Chapter

2

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MicroRNA-155 functions as a negative regulator of RhoA signaling in TGF-β-induced endothelial to mesenchymal transition

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Abstract

Endothelial to mesenchymal transition (EndoMT) has been proposed to be involved in the loss of microvascular capillaries in the pathophysiology of fibrosis and organ failure. In EndoMT, endothelial cells (EC) undergo a mesenchymal transition associated with the loss of cell-cell contacts and the acquisition of a synthetic, contractile phenotype. Here, we sought to identify microRNAs (miRNAs) that could play a central role in regulating EndoMT. In a TGF-β dependent *in vitro* model for EndoMT, we identified miRNAs that were differentially expressed in normoxic and hypoxic conditions. These studies identified miR-155 to be significantly upregulated in EndoMT, an effect that was enhanced under hypoxia, which further augments EndoMT. Silencing of miR-155 directly increased RhoA expression and activity in endothelial cells and affected phosphorylation of downstream LIMK. In contrast, overexpression of miR-155 counteracted RhoA function. Using a selective Rho kinase inhibitor, we could partly suppress EndoMT, strengthening the notion that RhoA plays a central role in EndoMT. Forced overexpression of miR-155 completely suppressed EndoMT, as evidenced by the maintenance of EC characteristics and blocking the acquisition of a mesenchymal phenotype, as compared to control cells. Our data demonstrate that miRNA-155 functions as a negative regulator of RhoA signaling in TGF-β-induced endothelial to mesenchymal transition.

Introduction

Endothelial to mesenchymal transition (EndoMT) is an essential process in embryonic development of the heart valves and involves dedifferentiation of endothelial cells towards a mesenchymal, synthetic phenotype[1,2]. At the same time, evidence is accumulating that EndoMT can also contribute to the loss of the microvasculature in the adult, limiting the long-term survival of transplanted organs[3] and leading to end organ failure in progressive cardiac and renal disease[4]. EndoMT involves the dissolution of endothelial tight junctions, reorganization of the cytoskeleton, acquisition of invasive and migratory properties and the production and deposition of extracellular matrix[5]. These phenotypic features are shared with the pro-fibrotic myofibroblast that drive fibrotic disorders. While myofibroblasts were thought to be derived from resident fibroblasts, epithelial cells or bone marrow-derived cells, evidence is accumulating that EndoMT provides another source of tissue myofibroblasts[4]. A common denominator in chronic diseases associated with the development of cardiac or kidney fibrosis is the reduced bioavailability of nitric oxide (NO)[6]. In mice, long term inhibition of NO synthesis with inhibitors of endothelial nitric oxide synthase (NOS3) leads to induction of TGF-β expression and fibrosis in the kidney and using cell-lineage tracing it was demonstrated that a fraction of the myofibroblasts was derived from endothelial cells (EC)[7]. Likewise, in an experimental model for cardiac fibrosis, it was demonstrated that a major fraction of the myofibroblasts was derived from EC in a TGF-β dependent fashion[8]. These studies strongly suggest that EndoMT may be an early event in the pathogenesis of fibrosis and are consistent with observations that fibrosis starts in the perivascular area[9]. Although the mesenchymal transition of EC to myofibroblastic cells may add to the excessive formation of scar tissue, the associated loss of microvascular EC and capillary density, that eventually leads to a state of chronic ischemia, may act as the real profibrotic switch. The tissue response to hypoxia further upregulates TGF-β expression, promotes the influx of inflammatory cells and thereby exacerbates the fibrotic process.

TGF-β is a well-established regulator of EndoMT[5]. TGF-β can bind to the heteromeric receptor complex of TGFβRI and TGFβRII, which results in the phosphorylation of SMAD2 and SMAD3. The phosphorylated SMAD2/3 complex subsequently associates with SMAD4 and translocates to the nucleus. This complex binds to SMAD-binding DNA elements and also to several regulatory molecules, thereby activating various transcriptional networks[10,11].

