normalization option of Proteome Discoverer 1.2 quantification mode.

Statistical analysis

Data are expressed as the mean ± SEM, and statistical evaluation was performed using Student's *t* test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni *t* test, or ANOVA for repeated measures where appropriate. Values of $P<0.05$ were considered statistically significant.

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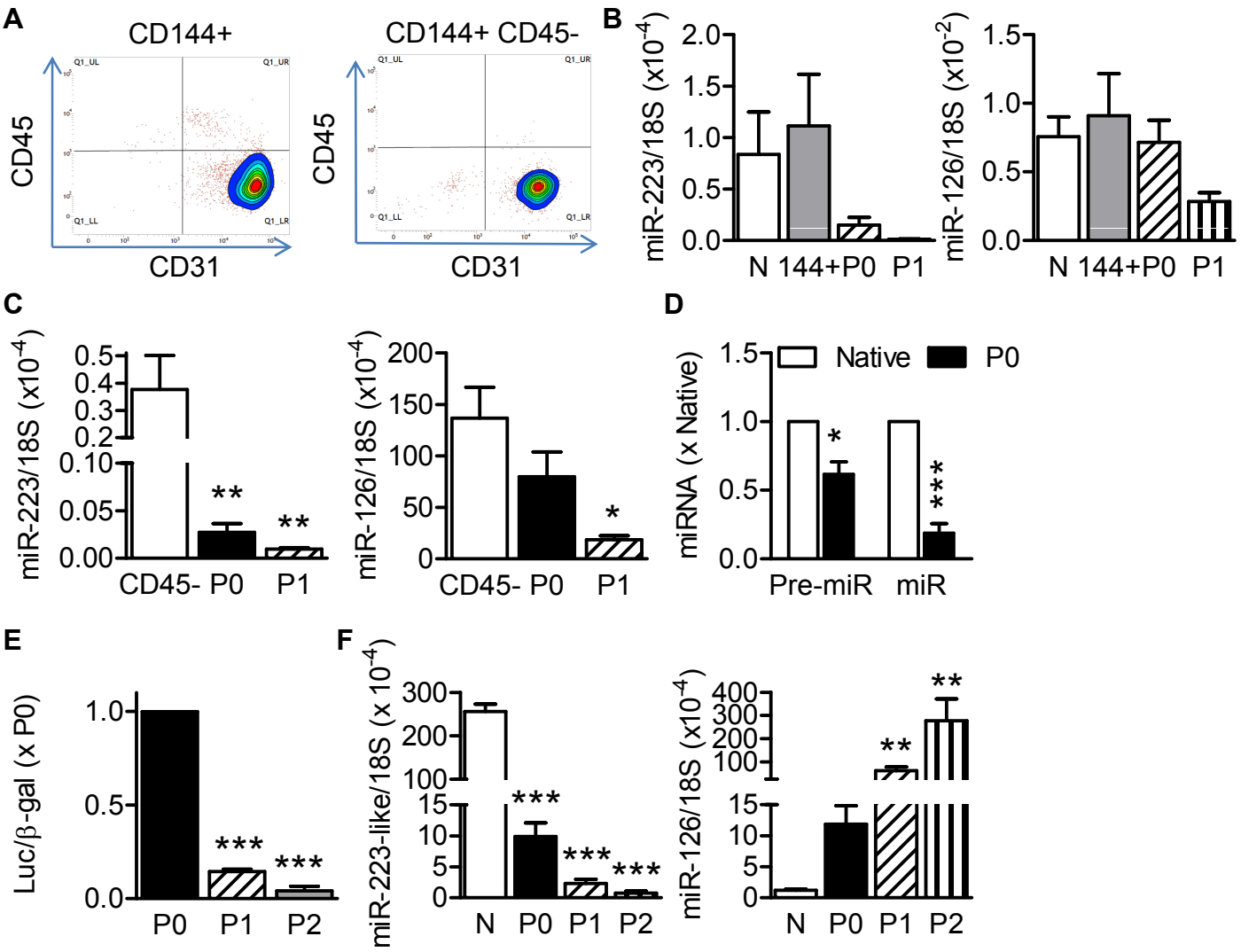
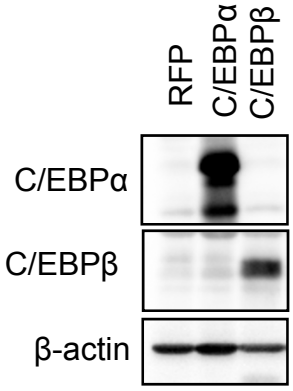
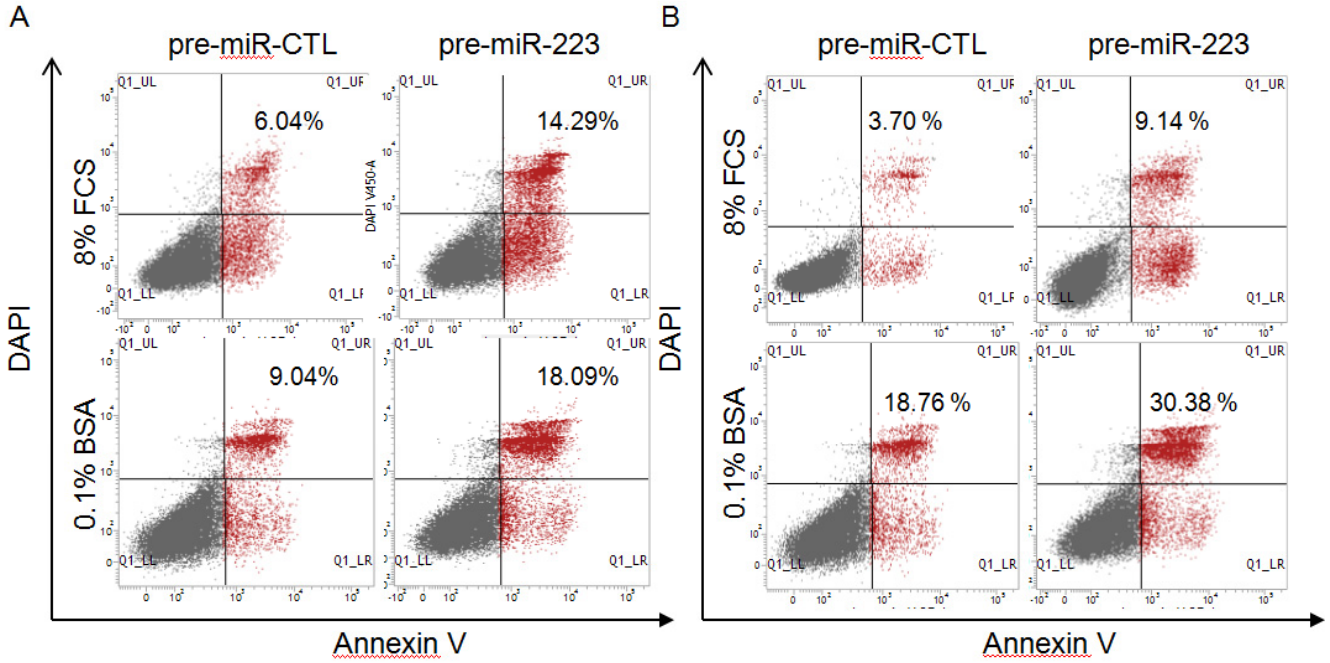


