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## Regular Article

# TGF $\beta$ Triggers miR-143/145 Transfer From Smooth Muscle Cells to Endothelial Cells, Thereby Modulating Vessel Stabilization

Montserrat Climent, Manuela Quintavalle, Michele Miragoli, Ju Chen, Gianluigi Condorelli, Leonardo Elia

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**Rationale:** The miR-143/145 cluster is highly expressed in smooth muscle cells (SMCs), where it regulates phenotypic switch and vascular homeostasis. Whether it plays a role in neighboring endothelial cells (ECs) is still unknown.

**Objective:** To determine whether SMCs control EC functions through passage of miR-143 and miR-145.

**Methods and Results:** We used cocultures of SMCs and ECs under different conditions, as well as intact vessels to assess the transfer of miR-143 and miR-145 from one cell type to another. Imaging of cocultured cells transduced with fluorescent miRNAs suggested that miRNA transfer involves membrane protrusions known as tunneling nanotubes. Furthermore, we showed that miRNA passage is modulated by the transforming growth factor (TGF)  $\beta$  pathway because both a specific TGF $\beta$  inhibitor (SB431542) and an shRNA against TGF $\beta$ RII suppressed the passage of miR-143/145 from SMCs to ECs. Moreover, miR-143 and miR-145 modulated angiogenesis by reducing the proliferation index of ECs and their capacity to form vessel-like structures when cultured on matrigel. We also identified hexokinase II (*HKII*) and integrin  $\beta$  8 (*ITGB8*)—2 genes essential for the angiogenic potential of ECs—as targets of miR-143 and miR-145, respectively. The inhibition of these genes modulated EC phenotype, similarly to miR-143 and miR-145 overexpression in ECs. These findings were confirmed by ex vivo and in vivo approaches, in which it was shown that TGF $\beta$  and vessel stress, respectively, triggered miR-143/145 transfer from SMCs to ECs.

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**Conclusions:** Our results demonstrate that miR-143 and miR-145 act as communication molecules between SMCs and ECs to modulate the angiogenic and vessel stabilization properties of ECs. (*Circ Res.* 2015;116:00-00. DOI: 10.1161/CIRCRESAHA.116.305178.)

**Key Words:** angiogenesis effect ■ endothelial cells ■ microRNAs ■ smooth muscle myocytes

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Vascular endothelium plays a fundamental role in modulating vessel biology and homeostasis.<sup>1</sup> Alteration of its function can induce the development of vessel pathologies, such as atherosclerosis.<sup>2</sup> Several events are involved in angiogenesis, including endothelial cell (EC) division, selective degradation of the basement membrane and the surrounding extracellular matrix, EC migration, and the formation of neovessels.<sup>3</sup> Similar events take place during embryogenesis, but in this context blood vessels are generated from the differentiation of EC precursors, the angioblasts, which associate to form primitive vessels in a process referred to as vasculogenesis.<sup>3</sup> In both cases, once blood vessels are generated, ECs undergo tissue-specific changes creating vessels functionally distinct from each other.<sup>4</sup>

The endothelium maturation process is guided also by signals from other cell types. Indeed, development of a vessel's

architecture involves the strict association between ECs and mural cells (pericytes and smooth muscle cells [SMCs]): under physiological conditions, communication between these cell types leads to the maturation and stabilization of the vessel.<sup>5,6</sup> These processes involve the action of different growth factors as well as a variety of heterotypic cellular interactions and they could be important mechanisms, by which also pathological signals are transferred from one cell type to another.

MicroRNAs (miRNAs, miRs) constitute a group of short, noncoding RNAs that direct inhibitory complexes to targeted mRNAs.<sup>7</sup> MiRNAs have been implicated in a wide variety of physiological and pathological processes, including those of vessels.<sup>8-11</sup> Recent studies have revealed that miRNAs are released by cells and exert these effects by modulating processes in recipient cells.<sup>12-14</sup> Thus, miRNAs have intercellular signal transduction capabilities.

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**Nonstandard Abbreviations and Acronyms**

<b>EC</b>	endothelial cell
<b>FBS</b>	fetal bovine serum
<b>MiR</b>	microRNA
<b>SMC</b>	smooth muscle cell
<b>TGF</b>	transforming growth factor
<b>TNTs</b>	tunneling nanotubes
<b>WT</b>	wild-type

MiR-143 and miR-145 are clustered, intergenic miRNAs located on chromosome 5 in humans and chromosome 18 in mice and rats. Other groups,<sup>15–17</sup> as well as ours,<sup>8,10,18</sup> have described the role of these miRNAs in the regulation of vascular homeostasis. In particular, we have demonstrated a critical role of the miR-143/145 cluster in SMC differentiation and vascular pathogenesis. A role for this miRNA cluster has also been suggested in ECs: in response to altered blood flow, their expression was shown to be induced through the upregulation of the KLF2 transcription factor with a subsequent transfer to SMCs through EC-released exosomes.<sup>14</sup> Kohlstedt et al<sup>19</sup> then showed the involvement of the AMP-activated protein kinase-p53 pathway in miR-143/145 maturation; miR-143 and miR-145 modulate EC response to shear stress, controlling their common target angiotensin-converting enzyme.

In this study, we demonstrate that SMCs communicate with ECs via miR-143 and miR-145. In particular, cell-to-cell EC/SMC contact induces the activation of miR-143/145 transcription in SMCs, and promotes the transfer of these miRNAs to ECs. Once within ECs, the miRNAs blunt the angiogenic potential of ECs. These findings indicate that the miR-143/145 cluster plays a fundamental role for EC/SMC interaction, modulating EC function and then vascular homeostasis.

**Methods****Primary Mouse SMC Isolation and Culture**

Aortas from euthanized mice were isolated, washed, cleaned of adventitia in phosphate-buffered saline, and then digested with 1 mg/mL of collagenase (Worthington) and 1 mg/mL of dispase II (Worthington) in 1× Hanks' balanced salt solution at 37°C for 1 hour. The reaction was stopped with DMEM supplemented with 10% fetal bovine serum (FBS), and cells centrifuged for 10 minutes at 1200 rpm. Pellets were resuspended in DMEM (Lonza) supplemented with 10% FBS (Lonza), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 100 U/mL penicillin-streptomycin, and plated in 1% gelatin precoated plates. Primary cells from passages 3 to 6 were used for the experiments. Wild-type (WT) and knockout cells were isolated from 8-week-old WT and miR-143/145 knockout mice.<sup>8</sup>

**Scanning Ion Conductance Microscopy**

Scanning ion conductance microscopy setup and its working in hopping mode has been previously described.<sup>20,21</sup> Briefly, a piezo-controller (ICnano Scanner Controller, Ionscope Ltd) controlled the 3-axis (*x*, *y*, and *z*) piezo translation stage (Physik Instruments, Germany) with 100- $\mu$ m closed-loop travel range in *x* and *y*, and 50  $\mu$ m in the *z* direction. The piezo-stage was driven by a high-voltage amplifier (Physik Instruments, Germany) connected to an ICnano scanner controller. The pipette electrode headstage was connected to a Multiclamp 700B (Molecular Devices). The scan head was placed onto an electric micromanipulator (Scientifica, United Kingdom) based on a motorized platform (Scientifica). Preparations were imaged with a Nikon TE-i inverted microscope (Nikon Corporation,

Japan). Nanopipettes ( $\approx 80$  M $\Omega$  tip-resistance) were pulled from borosilicate glass with O.D. 1.0 mm and I.D. 0.50 mm (Intracell, United Kingdom) using a laser puller P-2000 (Sutter Inc). Nanopipettes were filled with the same medium used for the cultures, that is, M200 medium (Life Technologies). Surface topographical images of the 2 cells (100 $\times$ 100  $\mu$ m, 512 $\times$ 512 pixels) were acquired by scanning ion conductance microscopy at 25°C in the same medium supplemented with 10  $\mu$ mol/L HEPES (Lonza) to keep CO<sub>2</sub> concentration constant during the loop images acquisition. Acquisition and analysis were performed with a customized software.