MicroRNAs (miRNAs) are small ~22 nucleotide regulatory RNAs that function as negative regulators of gene expression[12]. Since these molecules play a central role in regulating cellular differentiation, miRNAs may also be involved in EndoMT. Support for this notion can be derived from the fact that miRNAs have been described to regulate TGF-β signaling in epithelial to mesenchymal transition (EMT). Among the best characterized miRNAs, miR-200 has been found to regulate EMT, by targeting the transcriptional E-cadherin suppressors ZEB1 and SIP1, while overexpression of the miR-200 family prevents TGF-β-induced EMT[13]. Furthermore, miR-21 has been shown to be upregulated in EMT[14], and to be essential in cardiac fibrosis[15]. More recently, initial evidence detailing a role for miRNAs in EndoMT has come to light, with miR-21 silencing phosphatase and tensin homolog in EC resulting in activation of the Akt-pathway[16].

In the present study, we investigated TGF-β-induced EndoMT in mouse embryonic endothelial cells (MEEC), and determined the miRNA expression profile during this dedifferentiation process. Among the differentially expressed miRNAs, miR-155 was one of the most significantly upregulated miRNAs. While miR-155 is important in regulating diverse processes such as immune regulation and cancer[17], a role for this miR in EndoMT is currently unknown. Ras homolog gene family, member A (RhoA), which is a well-established target of miR-155[18], has been described as a regulator of EMT. In this study, we demonstrated that modulation of miR-155 levels in EC can affect EndoMT via targeting the RhoA pathway.

Materials and Methods

Cell Culture and induction of EndoMT

Generation of mouse embryonic endothelial cells (MEEC) was previously described[19]. MEEC were maintained on 1% (w/v) gelatin coating in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Breda, the Netherlands) supplemented with 10% fetal calf serum and 2 mM L-glutamine (Invitrogen). Cells were treated with 2 ng/mL TGF-β3 (Peprotech, London, UK) for 48 hours, unless indicated otherwise. For the culture of MEEC under hypoxic conditions, hypoxic chambers were flushed with nitrogen, resulting in oxygen levels of <1%. To effectively block RhoA activity, we used the Rho kinase inhibitor Y-27632 (Sigma-Aldrich, Zwijndrecht, the Netherlands) at a final concentration of 3.5 μg/mL. For the scratch assay, MEEC were scraped in a straight line with a 200 μL pipette tip. A reference point was marked with a tip marker. Cell migration after 24 hours of incubation was determined by microscopy and expressed as migration distance in μm.

MiRNA microarray

MEEC were stimulated with TGF-β along with an unstimulated control (n=3). To determine miRNA profiles we used NCode Multi-Species miRNA microarrays V2 (Invitrogen). For this, isolated RNA was poly-A tailed, followed by ligation of the capture sequences. Subsequently, the tagged miRNAs were purified and hybridized to the chips, after which fluorescently-labeled capture reagents were hybridized and signal intensity measured using an array reader. Data analysis was performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team. All data were normalized to miR-125b, as we have previously found miR-125b to be unresponsive to TGF-β stimulation in MEEC.

RNA isolation and qRT-PCR analysis

Total RNA from kidneys was isolated using Trizol reagent (Invitrogen). Reverse transcription was performed using a 5 minute 65ºC incubation of 250 ng total RNA with dNTPs (Invitrogen) and oligo(dT) (Invitrogen) or using specific Taqman® microRNA probes (Applied Biosystems, Foster City, CA, USA). cDNA was synthesized using a M-MLV First-Strand Synthesis system (Invitrogen). Validation of target genes was performed using SYBR Green Master Mix (Applied Biosystems) and the following primers: Twist (sense): ACGCAGTCGCTGAACGAGGC, Twist (antisense): GTACAGGAAGTCGATGTA-CC, α-SMA (sense): CGTGGCTATTCCTTCGTGAC, α-SMA (antisense): GCGT-