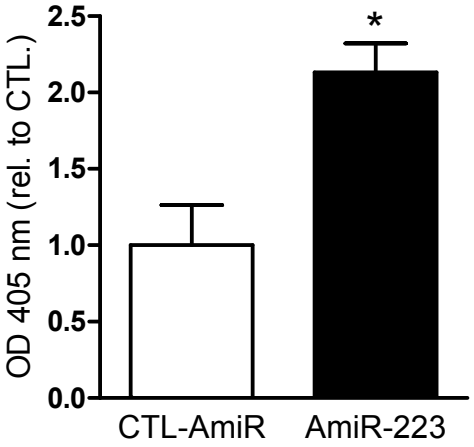
Online Figure I. **Rapid downregulation of miR-223 in cultured endothelial cells.** (A) FACS scatter blots showing CD31 and CD45 expression in freshly isolated human umbilical vein endothelial cells after positive selection by CD144 only (left) or in addition to CD45 depletion (left panel). Similar results were obtained in 3 additional cell batches. (B) MiR-223 and miR-126 expression in human umbilical vein endothelial cells immediately after isolation (native; N), after purification with CD144-beads (144+), in confluent primary cultures (P0) and after the first passage (P1). (C) MiR-223 and miR-126 expression in CD45 depleted human umbilical vein endothelial cells freshly isolated (CD45-) and cultured (P0 and P1). (D) Precursor (pre)-miR-223 and mature miR-223 levels in native and primary cultures (P0) of human umbilical vein endothelial cells. (E) The miR-223 promoter reporter construct and pcDNA3-CMV-beta-gal were co-transfected into different passages of endothelial cells and reporter gene activities were assessed after 2 days. (F) Comparison of miR-223-like and miR-126 expression in porcine aortic endothelial cells immediately after isolation (native; N), in confluent primary cultures (P0) and after the first (P1) and second (P2) passages. The graphs summarize data from 4-5 different cell batches; *P<0.05, **P<0.01, ***P<0.001 versus native or CD45- cells.