For cell identification, SMCs were labeled with Vybrant DiO cell-labeling solution (Life Technologies), following manufacturer's instruction, and then seeded with ECs.

**Bioinformatics**

Potential miR-143 and miR-145 targets were identified using the following algorithms: miRanda (<http://microrna.sanger.ac.uk/>); TargetScan (<http://www.targetscan.org/>); and PicTar (<http://pic-tar.bio.nyu.edu/>), as previously described.<sup>22</sup>

**Mouse Aorta Ex Vivo Isolation and Culture**

Aortas from euthanized mice were isolated, washed, and cleaned of the adventitia in phosphate-buffered saline, then cut in 2 and cultured in DMEM, as described above, with 10% FBS for 24 hours. They were then washed with phosphate-buffered saline and serum-starved (DMEM with 0.1% FBS) for 24 hours. After starvation, aortas were treated or not with TGF $\beta$ 1 for 24 hours (10 ng/mL), and digested with 1 mg/mL of collagenase and 1 mg/mL of dispase II in 1× Hanks' balanced salt solution at 37°C for 1 hour. The reaction was stopped with DMEM 10% FBS, and cells centrifuged 10 minutes at 1200 rpm. Pellets were resuspended in phosphate-buffered saline/0.2% FBS/0.5 mmol/L EDTA, and incubated with antiCD31 (Pecam1), clone MEC13.3 (Biolegend). Then, antirat magnetic beads (Life Technologies) were used to separate the endothelial layer from the SMC layer. After magnetic separation, EC and SMC fractions were analyzed by fluorescence-activated cell sorter, immuno-histochemistry, and quantitative reverse transcription polymerase chain reaction.

**Statistical Analysis**

Luciferase, RNA, protein, and proliferation values were compared using 2-tailed ANOVA test. A value of  $P \leq 0.05$  was considered to be statistically significant.

**Results****miR-143 and miR-145 Transfer From SMCs to ECs**

We performed coculture experiments with ECs and SMCs under different conditions. First, the 2 cell types were cocultured using an insert system, in which a polyethylene terephthalate membrane separated the 2 cell types in a way that made direct contact impossible (Online Figure IA). In this setup, miR-143/145 was not detected in ECs after 24 and 48 hours of coculture, indicating that under basal conditions, no transfer from SMCs to ECs took place (Online Figure IB and data not shown). Then, a coculture experiment was performed, in which the 2 cell types were separately seeded on the 2 sides of a polyethylene terephthalate membrane with a porosity of 1.0  $\mu$ m, which allows SMC protrusions to be in direct contact with ECs.<sup>23,24</sup> Using this system, we were able to detect miR-143/145 in ECs after 24 hours of coculture (Figure 1A). To determine whether the contact was inducing direct transfer or de novo transcription of miR-143/145 in ECs, we used a hybrid system, in which primary human ECs were cultured together with primary mouse SMCs. Levels of primary and precursor human miR-143/145 in ECs were unchanged, indicating that contact triggered only the transfer of mature

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miR-143/145 (Figure 1B and data not shown). In contrast, mature miRNAs were almost undetectable in ECs cocultured with miR-143/145-knockout SMCs (Figure 1C).

### TGF $\beta$ Triggers the Transfer of miR-143/145 From SMCs to ECs

In an effort to understand the molecular mechanism underlying the transfer of miR-143/145, we hypothesized that direct contact between SMCs and ECs induced the production of cytokines directly involved in this process. Thus, we measured the level of TNF $\alpha$ , TGF $\beta$ , IL-10, IL-6, and VEGF in conditioned medium from ECs cocultured or not with SMCs. We found that direct contact induced  $\approx 20\%$  to 30% increase in some of these cytokines (Figure 2A; Online Figure IIA). Because it is known that TGF $\beta$  signaling is critical for vessel stabilization, and its main source of production are ECs activated by contact with SMCs,<sup>6,25</sup> we hypothesized that TGF $\beta$  was a critical factor regulating the transfer of miR-143/145 to ECs. As previously shown,<sup>26</sup> we confirmed in our model that TGF $\beta$  is able to augment the differentiation status of SMCs by increasing SMC differentiation markers and miR-143/145 levels (Figure 2B and 2C). When EC/SMC cocultures were treated with a specific and widely used TGF $\beta$  pathway inhibitor (SB431542),<sup>27–30</sup> we observed a significant reduction in miR-143/145 transfer to ECs (Figure 2D). This result was further confirmed using SMCs transduced with an shRNA against TGF $\beta$ R2 (Figure 2E). The inhibitor and shRNA activities were evaluated by quantitative polymerase chain reaction, measuring the expression of the SMC-TGF $\beta$  readout genes *Serpine1* (*PAI-1*) and *Smad7* (Online Figure IIB and IIC).<sup>31,32</sup>

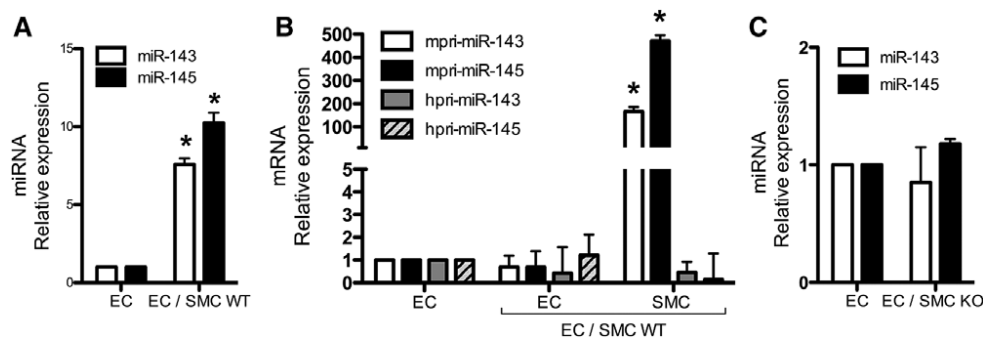
### Tunneling Nanotubes, Not Exosomes, Mediate the Transfer of miR-143/145 From SMCs to ECs

We then sought to determine the mechanism underlying the transfer of miR-143/145 from SMCs to ECs. To this end, we first cultured ECs in conditioned medium from direct cocultures. We found that conditioned medium did not induce changes in the levels of mature miR-143/145 in ECs (Figure 3A). We then assessed whether miRNA transfer was mediated by the release of exosomes. In fact, when SMCs were cultured with ECs, they secreted exosomes containing high levels of

miR-143/145: this effect was blunted when SMCs were treated with the exosome inhibitor GW4869 (Online Figure IIIA), and increased when treated with platelet-derived growth factor or FGF (Online Figure IIIB). These results raised the possibility that ECs were able to uptake SMC-derived exosomes. To address this possibility, we used a lentiviral system to generate SMCs expressing the exosome marker CD63 fused to GFP. However, ECs treated with GFP exosomes purified from the conditioned medium of ECs cocultured with these SMCs did not present any GFP signal at confocal microscopy (data not shown); moreover, ECs challenged with exosomes from SMCs treated with platelet-derived growth factor, FGF, or TGF $\beta$  did not present any statistically significant modulation of miR-143/145 (Figure 3B). In addition, the exosome inhibitors GW4869, NH<sub>4</sub>Cl, and chloroquine did not reduce miRNA transfer (Online Figure IIIC). Together, these results demonstrate that there is no paracrine transfer of the 2 miRNAs after cell-to-cell contact.