TCGTAGCTCTTCTCC, CD31 (sense): CTCCAACAGAGCCAGCAGTA, CD31 (antisense): TCACTTCTTCCGGGGTTCCTTATCTG, SDF-1 (sense): CGTGAG-GCCAGGGAAGAGT, SDF-1 (antisense): TGATGAGCATGGTGGGTTGA, Col1a1 (sense): TGACTGGAAGAGCGGAGAGT, Col1a1 (antisense): GTTCGG-GCTGATGTACCAGT, fibronectin (sense): ATGTGGACCCCTCCTGATAGT, fibronectin (antisense): GCCCAGTGATTTCAGCAAAGG, GAPDH (sense): ACT-CCCACTCTTCCACCTTC; GAPDH (antisense): CACCACCCTGTTGCTGTAG. Levels of expression were determined by normalizing to GAPDH.

Validation of miRNA levels was performed using Taqman® miR assays (Applied Biosystems) according to manufacturer's instructions (Invitrogen),RNU6B was utilized for normalization (sense U6: CTCGCTTCGGCAGCACA and antisense U6: AACGCTTCACGAATTTGCGT). Data were quantitated using the delta delta Ct method.

Western Blot

Western blot analyses were performed on cellular lysates harvested in lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% SDS, 0,5% deoxycholate, 0,5% Triton X-100) containing phosphatase and protease inhibitors. Following quantitation of protein determination, equal amounts of proteins were assessed using SDS-polyacrylamide gels (SDS-PAGE) under reducing conditions. Proteins were transferred to a PVDF membrane (Amersham, 's Hertogenbosch, the Netherlands). Primary antibodies were utilized to detect α-SMA (R&D, Minneapolis, MN, USA), β-actin (Abcam, Cambridge, UK), CD31 (Santa Cruz, Heidelberg, Germany) and RhoA (Cytoskeleton, Denver, Co, USA). Bound primary antibody was labeled with HRP-conjugated secondary antibody in blocking solution. Bound fragments were detected with chemiluminescent reagents (West Dura supersignal; ThermoFisher Scientific, Waltham, MA, USA) and exposed on Hyperfilm ECL (Amersham). Quantitative analysis of the protein bands was performed using ImageJ software and normalized to β-actin.

Antagonism and overexpression of miR-155

Design of Antagomirs

Cholesterol-conjugated RNA analogs, 'antagomirs', (Dharmacon RNA technologies, Lafayette, CO, USA) were synthesized as previously described[20]. For antagomir-155 the following sequence was used: 5'-$a_s c_s$cccuaucacaauuagcau$_s u_s a_s a_s$-Chol-3'. As a control a 'scramblemir' was used,

this RNA analog is constructed from a randomized nucleotide sequence which does not bind to any known microRNAs: 5'-$a_s u_s$gacuaucgcuauucgc$_s a_s u_s g_s$-Chol-3'. The lower case letters represent 2'-*O*Me-modified nucleotides; subscript 's' represents phosphorothiate linkage; 'Chol' represents a cholesterol-group linked through a hydroxyprolinol linkage. Antagomir or control scramblemir was added to the medium 16 hours before TGF-β stimulation at a concentration of 5 μg/mL.

Overexpression of miR-155

To overexpress miR-155, a synthetic pre-miR-155 precursor (Applied Biosystems) was introduced into the cells in a 24-well format with a final concentration of 100 nM. As a control, we used a negative control precursor molecule. Per well, 4 μL of Lipofectamine 2000 (Invitrogen) was added to facilitate transfection.

Immunocytochemistry

For immunocytochemistry, MEEC were seeded into 4-well chamber slides. At the end of incubation, cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 5 min. The cells were subsequently washed again, and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. Cells were then blocked with 10% normal goat serum in PBS for 30 min at room temperature, and incubated for 1 hr with primary antibodies. The following primary antibodies were used: F-actin was visualized with 2 μg/mL phalloidin-TRITC (Sigma-Aldrich), pLIMK1/pLIMK2 (Cell Signaling Technology, Leiden, the Netherlands), α-SMA (Sigma-Aldrich). Cells were subsequently washed and incubated for 1 hour at room temperature with the appropriate secondary antibodies. The stained cells were covered with Vectashield® mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Immunostaining was visualized by fluorescence microscopy (Leica DM1-6000).