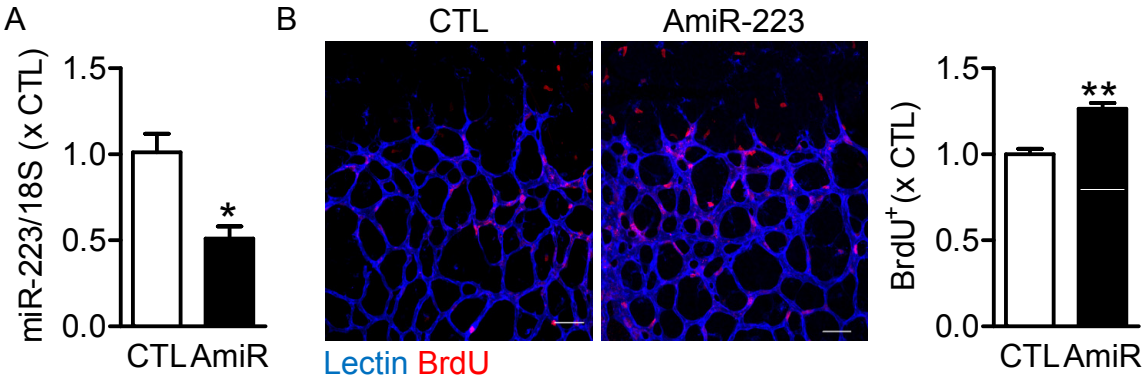
Online Figure II. **Electroporation mediated gene expression in HUVEC.** C/EBP α and β overexpression in human endothelial cells as determined by Western blotting two days after electroporation.



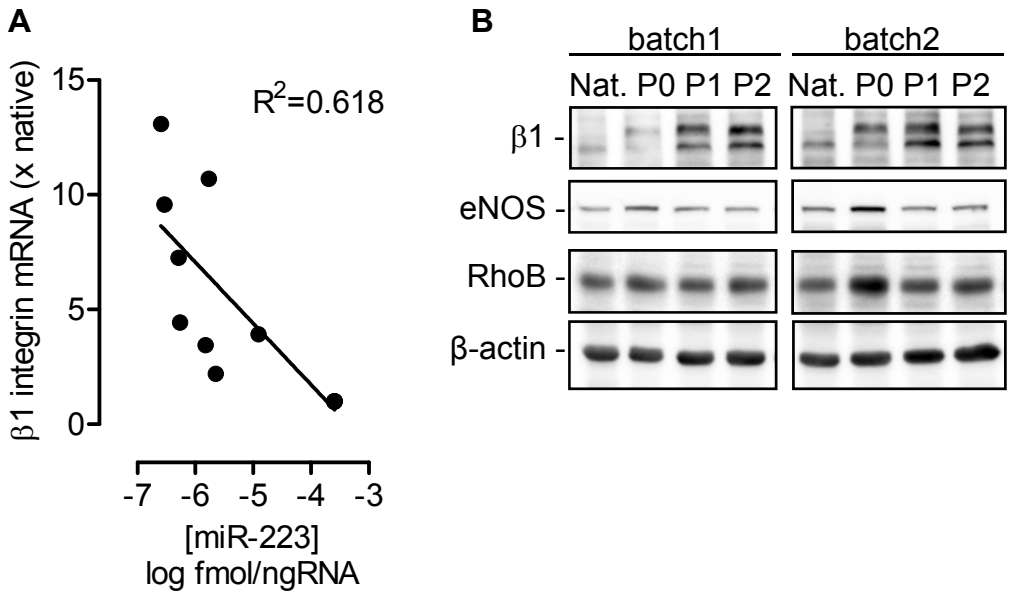
Online Figure III. Effect of miR-223 on endothelial cells apoptosis. (A) human umbilical vein endothelial cells (HUVEC) and (B) mouse lung endothelial cells (MLEC) were transfected with control pre-mir or pre-miR-223 and cultured in either basic medium (8% FCS) or serum-deprived medium (0.1% BSA) for 24 hours. FACS scatter evaluated cell apoptosis by Annexin V and DAPI double staining. Comparable results were obtained using 3 additional cell batches.