We then evaluated whether miRNA transfer was dependent on specialized membrane proteins. In particular, as the role of gap junctions in mediating miRNA transfer has been suggested,<sup>33,34</sup> we tested whether they played a role also in this model. Treating cells with oleic acid or heptanol—2 well-known gap junction uncoupler agents—was not accompanied by miRNA transfer inhibition (Online Figure IIID). In addition, transduction of SMCs with shRNAs targeting connexin 43 (*Cx43*) was not accompanied by a decrease of miRNA transfer (Online Figure IIIE). These results indicated that gap junctions are not implicated in miRNA transfer.

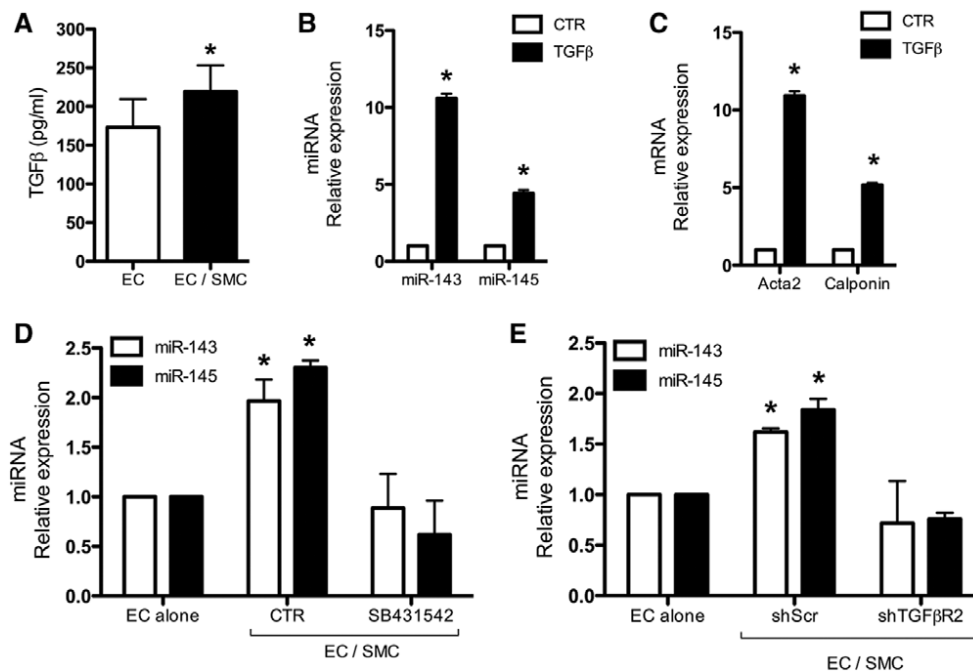
We subsequently hypothesized that tunneling nanotubes (TNTs) were involved in EC/SMC miRNA transfer. TNTs are small plasma-membrane protrusions recently found to be important for cell communication.<sup>35–37</sup> To test this hypothesis, we used a system, in which ECs and SMCs were cocultured and then separated using anti-Pecam1 magnetic beads. This approach was used because the inhibition of TNTs with Latrunculin A (LatA), which modifies the cytoskeleton, reduces the ability of cells to grow on polyethylene terephthalate membranes. The purity of ECs (which reached 99%) was determined by staining the separated cells for  $\alpha$  smooth muscle actin and performing fluorescence-activated



**Figure 1. Transfer of miR-143 and miR-145 from smooth muscle cells (SMCs) to endothelial cells (ECs).** **A**, Mature miR-143 and miR-145 and **(B)** human and mouse pri-miR-143 and pri-miR-145 were measured in ECs (HUVECs) after culture with wild-type (WT) SMCs. **C**, Mature miR-143 and miR-145 were measured in ECs after 24 hours of coculture with miR-143/145 knockout (KO) SMCs. For quantitative polymerase chain reaction, U6 snRNA was used as internal control. All measurements were calculated as percent of control (EC) and error bars calculated as propagated SEM of triplicate measurements from each experiment. \* $P < 0.05$  (each experiment was replicated at least 3 $\times$ ).

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**Figure 2. Transforming growth factor (TGF)  $\beta$  triggers the transfer of miR-143 and miR-145 from smooth muscle cells (SMCs) to endothelial cells (ECs).** **A**, Medium from EC/SMC (HUVEC/murine SMCs) coculture was collected after 24 hours and the level of hTGF $\beta$ 1 measured by ELISA. **B**, Mature miR-143 and miR-145 and **(C)** smooth muscle differentiation genes (*Acta2* and *Calponin*) were analyzed in SMCs treated with 10 ng/mL of TGF $\beta$  for 24 hours. **D**, MiR-143 and miR-145 were measured in ECs (H5V) after coculture with SMCs without or with the TGF $\beta$  inhibitor SB431542, at 24 hours, **(E)** and in ECs (HUVEC) with SMCs transduced with a lentivirus expressing an shRNA scrambled sequence (shScr) or shRNA against TGF $\beta$  receptor 2 (shTGF $\beta$ R2). Measurements in **A** and error bars calculated as propagated SEM of triplicate measurements from each experiment. Data for **B–E** were plotted and analyzed as described in Figure 1. \* $P < 0.05$  (each experiment was replicated at least 3x). CTR indicates control.

cell sorter analysis (data not shown), as previously reported by other groups.<sup>38</sup> We found that inhibiting coculture cell contact with LatA (Online Figure IVA) reduced the transfer of miR-143/145 from SMCs to ECs (Figure 3C).

TNTs are heterogeneous structures that express F-actin and other proteins.<sup>39</sup> When we analyzed the origin of the TNTs with cell type-specific staining, we found that they grew out of ECs and SMCs, and formed homocellular and heterocellular contacts (Figure 3D). We detected the presence of tubulin, F-actin, and the small GTPase Cdc42 in TNTs, but never the expression of Cx43 (Online Figure IVB).