RhoA activity assay

RhoA activity was determined using G-lisa (Cytoskeleton). The assay was performed according to the protocol of the manufacturer.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's t-test. $P<0.05$ was considered statistically significant (* = >0.05, ** = $p<0.01$, *** = $p<0.001$).

Results

TGF-β induces EndoMT in MEEC

We stimulated MEEC with TGF-β to recapitulate EndoMT *in vitro* and assessed whether they displayed mesenchymal characteristics. When MEEC were stimulated with TGF-β for 48 hr they displayed a characteristic morphological transition towards a spindle-like myofibroblast-like phenotype (Figure 1A-B). A hallmark feature of mesenchymal transition is the expression of α-SMA containing stress fibers. Using immune histochemical staining we confirmed this in the TGF-β treated MEEC (Figure 1C-D). Likewise, F-actin containing stress fibers are critical for migration and myofibroblast contraction. When we visualized F-actin by immunostaining with phalloidin we observed marked TGF-β induced stress fiber formation (Figure 1E-F).
The TGF-β induced mesenchymal transition of MEEC was associated with

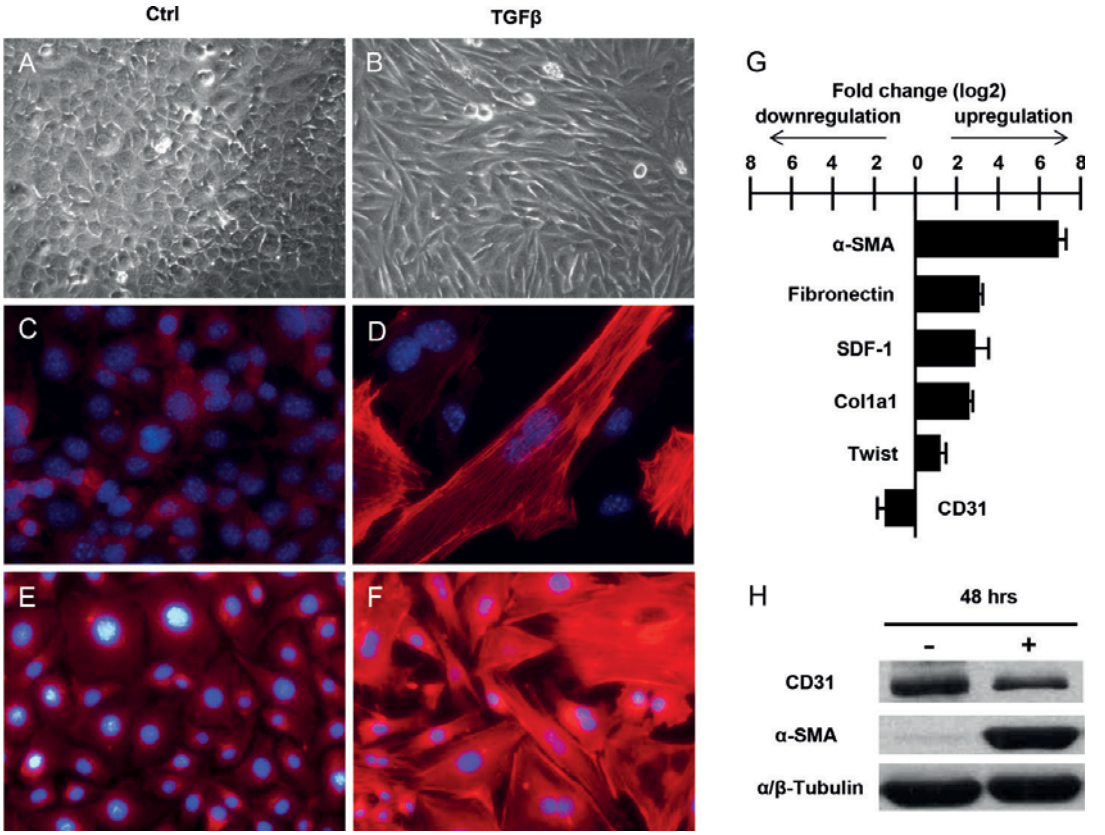

Figure 1. TGF-β induces EndoMT in MEEC. A-B, Representative microscopic images of control MEEC (A) and those stimulated with TGF-β (B) show the morphological transition. C-D, immunofluorescence shows the formation of -SMA containing stress fibers. E-F, immunofluorescent images show the formation of F-actin containing stress fibers. G, qRT-PCR analysis of EC marker CD31 and myofibroblast markers α-SMA, Fn, col1a1, SDF-1 and Twist. Data is normalized on GAPDH mRNA levels. H, Western Blot shows increased expression of α-SMA and decreased expression of CD31.

a concomitant increase in expression of mRNAs encoding myofibroblast markers α-SMA, fibronectin (Fn), collagen1a1 (col1a1) and Twist[21] (Figure 1G). Furthermore, stromal cell derived factor-1 mRNA (SDF-1) was up regulated as previously described in EMT[22]. In contrast, the expression of EC intercellular junction protein CD31 was decreased. Western blot analysis confirmed the observed changes in CD31 and α-SMA mRNA expression at the level of protein expression (Figure 1H).

