SUPPLEMENTAL MATERIAL

TGFβ triggers miR-143/145 transfer from smooth muscle cells to endothelial cells, thereby modulating vessel stabilization.

Short Title: Climent, miR143/145 modulates EC biology

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Detailed Methods

Animals

8 weeks old C57/b6 mice were used for the experiments. Special attention was paid to animal welfare and to minimize the number of animals used and their suffering.

For treatments, cells were washed with PBS, serum-starved for 24 h, and then treated with 10 ng/ml TGFβ (Peprotech), PDGF (Sigma), FGF, TNFα, or EGF (Peprotech). The TGFβ inhibitor SB431542 (Sigma) was used at a concentration of 10 µM. Latrunculin A (LatA) (Sigma) was added at the final concentration of 0.5 µM. For conditioned media assay, medium was collected after 24 h of culture and added to the selected cells. Exosome inhibitors were used at the following concentrations: GW4869 (2 µM), NH₄Cl (200 µM), and chloroquine (0.4 µM).

Smooth muscle cells expressing CD63-GFP and treatments
For the study of exosomes, lentiviral particles encoding CD63-GFP (Lenti-CD63-GFP) were purchased from SBI and used to transduce SMCs. CD63-GFP-SMCs were used to study the secretion of CD63-vesicles using different stimuli (PDGF, FGF, TGFβ). After treatments,
conditioned medium containing the CD63-GFP vesicles were either used to evaluate the fluorescence in the medium or added to ECs in order to detect the uptake of these vesicles. Exosomes were purified with ExoQUICK solution (SBI) following manufacturer’s instructions.

**Ago2 immunoprecipitation**

ECs transfected with 50nM of the control, miR-143, or miR-145 mimics (Dharmacon) were washed with 1xPBS and UV-crosslinked for 30 sec at 400 mJ. Afterwards, cells were harvested and washed twice with 1xPBS. Cell pellets were resuspended and incubated 5 min on ice in 700 µl of lysis buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5% NP-40, 5 mM DTT) supplemented with protease inhibitor cocktail (Roche) and 100 U/ml RNase inhibitor (Life Technologies). Thereafter, lysates were centrifuged for 10 min at 10,000 g and cytoplasmic lysates recovered. Magnetic beads were washed and pre-blocked in PBS with 0.5% BSA and then anti-Ago2 (Ascension) was added and incubated 1 h at 4ºC with rotation. 10% of the cytoplasmic lysate was kept as input and the rest was incubated with the Ago2-beads O/N at 4ºC in rotation. Tubes were then placed in the magnet, and beads washed three times. Finally, 1 ml of Purezol (BioRad) was added to the samples for RNA extraction.

**Luciferase reporter assay**

HKII-3'-UTR and ITGβ8-3'-UTR plasmids were co-transfected with the miR-143 or miR-145 mimics (Dharmacon) and relative controls, using lipofectamine 2000 (Life Technologies). 3'UTR site-specific mutagenesis at the predicted sites for each target was performed using the QuikChange Site-Directed Mutagenesis Kit, as described by the manufacturer (Stratagene). Cells were harvested 48 h after transfection and analyzed with the dual-luciferase reporter assay kit (Promega), according to manufacturer’s protocol.

**RNA extraction and qRT-PCR**

Total RNA was isolated using Purezol reagent (BioRad) according to manufacturer’s protocol. For gene expression, RNA was reverse transcribed with High capacity cDNA archive kit (Applied Biosystems) and qRT-PCR was performed using Select SYBR Green (Applied Biosystems). PCR primers are listed in Table I. For miRNA expression, RNA was reverse transcribed using the miRCURY LNA™ Universal RT (Exiqon). SYBR Green was used to perform the quantitative real-time PCR for mature miR-143, miR-145, miR-15, and miR-16 (Exiqon), and U6, as internal control. For exosome quantification we used a spike-in miRNA (Exiqon) as internal control as previously described.4

**Western blot**

Cells were lysed in RIPA buffer and sonicated for 5 cycles (30 sec on, 30 sec off) using a Biorupture™ Next Gen (Diagenode). After quantification using DC™ Protein Assay (BioRad), the same quantity of protein was separated in 4–12% NuPAGE® Bis-Tris precast gel (Life Technologies) and transferred to a nitrocellulose membrane (Biorad). Afterwards, anti-HKII (Santa Cruz Biotechnologies), anti-ITGβ8 (Santa Cruz Biotechnologies), and anti-GAPDH (Santa Cruz Biotechnologies) were incubated overnight at 4ºC, and secondary peroxidase-conjugated antibodies incubated for 1 h at room temperature. Immobilon Western Chemiluminiscent HRP (Millipore) was used for protein visualization using a ChemiDoc (BioRad). Bands were quantified using ImageJ software and results are expressed relative to the control.