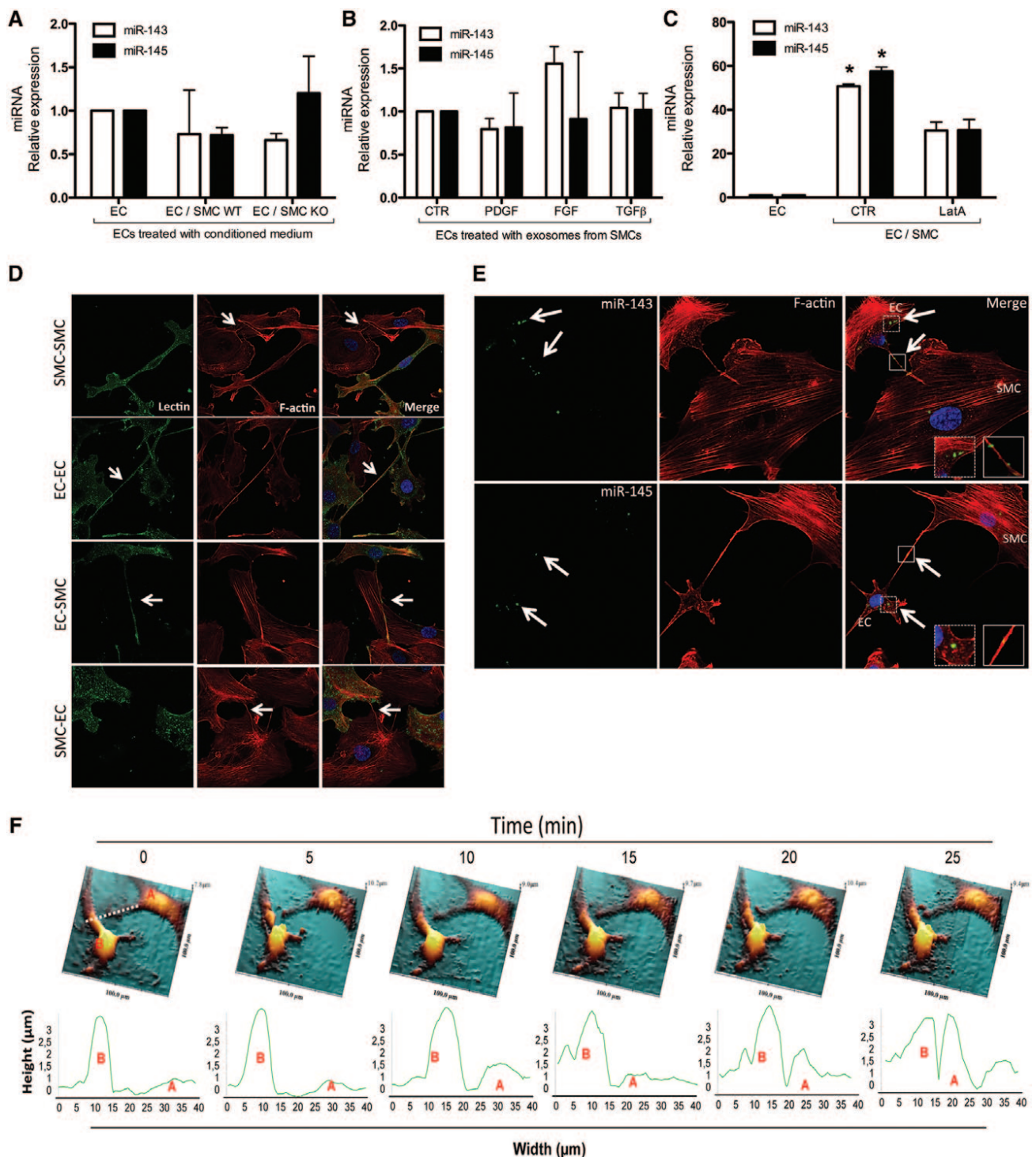
We then studied TNT formation using scanning ion conductance microscopy. After seeding, the cells created TNT contacts in <30 minutes (Figure 3F, top). Detailed analysis of the TNT formation profile indicated that the total cell volume was not changed and that there was a continuous membrane profile at the cell-to-cell junction point, indicative of direct cytoplasmic communication between the interacting cells (Figure 3F, bottom). This was further supported at the ultrastructural level by scanning electron microscopy (Online Figure V).

Finally, to confirm that TNTs probably vehicle miR-143/145 from SMCs to ECs, we transduced primary SMCs with fluorescently labeled miRNA mimics and then cocultured them with ECs: fluorescent signals were indeed detected in TNTs and ECs (Figure 3E and Online Movies I and II). Together, these results strongly support the notion that, after the activation of SMCs by ECs, miR-143, and miR-145 are transferred from SMCs to ECs possibly via TNTs.

### Antiangiogenic Effects of miR-143/145 on ECs

Angiogenesis is a complex process; in which a tight coordination of cues (cytokines, growth factors, etc.) controls EC migration and proliferation.<sup>40,41</sup> When EC-lined tubes are formed, the structures are stabilized by supporting cells, such as SMCs and pericytes.<sup>42</sup> In this state, ECs do not possess strong angiogenic capabilities, and cell growth potential is, therefore, limited.<sup>43</sup>

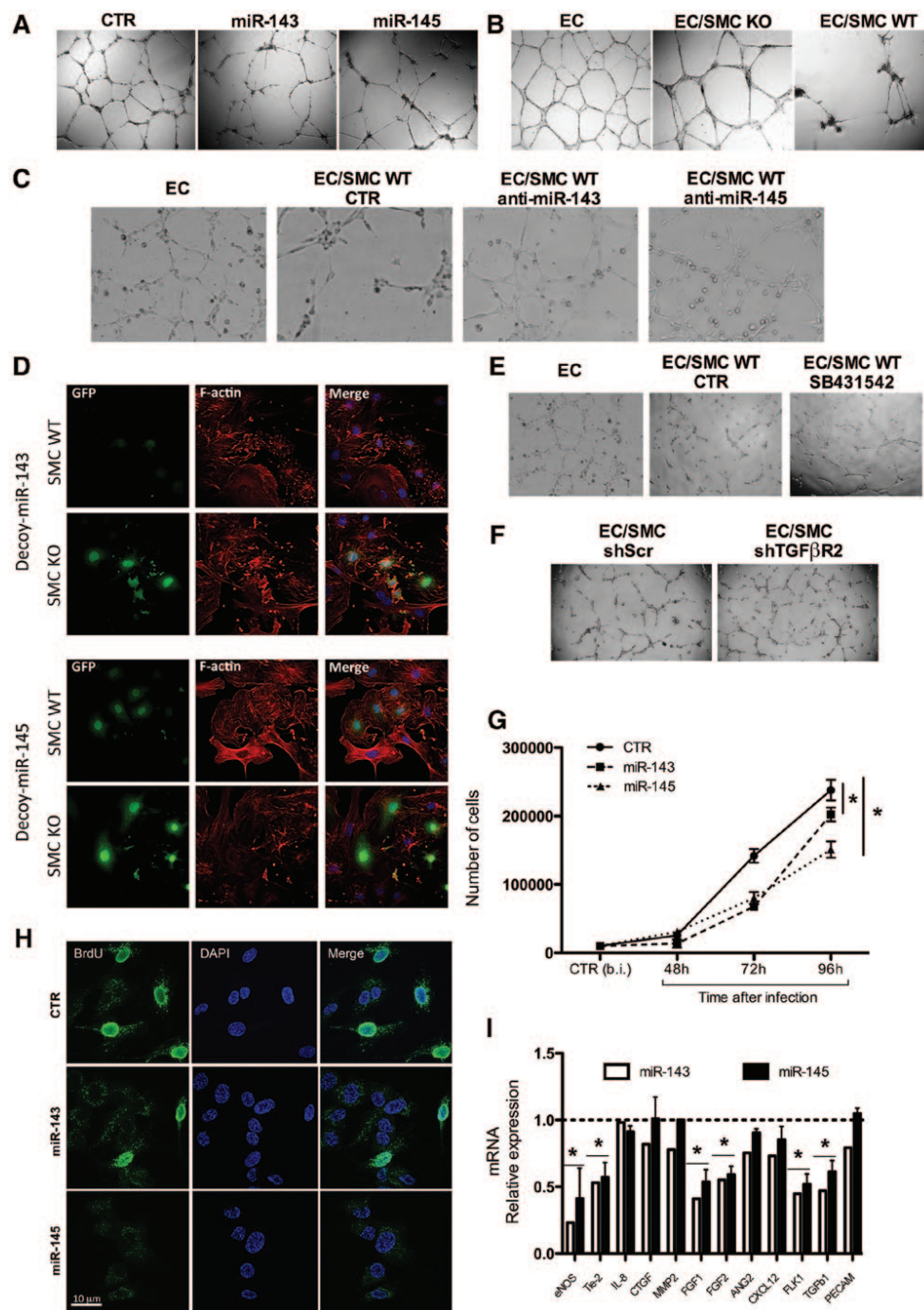
Because we observed that the TGF $\beta$  pathway, which is important for vessel stabilization,<sup>6,25</sup> significantly regulated miR-143/145 transfer to ECs, we asked whether miR-143/145 might have an antiangiogenic role on ECs. To test this hypothesis, we performed a vessel formation assay by plating ECs overexpressing either miR-143 or miR-145 on matrigel and then measuring their ability to form vessel-like structures. The levels of overexpressed miRNA were similar to those observed after direct coculture and separation with anti-Pecam1 beads (Online Figure VIA). As expected, miR-143- and miR-145-expressing ECs lost their angiogenic potential compared with nonexpressing controls (Figure 4A). To demonstrate the functional capacity of the transferred miR-143/145 in cocultured ECs, we repeated the experiments by coculturing ECs with either WT SMCs or miR-143/145-knockout SMCs on matrigel. Although there was no difference in vessel formation after 6 hours of coculture (data not shown), ECs cocultured with WT SMCs lost their ability to form vessels after 16 hours (Figure 4B), confirming the antiangiogenic role of miR-143/145. In contrast, coculture with miR-143/145-knockout SMCs did not inhibit the formation of capillary-like