Hypoxia augments TGF-β induced EndoMT in MEEC

Given that hypoxia is considered to be an HIF-1α dependent accelerator of EndoMT *in vivo*[5], we investigated whether hypoxia also augmented EndoMT in MEEC. MEEC were cultured under normoxic and hypoxic conditions (<1% oxygen) in the presence or absence of TGF-β. Following our expectation, hypoxia induced a cellular disorganization that, when combined with TGF-β stimulation resulted in a more profound morphological change towards a myofibroblastic phenotype (Figure 2A-B). Moreover, the combination of hypoxia and TGF-β stimulation led to marked changes in F-actin immunostaining, as evidenced by a highly disorganized staining pattern together with increased stress fiber formation (Figure 2C-D). Further supporting the exacerbation of the myofibroblast phenotype in hypoxia we demonstrated that hypoxic MEEC treated with TGF-β revealed an earlier increase in α-SMA expression (Figure 2I).

Finally, to validate increased production and deposition of extracellular matrix (ECM) under conditions of hypoxia, we performed Sirius Red staining on unstimulated MEEC and MEEC treated with TGF-β for 48 hr. As shown in figure 2J, TGF-β treatment led to an almost two fold increase in ECM production that was even furthers pronounced in hypoxia (from 4.51 ± 0.20 to 6.42 ± 0.28, $p<0.0005$). Together these data confirmed the mesenchymal transition of MEEC following treatment with TGF-β and validated the use of these cells to study mechanisms in EndoMT.

MiRNA expression in TGF-β induced EndoMT

To identify miRNAs that are differentially expressed during EndoMT, we isolated total RNA from MEEC stimulated with TGF-β for 48 hr or unstimulated control cells and processed the samples to determine their miRNA profiles using miRNA microarrays (n=3 per condition). From the 427 miRNAs assessed, 62 miRNAs displayed differential expression during EndoMT. Of these, 51 miRNAs were significantly downregulated during the dedifferentiation process, while 11 miRNAs were upregulated during the conversion to the pro-fibrotic state. Next, we validated the differential