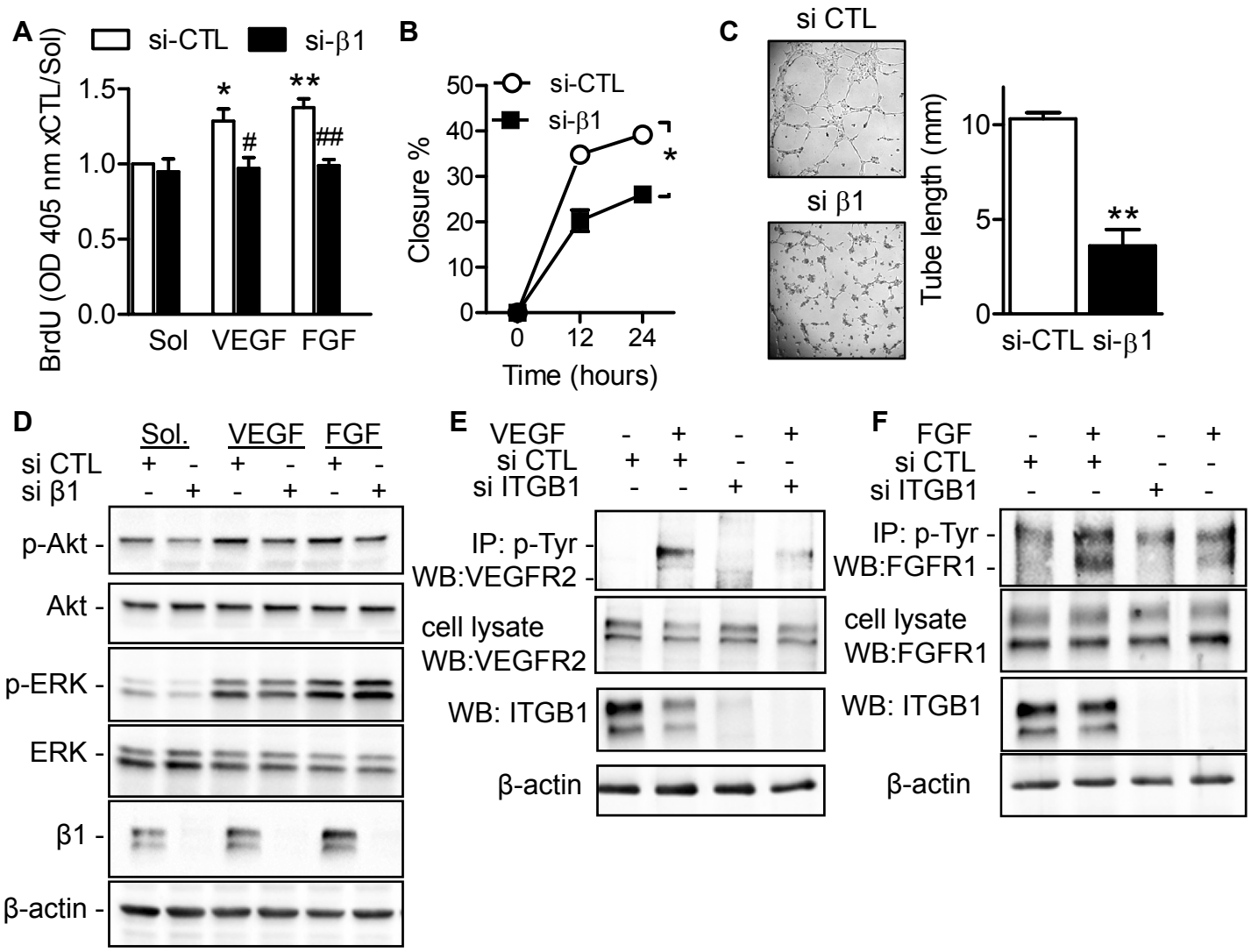
Online Figure IV. MiR-223 inhibition promotes the proliferation of freshly isolated mouse lung endothelial cells. Lung endothelial cells were isolated from wild-type mice treated with a control antagomir or antagomir-223 (AmiR-223). BrdU incorporation was assessed after 24 hours in culture. The graph summarizes data obtained from 3 animals with each experiment performed in quadruplicate; *P<0.05 vs CTL-AmiR.