**Figure 3. Mechanism of miR-143 and miR-145 transfer from smooth muscle cells (SMCs) and endothelial cells (ECs).** Levels of miR-143 and miR-145 were analyzed: **(A)** in ECs (HUVEC) incubated 24 hours with conditioned medium from cocultures of ECs and wild-type (WT) or **miR-143/5-KO** SMCs; **(B)** in ECs (HUVEC) challenged with exosomes purified from SMCs treated with platelet-derived growth factor (PDGF), FGF, or transforming growth factor (TGF)  $\beta$ ; and **(C)** in ECs (HUVECs) first cocultured with WT SMCs, with or without Latrunculin A (LatA) for 24 hours, and then purified using anti-Pecam1 magnetic beads. **D**, Confocal images of Lectin- and F-actin-stained ECs and WT SMCs for identification of tunneling nanotubes (TNT); **(E)** Alexa-488-miR-143 and Alexa-488-miR-145 in TNTs between SMCs and ECs: white arrows indicate fluorescent miRNA mimics within TNTs and ECs. **F**, Time-course of TNT formation (range, 40  $\mu$ m). **Top**, Scanning ion conductance microscopy color-coded topographical images taken in loop mode every 5 minutes. **Bottom**, height profiles along the dashed green line, showing a nanotube forming from an SMC **(A)** and fusing with an EC **(B)**. Data for **A–C** were plotted and analyzed as described in Figure 1. \* $P < 0.05$  (each experiment was replicated at least 3 $\times$ ). CTR indicates control; and KO, knockout.

structures by ECs. Coherently, ECs transduced with anti-miR-143 and anti-miR-145 LNA oligonucleotides and then cocultured with WT SMCs had an increased capacity to form

vessel-like structures on matrigel (Figure 4C). To further corroborate these findings, we generated 2 decoy systems; in which synthetic untranslated regions harboring 2 tandem



**Figure 4. Effects of miR-143 and miR-145 on endothelial cells (ECs).** Ninety-six or 48 well plates were precoated with GelTrex and EC tube formation assay performed with: (A) control (CTR) ECs (HUVEC) or ECs overexpressing miR-143 or miR-145; (B) ECs (HUVECs) alone or in coculture with wild-type (WT) or miR-143/145-KO smooth muscle cells (SMCs); and (C) ECs (HUVECs) transduced with anti-miR-143 and anti-miR-145 LNA oligonucleotides and then cocultured with WT or miR-143/145-KO SMCs (as control). D, ECs transduced with a decoy system: Decoy-miR-143 or decoy-miR-145 ECs (H5V) were cocultured with WT or knockout (KO) SMCs and stained for F-actin after 72 hours. Tube formation assay on ECs (HUVECs) isolated from coculture (E) with WT SMCs without or with the transforming growth factor (TGF)  $\beta$  inhibitor SB431542 or (F) with WT SMCs transduced with a lentivirus expressing an shRNA sequence scrambled or against TGF $\beta$ R2. G, Growth curve of CTR ECs and those overexpressing miR-143 or miR-145 at different time points. H, BrdU incorporation in CTR ECs and ECs overexpressing miR-143 or miR-145, and relative quantification (magnification:  $\times 20$ ). I, quantitative reverse transcription polymerase chain reaction analysis of endothelial markers in CTR ECs and ECs overexpressing miR-143 or miR-145. The threshold (obtained from CTR ECs) is indicated by the dotted line. Error bars in C and D were calculated as propagated SEM of triplicate measurements from each experiment. Data in E were plotted and analyzed as described in Figure 1. \* $P < 0.05$  (each experiment was replicated at least 3 $\times$ ). b.i. indicates before infection.

miR-143 or miR-145 complementary sequences were placed 3' to the GFP gene. ECs transduced with these decoys were cocultured with either WT or knockout SMCs: after 72 hours,

a clear reduction of the GFP signal was observed in EC/WT SMC cocultures compared with the EC/knockout SMC ones (Figure 4D; Online Figure VIB). Moreover, ECs cultured

with WT SMCs treated with the TGF $\beta$  inhibitor SB431542 or SMCs transduced with an shRNA sequence against TGF $\beta$ R2 show an increased ability to form capillary-like structures on matrigel (Figure 4E and 4F).

In addition, ECs overexpressing miR-143 or miR-145 had a reduced proliferation index, as assessed by growth curve and BrdU incorporation analyses (Figure 4G and 4H; Online Figure VIC). To further validate the antiangiogenic role of these 2 miRNAs in ECs, we measured the mRNA levels of different EC angiogenic markers. We found a significant downregulation of many of them (Figure 4I). Taken together, this set of experiments indicates that the miR-143/145 cluster regulates EC growth properties on direct contact with SMCs during vessel formation.

### Identification of miR-143 and miR-145 Targets in ECs

Having established that the transfer of miR-143 and miR-145 from SMCs exerts a powerful biological effect on ECs by inhibiting their growth, we sought to identify the targets of these miRNAs in ECs. Through a bioinformatics approach, we found that hexokinase II (HKII) and integrin  $\beta$  8 (ITG $\beta$ 8) were potential binding partners for miR-143 and miR-145, respectively. These genes have highly conserved seed sequences (Online Figure VID). Transduction of primary ECs with lentiviral miR143- and miR-145-encoding vectors decreased the abundance of the target proteins and their mRNA levels (Figure 5A and 5B). Luciferase assay with 3' untranslated region-binding sites confirmed that HKII and ITG $\beta$ 8 mRNAs were directly targeted by miR143 and miR-145; deregulation was not observed when the seed sequences were mutated (Figure 5C). In addition, binding affinity of the 2 miRNAs to their targets was confirmed by Ago2-immunoprecipitation experiments (Figure 5D).