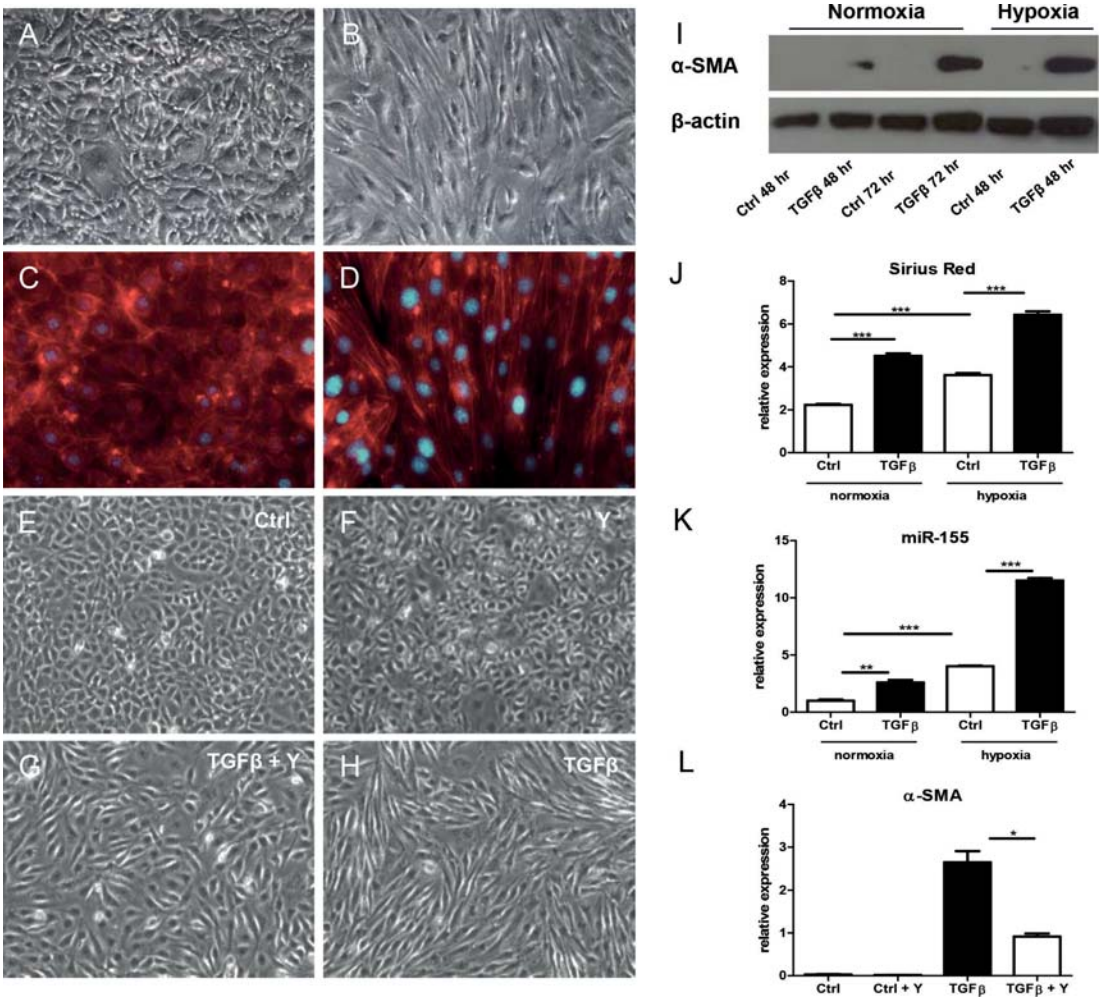

Figure 2. Hypoxia augments, and RhoA is essential for, EndoMT.
A-B, representative microscopic images of cells cultured under hypoxic conditions in the presence or absence of TGF-β. C-D, representative microscopic images of fluorescence of F-actin. E-H, Rho/ROCK inhibitor Y-27632 suppresses EndoMT. Representative microscopic images of unstimulated (E), Y-27632 treated (F), TGF-β and Y-27632 treated (G) and TGF-β treated (H) MEEC. I, Western Blot shows expression of α-SMA in conditions of normoxia and hypoxia. Time points as indicated. J, Sirius Red staining shows that TGF-β stimulation increases extracellular matrix production and is augmented by hypoxia. Expression is relative to cell number. K, qRT-PCR analysis shows that miR-155 expression is increased in EndoMT and augmented by hypoxia. L, qRT-PCR analysis shows reduced α-SMA levels when Y-27632 is added to TGF-β treated cells.

expression of a selection of miRNAs by qPCR and confirmed the differential expression for all tested miRNAs (Table 1).
In our search for miRNAs that regulate EndoMT we selected miR-155 for further study as this miRNA is was one of the few up regulated miRNAs and was previously described to modulate TGF-β-induced EMT through RhoA[18]. Given that hypoxia was shown to exacerbate EndoMT, we measured miR-

155 levels both under normoxic and hypoxic conditions in the presence or absence of TGF-β. We found that hypoxia further increased miR-155 levels (Figure 2K), suggesting that TGF-β and hypoxia have a synergistic effect on miR-155 expression levels and that miR-155 expression quantitatively follows EndoMT.