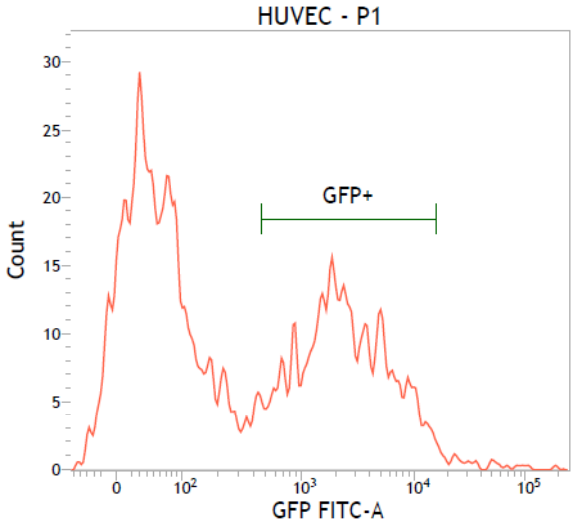
Online Figure V. Effect of miR-223 knockdown on endothelial cell proliferation in P5 retina. Mice (P2) were treated (i.p.) with control antagomir (CTL, 8mg/kg) or antagomir-223 (AmiR, 8mg/kg) daily until postnatal day 4. BrdU (50 mg/kg) was administrated (i.p.) 3 hours before sacrifice and the lungs and retina were isolated. (A) The efficiency of antagomiR-223 was determined by assessing miR-223 levels in freshly isolated mouse lung endothelial cells (CD31+). (B) Retinal whole mounts showing BrdU positive staining (red) in the developing vascular plexus (labeled with Isolectin B4, blue); bar=20 μm. The graphs summarize the data from 3-4 mice per group; *P<0.05, **P<0.01 versus CTL.



Online Figure VI. Expression of miR-223 and its targets in cultured human umbilical vein endothelial cells. (A) Correlation of miR-223 and β1 integrin mRNA expression in three different batches of human endothelial cells over 3 passages**.** (B) Expression of β1 integrin, eNOS and RhoB in two separate batches of native (Nat), primary (P0), first (P1), and second (P2) passaged human endothelial cells.



Online Figure VII. **Consequences of β1 integrin downregulation on *in vitro* angiogenesis and growth factor signaling**. Effect of siRNA mediated knockdown of β1 integrin (si-β1) on (A) endothelial cell proliferation in the presence of 2% FCS and solvent (Sol), VEGF (30 ng/mL) or bFGF (30 ng/mL), (B) endothelial cell migration in the scratch wound assay in the presence of 8% FCS, and (C) tube formation on Matrigel in the presence of 0.1% BSA. (D) Western blots showing the consequences of β1 integrin downregulation on the phosphorylation of Akt and ERK1/2 following cell stimulation with solvent, VEGF or bFGF (each 30 ng/mL, 15 minutes). (E&F) Consequences of β1 integrin downregulation on the growth factor-induced (each 30 ng/mL, 5 minutes) tyrosine phosphorylation of the (E) VEGFR2 and (F) FGFR1. IP=immunoprecipitate, WB=western blot. The graphs summarize data from 3 independent experiments; *P<0.05, **P<0.01 versus si-CTL/solvent; #P0.05 ## P<0.01, versus si-CTL with the same stimulation.



Online Figure VIII. **Electroporation mediated gene expression in human endothelial cells (1st passage).** Transfection efficiency (about 40%) as determined by FACS analysis for GFP positive cells two days after transfection with a peGFP-CYP2C8 plasmid.

Online Table I. Proteins downregulated by pre-miR-223 overexpression in human endothelial cells. The results summarize data obtained using 3 different cell batches and data are given relative to values from pre-miR-CTL treated cells.