We then tested whether HKII and ITG $\beta$ 8 were implicated in angiogenic regulation in ECs. In loss-of-function experiments, we determined that primary ECs knocked-down for HKII and ITG $\beta$ 8 had a reduced capacity to form vessel-like structures when cultured on matrigel (Figure 5E), and a reduced proliferation capacity (Figure 5F and 5G; Online Figure VIE). Thus, HKII and ITG $\beta$ 8 are 2 important miR-143/145 targets that regulate the angiogenic capability of ECs.

### Transfer of miR-143/145 From SMCs to ECs Ex Vivo and In Vivo

To determine whether the in vitro results were applicable also in an ex vivo setting, we performed experiments using aortas excised from WT mice. Vessels were cultured in normal medium for 24 hours and then, after 16 hours of serum starvation, were treated with or without TGF $\beta$  for 24 hours. Aortas were then dissected and digested for EC isolation with anti-Pecam1 magnetic beads. The purity of EC separation was >99% (Online Figure VIIA and VIIB), and a comparison of Pecam1 and different smooth muscle differentiation marker (*Acta2*, *Calponin*, and *Myh6*) mRNA levels in the positive and negative fractions confirmed the quality of the separation process (Online Figure VIIIA and VIIB). MiR-143 and miR-145 were found significantly increased in isolated ECs from TGF $\beta$ -treated vessels, confirming the in vitro results

(Figure 6A). No differences in pri-miR-143/145 levels were detected (Figure 6B), suggesting that TGF $\beta$  triggers only the transfer of mature miR-143/145 from SMCs to ECs.

To further corroborate these findings in vivo, we used our mouse model, in which the miR-143/145 cluster can be specifically deleted in the SMC layer on induction with tamoxifen (Online Figure VIIC and VIID). Trans-aortic constriction-induced pressure overload increased miR-143/145 expression in the endothelium of coronary arteries of WT mice, an effect absent in the SMC-specific miR-143/145 knockout mice (Figure 6C). HKII and ITG $\beta$ 8, the 2 identified miR-143/145 targets, were increased in the EC layer of knockout vessels when compared with WT (Figure 6D and 6E). We also found that in vivo migration was significantly increased in ECs from knockout mice when we performed a matrigel plug assay (Figure 6F; Online Figure VIIE).

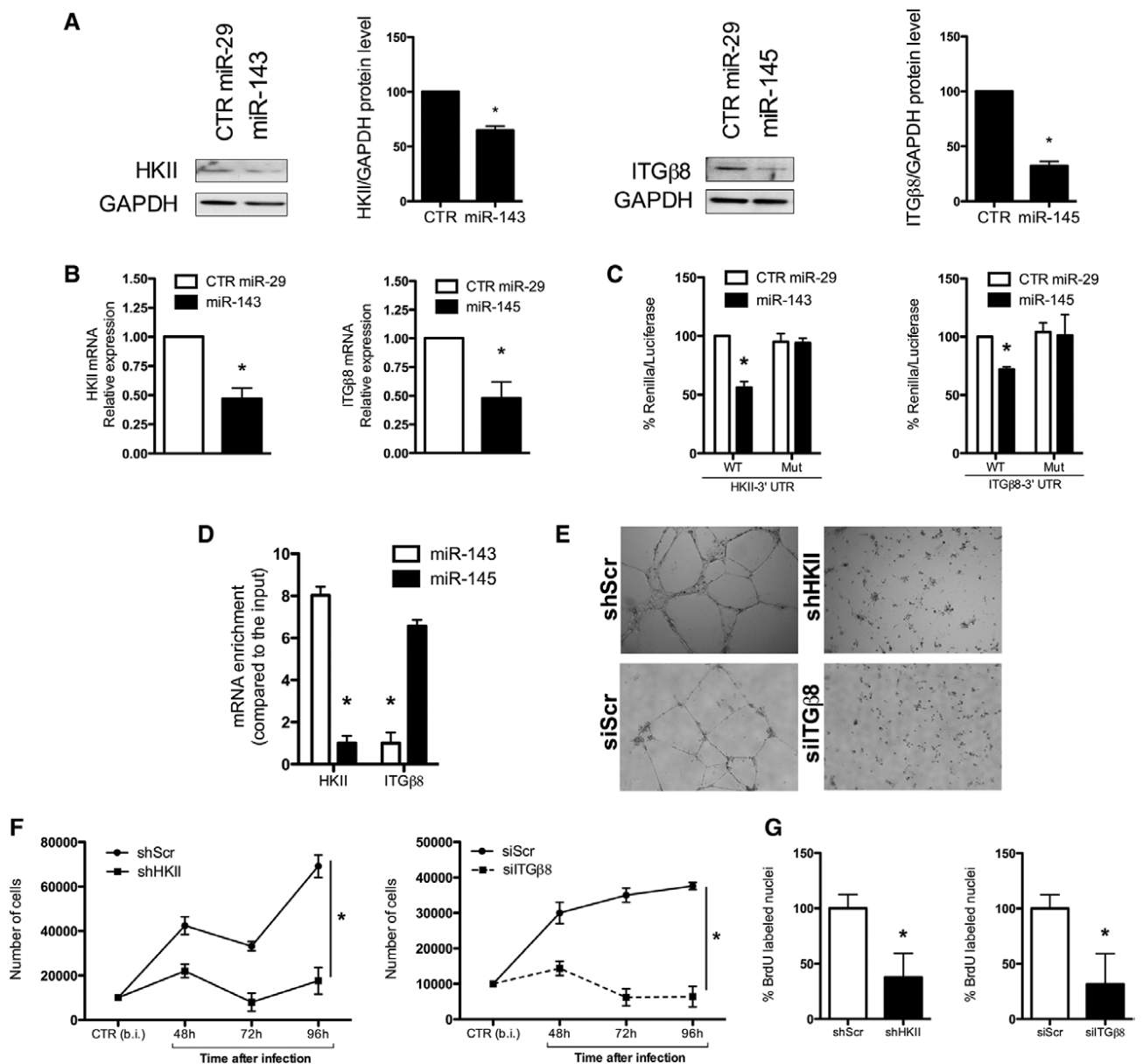
As a whole, these ex vivo and in vivo results further support the notion that the TGF $\beta$  signaling cascade triggers the transfer of the miR-143/145 cluster to ECs from SMCs, and that this transfer reduces EC migration.

## Discussion

Vasculogenesis is the process by which mesoderm-derived endothelial precursor cells (angioblasts) form de novo vessels; it is responsible for the formation of the first, primitive blood vessels in the embryo. In contrast, physiological and pathological blood vessel growth later on in life is predominantly, if not exclusively, achieved through angiogenesis-dependent mainly on mature ECs.<sup>44</sup> In adulthood, the formation and function of these vessels support the life of organs; the functional and structural organization of vessels are maintained by the direct interaction between ECs and mural cells. However, ECs are highly heterogeneous in terms of functional properties and gene expression profiles. This heterogeneity depends on the differentiation and proliferation status.<sup>45</sup>

The miR-143/145 cluster has been observed to play a fundamental role governing SMC differentiation during physiological and pathological events.<sup>8,10,15-18</sup> Beside its ability to regulate SMC homeostasis, it is still not well understood how the miR-143/145 cluster regulates EC functions. In this report, we demonstrate that the modulation of the angiogenic potential of ECs is also because of the direct transfer of miR-143/145 from SMCs. It is known that EC/SMC contact triggers SMC activation through the TGF $\beta$  pathway,<sup>25</sup> but the effect of stimulated SMCs on ECs is still unclear. Here, we confirm that the level of miR-143/145 is increased in differentiated SMCs,<sup>26</sup> and that this triggers the transfer of these small RNAs to neighboring ECs. Furthermore, overexpression of either miR-143 or miR-145 in ECs reduced the ability of ECs to form capillary-like structures and decreased their proliferation index. This effect was further confirmed in other experiments, such as coculture of ECs with WT SMCs or miR-143/145 knockout SMCs: as a matter of fact, WT, but not knockout, SMCs were able to reduce the angiogenic capacity of ECs.