Table 1. Differential miRNA expression in TGF-β induced EndoMT in MEEC. Data is normalized on miR-125b expression, which is the most highly expressed miRNA in MEEC and stable during TGF-β stimulation. Absolute values indicated in first two columns as compared to miR-125b expression level. Third and fourth column show differential expression as determined by respectively microarray and qPCR. – is downregulation, + is upregulation.

miRNA	TGF-β		Fold Change	
	-	+	array	qRT-PCR
miR-125b	1	1	1	1
miR-424	0.13	0.03	-4.3	-2.5
miR-696	0.51	0.21	-2.4	1
miR-93	0.21	0.09	-2.3	n/a
miR-130a	0.19	0.09	-2.0	n/a
miR-15a	0.13	0.07	-1.9	n/a
miR-15b	0.13	0.07	-1.9	n/a
miR-106b	0.16	0.09	-1.8	n/a
miR-22 1	0.14	0.08	-1.8	n/a
miR-20a	0.13	0.08	-1.6	n/a
miR-16	0.34	0.21	-1.6	-2.0
miR-31	0.26	0.18	-1.4	n/a
miR-29a	0.31	0.22	-1.4	-1.5
miR-214	0.06	0.09	+1.5	+2.8
miR-155	0.06	0.08	+1.3	+1.5
miR-199a	0.08	0.10	+1.3	+2.0
miR-199a*	0.09	0.12	+1.3	+2.0
miR-22	0.17	0.22	+1.3	+1.5

Rho/Rock inhibition partly inhibits EndoMT

Since RhoA is a validated target of miR-155 in EMT, we first investigated whether there is also a role for RhoA in EndoMT in MEEC. Using the Rho kinase inhibitor Y-27632 we were able to partly suppress TGF-β induced EndoMT as indicated by a preservation of endothelial cell morphology (Figure

2E-H). In addition, α-SMA mRNA levels were significantly lower (Figure 2L). This indicates that RhoA is essential for full transition of endothelial cells towards a mesenchymal phenotype.

Silencing of miR-155 affects expression of α-SMA

Previously, we demonstrated that antagomirs can be used to specifically silence miRNA expression in EC both *in vitro* as well as *in vivo*[23]. To study the effect of miR-155 silencing during EndoMT, we employed an antagomir to miR-155 and a control scramblemir that was designed to lack complementary to the mouse transcriptome. When we assessed the effect of silencing of