**Cell transfection**

Primary mouse SMCs: cells were plated in 6-well plate at 1x10^5 cells/well. For oligo miR-143 and -145 mimics conjugated with Alexa-488 (IDT), cells were transfected at the concentration of 100 nM using Lipofectamine LTX (Invitrogen). For Cx43 and TGFβR2 knockdown, pLKO-shCx43 and -TGFβR2 were used (Respectively: Sigma #TRCN0000068477 and
#TRCN0000294529) with the corresponding control pLKO-shScr (Sigma # SHC002). Primary murine SMCs with stable expression of the shCx43 and shTGFβR2 were generated using a virus MOI of 5 together with 5 μg/ml of polybrene (Sigma). HUVECs: cells were plated in 12-well plates at 3x10⁴ cells/well. For anti-miR experiments, cells were transfected with anti-miR-143 and -145 or control scrambled oligonucleotides (Exiqon) at 100 nM using Lipofectamine RNAiMAX (Life Technologies). For siRNA experiments, cells were transfected with specific siRNA against ITGβ8 (IDT # HSC.RNAI.N000002214.12.1) at 80 nM using Lipofectamine RNAiMAX (Life Technologies). On the other hand, knockdown of HKII was obtained infecting the cells with lentiviral particles expressing the specific shRNAs (Sigma # TRCN0000037671).

**Scanning electron microscopy**

Cells were fixed with 2.5% glutaraldehyde in 0.1 M tampon cacodylate (pH 7.2) for 30 min at room temperature (RT). Samples were washed with phosphate buffered saline (PBS) at RT and dehydrated through a series of different concentration of alcohols. The samples were covered with few nanometers of gold with a coating device. The cell structure and topography were observed with a scanning electron microscope (SEM) Supra™ 40 (Zeiss, DE) equipped with InLens detector and operated with an acceleration of 3 kV.

**Generation of lentiviral vectors harboring miRNAs and decoys**

For gain- and loss-of-function of miR-143 and miR-145 in ECs, production of Lenti-Empty (CTR), Lenti-miR-143, Lenti-miR-145, Lenti-Decoy-miR-143, and Lenti-Decoy-miR-145 was performed as previously described.

**Co-culture with the decoy system**

ECs (H5V) were transduced with Lenti-Decoy-miR-143 and -145. 5x10³ cells were plated in a 12-well plate together with 20x10³ WT or miR-143/145-KO SMCs on coverslips. After 72 h of co-culture, cells were fixed and stained for F-actin with Phalloidin-Alexa-594 (Life Technologies).

**In situ hybridization**

In situ hybridization was performed with microRNA ISH Optimization Kit (Exiqon), following the manufacturer’s indications.

**Proliferation assay**

ECs were plated at 1x10⁴ cells/well in 6-wells plates. After 24 h, cells were transduced or transfected with the respective lentiviral particles or siRNA. Cell proliferation was determined at 48, 72, and 96 hours after transduction/transfection.

**BrdU incorporation assay**

EC cultures were treated with BrdU (Life Technologies) for 24 h, following the manufacturer’s protocol. Then, cells were fixed with 4% paraformaldehyde and permeabilized with PBS/1% Triton-X100, treated with HCl, and stained with anti-BrdU antibody (Santa Cruz Biotechnology).

**Immunofluorescence**

To determine the formation of the TNTs and characterize the proteins involved in their formation during ECs/SMCs co-cultures, the following reagents were used: anti-lectin-FITC (BS-1 from Sigma), anti-αSMA (Sigma), anti-Cx43 (Sigma), anti-tubulin (Cell Signaling), anti-Cdc42 (Cell Signaling), Phalloidin-Alexa-594 or -647 (Life Technologies).
**Confocal analysis**

Confocal images were obtained with a microscope (IX81-FV1000; Olympus) using FluoView software (Olympus) at room temperature.

**Live imaging**

The experiments were performed plating HUVECs and primary SMCs on glass plates at the ratio 1:1. SMCs were transfected with oligo miR-143-Alexa-488 or miR-145-Alexa-488 24 h before the imaging, while HUVECs were labeled with CellTracker™ Red CMTPX Dye (Life Technologies) following manufacturer’s protocol. Once in co-culture, pictures were taken every 3 minutes using the CellR system (Olympus) with a 40X objective in a humidified 5% CO₂ atmosphere at 37º C. Video reconstructions were realized using Fiji software.

**Flow Cytometry analysis**

Evaluation of the EC purity after Pecam1-beads separation was performed with intra-cellular staining of both EC and SMC fractions using an αSMA-FITC antibody (Sigma). Measurements were obtained with a FACS Canto II instrument (BD Bioscience).

**Tube formation assay**

Plates pre-coated with Geltrex® Matrix (Life Technologies) were used. EC tube formation assay was performed seeding the ECs at 1x10⁴ cells/well into 96-well plates or in 48-well plate for the co-culture seeding 6x10⁴ HUVEC and 3x10⁴ SMCs per well. The degree of tube formation was evaluated using an inverted microscope after 24 h incubation at 37ºC and 5% CO₂.