This phenomenon might have implications also for tumor biology. Many studies have shown the role of the miR-143/145 cluster in cancer development.<sup>46-48</sup> Under pathological conditions, such as wound healing and malignancies, angiogenesis

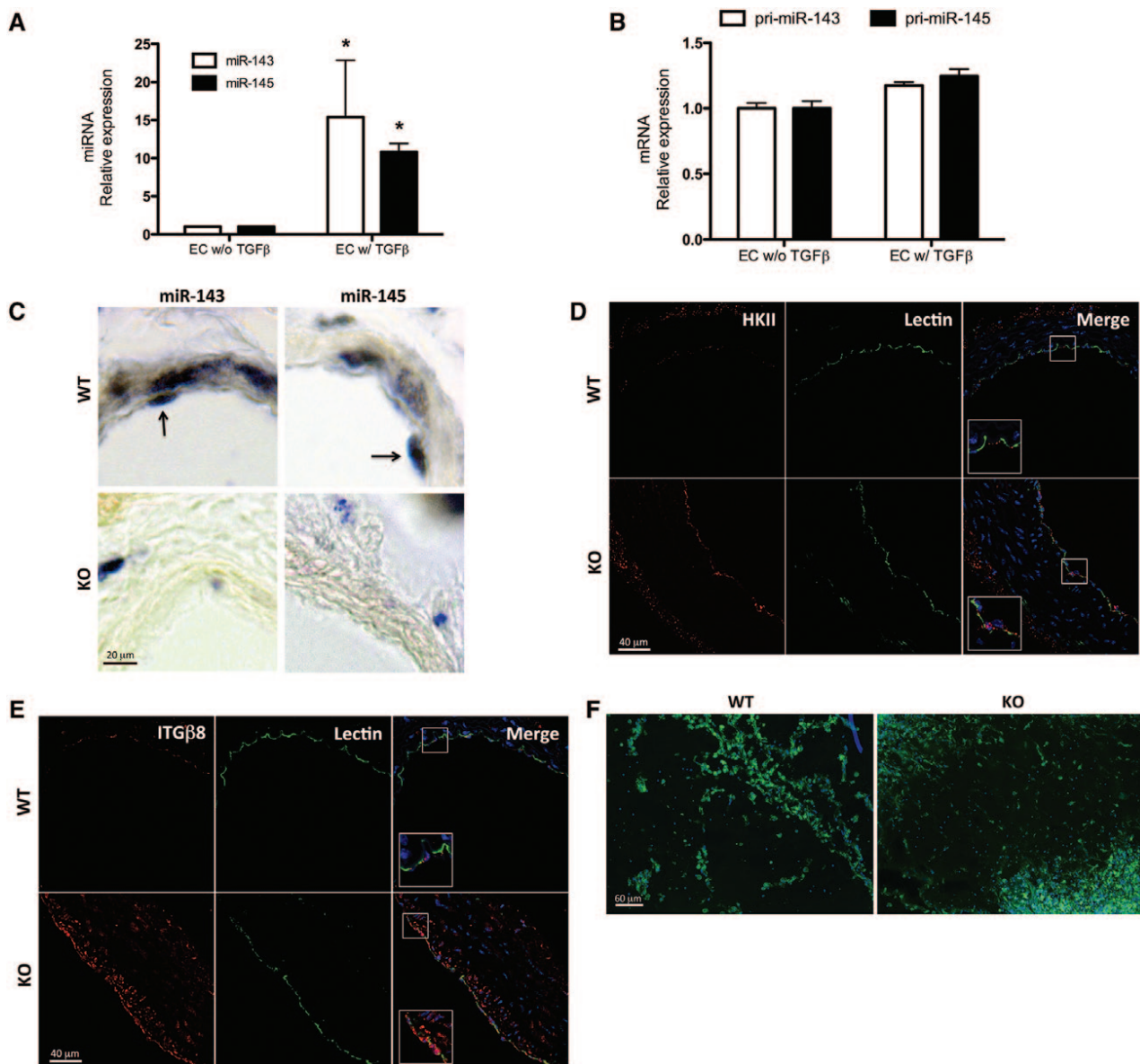


**Figure 5. Targets of miR-143 and miR-145 in endothelial cells (ECs).** **A**, Representative immunoblots for the target genes hexokinase II (HKII) and integrin  $\beta$  8 (ITG $\beta$ 8) in control (CTR) ECs (H5V) and ECs overexpressing miR-143, miR-145, or miR-29, and relative quantification normalized to GAPDH. **B**, RNA analysis of *HKII* and *ITG $\beta$ 8* RNAs with quantitative reverse transcription polymerase chain reaction (qRT-PCR). **C**, Luciferase reporter assay (all measurements were calculated as percent of control (wild-type [WT] 3' untranslated region [UTR]) and error bars calculated as propagated SEM of triplicate measurements from each experiment) on ECs (H5V), performed by cotransfection of (20 nmol/L) miR-143, miR-145, or CTR (miR-29) with a renilla reporter gene linked to (10 ng) wild-type (WT) or mutated (Mut) 3' UTRs of *HKII* and *ITG $\beta$ 8*. **D**, miR-143 or miR-145 mimics were transfected in ECs (H5V) and 48 hours later cells were lysed for Ago2-immunoprecipitation assay. RNA levels for *HKII* and *ITG $\beta$ 8* were analyzed by qRT-PCR. ECs (HUVEC) were transfected with shSCR, shHKII, siSCR, or siITG $\beta$ 8 and assayed for: **(E)** tube formation; **(F)** growth curve; and **(G)** BrdU incorporation. Data in **B** and **D** were plotted and analyzed, respectively as described in Figure 1. \* $P < 0.05$  (each experiment was replicated at least 3x).

is in fact reactivated.<sup>49</sup> As tumor cells grow, the formation of new immature blood vessels increases together with the request for supplies. In the normal vasculature, pericytes and SMCs are important for the stabilization of ECs and mediate EC survival together with the maturation of the vessels; mural cells modulate these events by direct cell contact and paracrine signaling.<sup>50</sup> In contrast, pericytes and SMCs are usually absent in the tumor vasculature or have loose associations with ECs, leaving most of the tumor's microvessels immature.<sup>42,51,52</sup> On the basis of our data, it can be speculated that to maintain the

proangiogenic phenotype of ECs, the level of miR-143/145 in the surrounding SMCs has to be low. Interestingly, tumors frequently have decreased levels of these miRNAs compared with normal samples of the same tissue.<sup>46,48</sup> Thus, it is possible that downregulation of the miR-143/145 cluster is associated with increased angiogenesis in tumors because the stabilizing effect on ECs is decreased or lost.