Protein		Mean±SEM	P value
RRS1	ribosome biogenesis regulator homolog	0.29±0.27	0.003
RHOB	ras homolog family member B	0.37±0.10	<0.001
ADAS	alkylglycerone phosphate synthase	0.46±0.38	<0.001
CTGF	connective tissue growth factor	0.48±0.00	<0.001
FXR2	fragile X mental retardation, autosomal homolog 2	0.49±0.09	<0.001
BSG	basigin	0.48±0.44	0.059
LAMB1	laminin, beta 1	0.51±0.14	<0.001
LIMS3	LIM and senescent cell antigen-like domains 3	0.51±0.35	0.03
FN1	fibronectin 1	0.52±0.06	<0.001
HSPG2	perlecan (heparan sulfate proteoglycan 2)	0.51±0.03	<0.001
PURB	purine-rich element binding protein B	0.54±0.44	0.084
PODXL	podocalyxin-like	0.56±0.02	<0.001
RECQ1	RecQ protein-like (DNA helicase Q1-like)	0.58±0.09	<0.001
LAMC1	laminin, gamma 1	0.59±0.07	<0.001
MYH10	myosin, heavy chain 10, non-muscle	0.63±0.05	<0.001
PLEC1	plectin	0.63±0.08	<0.001
ITGAV	integrin, alpha V	0.64±0.14	<0.001
ITGA5	integrin, alpha 5	0.65±0.07	0.083
ESYT1	extended synaptotagmin-like protein 1	0.65±0.06	<0.001
ITGB1	integrin, beta 1	0.66±0.05	<0.001
TOMM70A	translocase of outer mitochondrial membrane 70 homolog A	0.66±0.00	<0.001
SAMM50	sorting and assembly machinery component 50 homolog	0.66±0.03	<0.001
VDAC3	voltage-dependent anion channel 3	0.68±0.04	<0.001
ATP2A2	ATPase, Ca^{2}+ transporting, cardiac muscle, slow twitch 2	0.69±0.02	<0.001
CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	0.69±0.06	<0.001
COL4A3	collagen, type IV, alpha 3	0.70±0.07	<0.001
TUBB6	tubulin, beta 6 class V	0.70±0.07	<0.001
HK1	hexokinase 1	0.70±0.04	<0.001
HNRNPM	heterogeneous nuclear ribonucleoprotein M	0.71±0.02	<0.001
SNRNP200	small nuclear ribonucleoprotein 200kDa (U5)	0.71±0.10	0.0054
DDX5	DEAD (Asp-Glu-Ala-Asp) box helicase 5	0.71±0.10	0.0020
NOS3	nitric oxide synthase 3 (endothelial cell)	0.71±0.09	<0.001

Online Table II. Proteins upregulated by pre-miR-223 overexpression in human endothelial cells. The results summarize data obtained using 3 different cell batches shown relative to pre-miR-CTL treated cells.

Protein		Mean±SEM	P value
ANXA6	annexin A6	1.51±0.39	0.42
SEC13	SEC13 homolog	1.61±0.01	<0.001
WARS	tryptophanyl-tRNA synthetase	1.61±0.17	<0.001
MIF	macrophage migration inhibitory factor	1.64±0.30	0.040
PGAM1	phosphoglycerate mutase 1	1.67±0.37	0.013
PAPSS2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	1.70±0.03	<0.001
FKBP4	FK506 binding protein 4, 59kDa	1.70±0.18	<0.001
ERLIN1	ER lipid raft associated 1	1.70±0.31	0.027
METAP2	methionyl aminopeptidase 2	1.71±0.16	<0.001
TPT1	tumor protein, translationally-controlled 1	1.71±0.46	0.024
GSTP1	glutathione S-transferase pi 1	1.72±0.52	0.034
ASNS	asparagine synthetase	1.77±0.43	0.013
ANXA1	annexin A1	1.78±0.40	0.047
CRIP2	cysteine-rich protein 2	1.78±0.06	<0.001
SERPINB6	serpin peptidase inhibitor, clade B (ovalbumin), member 6	1.79±0.53	0.026
PFN1	profilin 1	1.81±0.03	<0.001
PFAS	phosphoribosylformylglycinamidine synthase	1.82±0.55	0.027
ANXA4	annexin A4	1.82±0.63	0.042
RPS6	ribosomal protein S6	1.83±0.56	0.028
C6orf115	chromosome 6 open reading frame 115	1.89±0.48	0.012
TXN	thioredoxin	1.92±0.62	0.057
PGD	Phosphogluconate dehydrogenase	1.94±0.49	0.010
GNG11	guanine nucleotide binding protein (G protein), gamma 11	2.05±0.23	<0.001
UCHL1	ubiquitin carboxyl-terminal esterase L1	2.13±0.65	0.016
SORD	sorbitol dehydrogenase	2.16±0.41	0.002
LAP3	leucine aminopeptidase 3	2.20±0.24	<0.001
TKT	transketolase	2.31±0.48	0.0024
GMPR	guanosine monophosphate reductase	2.48±0.55	0.0026
BMX	BMX non-receptor tyrosine kinase	2.65±1.26	0.16
NMI	N-myc (and STAT) interactor	5.29±1.82	0.0045
HSD17B10	hydroxysteroid (17-beta) dehydrogenase 10	35.84±34.15	0.093
MX1	myxovirus (influenza virus) resistance 1	53.94±20.81	0.003