**Enzyme-linked immunosorbent assay (ELISA)**

Conditioned media were collected for measuring the presence of different secreted proteins: VEGF, TNFa, IL-10, IL-6, and TGFβ1 (R&D Systems). Samples were diluted so that the optical density fell within the optimal portion of a log standard curve. TGFβ1 was assayed on acid-activated samples, following the manufacturer’s protocol.
Online Figure I. EC/SMC co-culture with no physical contact. (A) Cartoon illustrating how co-culture experiments were performed. (B) Mature miR-143 and miR-145 were measured on ECs (HUVEC) after 24 h of no-contact co-culture with WT and miR-143/5 KO SMCs. Data for B are plotted and analyzed as described in Figure 1.
**Online Figure II.** Cytokines secreted by ECs in co-culture with SMCs. (A) Medium from the EC/SMC co-culture was collected after 24 h and the levels of hVEGF, hTNFα, hIL-6, and hIL-10 measured by ELISA. (B) TGFβ readout genes (PAI-1 and Smad7) were measured in SMCs CTR or treated with the TGFβ inhibitor SB431542 after co-culture. (C) TGFβR2, PAI-1 and Smad7 RNA levels in SMCs transduced with a shRNA against TGFβR2 after co-culture. *, P<0.05. Data are plotted and analyzed as described in Figure 2.
Online Figure III. miR-143 and miR-145 secretion and transfer inhibition by specific agents. (A) MiR-143, -145, -15, and -16 were measured in exosomes from EC/SMC co-culture treated or not with the exosome inhibitor GW4869. (B) MiR-143 and -145 were analyzed in exosomes of SMCs treated with PDGF or FGF and in the relative conditioned medium. (C) MiR-143 and -145 were measured in ECs (HUVEC) after co-culture with WT SMCs treated or not with different exosomes inhibitors (Chloroq: chloroquine; NH4: NH4Cl; GW: GW4869). (D) MiR-143 and -145 were measured in EC/SMC co-cultures after treatment with gap junction uncoupler agents (heptanol and oleic acid). (E) MiR-143 and -145 were measured in EC/SMC co-cultures transduced with a lentiviral vectors expressing shRNA against Cx43. *, P<0.05. Data are plotted and analyzed as described in Figure 1.
Online Figure IV. Characterization of tunneling nanotubes. (A) Lectin-FITC and Phalloidin-Alexa-594 staining on EC/SMC co-culture following LatA treatment. White arrows indicate TNTs (Magnification: 40x); (B) F-actin together with Cdc42 or Cx43 or Tubulin (Magnification: 40x).
Online Figure V

Online Figure V. TNT characterization by scanning electron microscopy (SEM). Representative image of a TNT formed between an SMC and an EC.
Online Figure VI

**A**

![Bar chart showing mRNA relative expression](image)

**B**

![Bar chart showing fluorescent intensity/number of cells](image)

**C**

![Bar chart showing % BrdU labeled nuclei](image)

**D**

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*Indicates significance at p < 0.05.
Online Figure VI. miR-143 and -145 activity on ECs. (A) MiR-143 and -145 levels in over-expressing ECs. (B) Quantification of GFP signal for decoy experiments shown in Figure 4D. (C) Quantification of BrdU staining shown in Figure 4G. (D) Conservation of HKII and ITGβ8 seed sequences in different species. (E) BrdU staining in ECs (HUVEC) transduced with SCR (scrambled) sequence or siRNAs vs HKII and ITGβ8 (magnification: 20x).
Online Figure VII. Evaluation of EC purity following separation with Pecam1 beads. (A) EC and SMC layers from aorta were separated using Pecam1 beads. After separation, intracellular staining was performed on EC and SMC fractions using an αSMA-FITC conjugated antibody; (B) Immunofluorescence of EC and SMC fractions following separation with anti-Pecam1 beads. White arrows indicate auto-fluorescence of magnetic beads.
Online Figure VIII. Inducible miR-143/145 SMC-specific knockout mouse. (A and B) Levels of Pecam1 and Acta2 mRNAs in EC and SMC fractions after cell isolation were analyzed with qRT-PCR. (C) Strategy design for mouse generation. (D) MiR-143 and -145 levels on aortas of WT (cre+) and KO (floxed/cre+) mice upon tamoxifen induction. (E) Quantification of the plug matrigel assay reported in Figure 6F.
**Supplemental Table**

**Supplementary table I. Primers used in this study**

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*m*=mouse; *h*=human
Supplemental References


Video Legends

**Online Video I.** Co-culture of primary SMCs transfected with miR-143-Alexa 488 and ECs (HUVEC) pre-labeled with CellTracker™ Red CMTPX Dye (Life Technologies).

**Online Video II.** Co-culture of primary SMCs transfected with miR-145-Alexa 488 and ECs (HUVEC) pre-labeled with CellTracker™ Red CMTPX Dye (Life Technologies).