Another interesting aspect of our results relates to the role of the TNT plasma-membrane structures in mediating the transfer of miRNAs in cell-to-cell communication. TNTs are



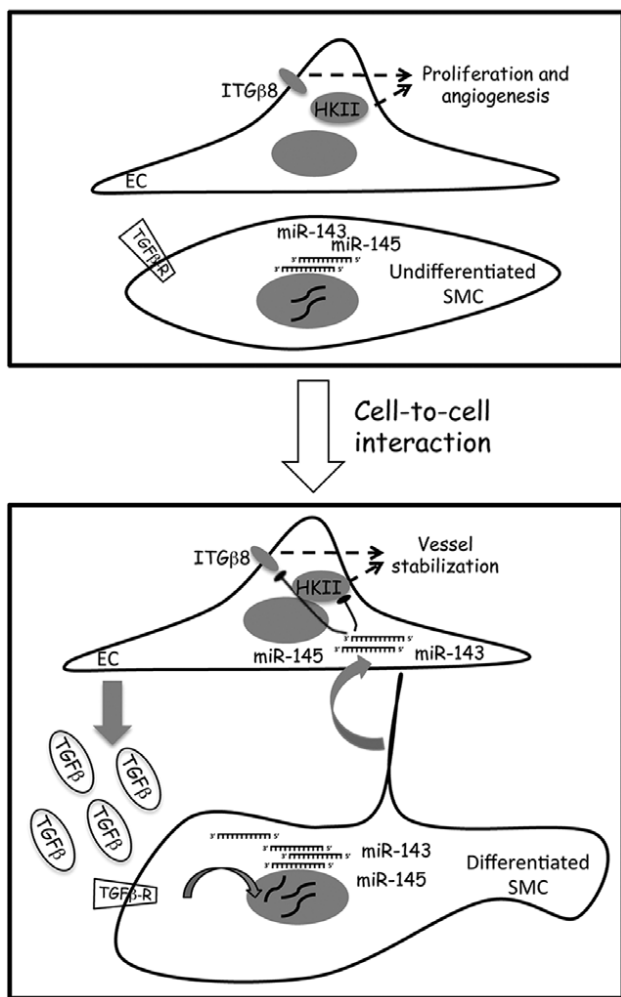
**Figure 6. miR-143/145 transfer ex vivo and in vivo.** Quantitative polymerase chain reaction for (A) mature miR-143 and miR-145, and (B) pri-miR-143 and pri-miR-145 in the endothelial cell (EC) fraction of wild-type (WT) vessels exposed to transforming growth factor (TGF)  $\beta$ . C, In situ hybridization for miR-143 and miR-145 in coronary arteries of WT (cre+) and miR-143/145 knockout (KO; floxed/cre+) mice after transaortic constriction; black arrows indicate positive ECs (magnification:  $\times 100$ ). D and E, Staining for hexokinase II (HKII) and integrin  $\beta$  8 (ITG $\beta$ 8) in WT and KO aortas after tamoxifen injection (magnification:  $\times 20$ ). F, Representative images of Lectin (green) and 4',6-diamidino-2-phenylindole (blue) staining of matrigel plugs implanted into mice of the indicated genotypes (magnification:  $\times 4$ ). Data in A and B were plotted and analyzed as described in Figure 1. \* $P < 0.05$  (each experiment was replicated at least 3 $\times$ ).

line-like membrane tubes able to connect 2 cells. The diameter of TNTs ranges from 50 to 200 nm and some are as long as the diameter of some cells. These structures have been observed in many different cell types, such as tumor cells, natural killer (NK) cells, cardiomyocytes, and ECs.<sup>35,36,53</sup> That TNTs might mediate the transfer of the miR-143/145 cluster from SMCs to ECs is here demonstrated by various lines of evidence (1) exosomes or other secreted structures are not implicated in the transfer; (2) inhibition of cell contact through gap junction inhibition does not reduce miR-143/145 passage to ECs; (3) with the use of a TNT inhibitor (LatA), we were able to significantly reduce miRNA transfer from SMCs to ECs, demonstrating that these structures are

probably important routes for the passage of small RNAs between cells; and (4) observation of fluorescent miR-143 and miR-145 in TNTs.

We also demonstrate that inhibition of 2 direct EC targets of miR-143/145, *HKII* and *ITG $\beta$ 8*, is sufficient for reducing the angiogenic phenotype of ECs. These 2 targets were previously demonstrated to be regulated by the miR-143/145 cluster in cancer cells<sup>54</sup> and human corneal epithelial cells,<sup>55</sup> respectively. Our data, although confirming the role of miR-143/145 in regulating these 2 targets also in ECs, demonstrate their importance in modulating EC biology.

In vessel maturation and stabilization, the specialization of vessel-wall cells is an important, yet not totally



**Figure 7. Model of the role of miR-143 and miR-145 in endothelial cells (ECs) on direct transfer from smooth muscle cells (SMCs).** After contact with SMCs, ECs secrete transforming growth factor (TGF)  $\beta$ . This, in turn, stimulates SMC differentiation and miR-143/145 upregulation, triggering also the transfer of the miRNAs to ECs via tunneling nanotubes. In ECs, miR-143 and miR-145 negatively modulate hexokinase II (HKII) and integrin  $\beta$  8 (ITG $\beta$ 8) expression, leading to reduced angiogenic capabilities and a stabilization of the endothelium.

understood, event.<sup>56</sup> Vessel maturation involves the phenotypic specialization of ECs and mural cells, stabilized by the production of extracellular matrix. This process is guided mainly by homotypic and heterotypic cell contacts.<sup>56</sup> Different pathways are involved in this biological process: sphingosine-1-phosphate-1 endothelial differentiation sphingolipid G-protein-coupled receptor-1,<sup>57</sup> AngI-Tie2,<sup>58</sup> platelet-derived growth factor B—PDGF receptor- $\beta$ ,<sup>59</sup> and TGF $\beta$ .<sup>60</sup> Apart from these indications, there is scant information on the involvement of miRNAs in EC/SMC communication during vessel stabilization. Here, we prove also in vivo that miR-143/145 cluster modulates EC function through direct transfer from SMCs.

Our work demonstrates the effect of the miR-143/145 cluster on EC stabilization, a process that depends on the direct transfer of these miRNAs from differentiated SMCs induced by TGF $\beta$  (Figure 7). This finding could have relevant implications for pathological and therapeutic angiogenesis.

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## Disclosures

None.

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## Novelty and Significance

### What Is Known?

- MiR-143/145 cluster regulates smooth muscle cell (SMC) physiology and vascular homeostasis.
- Transforming growth factor  $\beta$  modulates SMC differentiation toward a contractile phenotype.
- MiR-143/145 can be transcribed by endothelial cells (ECs) in response to an altered laminar shear stress.

### What New Information Does This Article Contribute?

- Transforming growth factor  $\beta$  signaling triggers miR-143/145 transfer from SMCs to ECs.
- MicroRNA transfer may take place through thin membrane structures called tunneling nanotubes although definitive proof remains to be shown.

- On endothelial uptake miR-143/145 modulate angiogenic capabilities, favoring vessel stabilization.

In this study, we demonstrate that miR-143 and miR-145 function as signaling molecules between SMCs and ECs. MiRNA passage is triggered by direct cell-to-cell contact and is modulated by the transforming growth factor  $\beta$  pathway. Our results suggest that both miRNAs may travel through tunneling nanotubes. Once in ECs, both miRNAs modulate gene expression leading to reduced proliferative and angiogenic properties. Once modulated by miRNAs, the EC phenotype, through a direct interaction with SMCs, leads to better vessel stabilization. These results provide new insights on the role of these miRNAs in neovascularization.

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