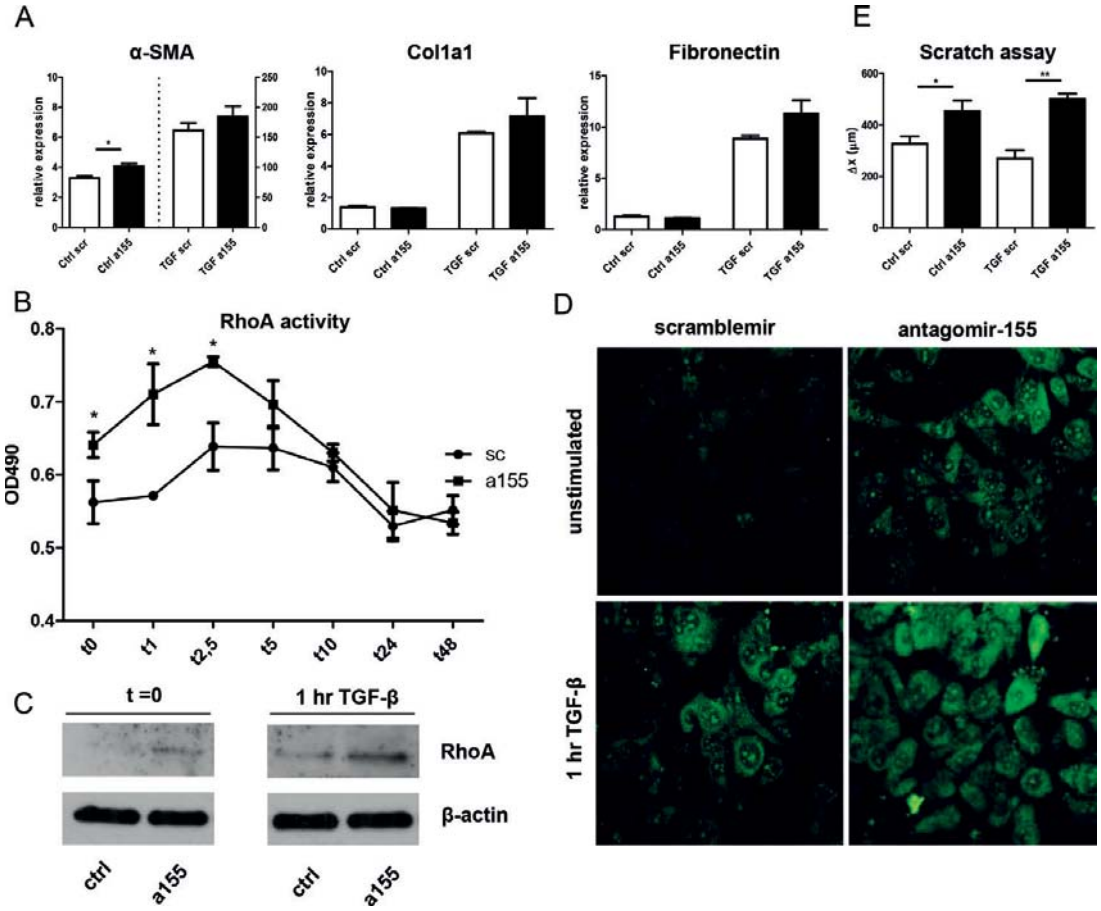

Figure 3. Effect of miR-155 silencing on EndoMT and RhoA signaling.
A, α-SMA, col1a1 and fibronectin mRNA levels, measured by qRT-PCR. TGF-β induced α-SMA levels are plotted on right y-axis. B, RhoA activity at different timepoints after TGF-β stimulation (plotted on x-axis) in antagomir-155 or scramblemir control treated MEEC. C, Western blot for RhoA. Antagomir-155 vs scramblemir control at baseline and 1 hr after TGF-β stimulation. D, representative microscopic images show increased pLIMK expression because of blocking miR-155 in both unstimulated or 1 hr TGF-β stimulated cells. E, increased migratory capacity of MEEC as a result of miR-155 silencing. Migration distance in μm was measured in a scratch assay.

miR-155 on markers of EndoMT 48 hours after treatment we observed only a minor, not significant trend in the expression of α-SMA, collagen 1a1 and fibronectin mRNA (Figure 3A). Interestingly, in the absence of TGF-β, silencing of miR-155 elevated α-SMA expression (from 3.29 ± 0.29 to 4.07 ± 0.3, $p<0.05$) suggesting a repressive role for miR-155 in the basal regulation of cytoskeletal organization.

MiR-155 regulates RhoA expression and activity in MEEC

As the Rho GTPase RhoA is a central regulator of stress fiber formation[24] we sought to determine if, similar to what has been described for EMT, miR-155 can also regulate RhoA in EndoMT. Since the conversion of RhoA to the GTP-bound state is known to be a rapid process following stimulation with TGF-β[25], we assessed the dynamics of RhoA activity following TGF-β stimulation in antagomir-155 and scramblemir treated cells. Figure 3B shows that silencing miR-155 indeed leads to a significant upregulation of the activity of RhoA activity in MEEC both before and at 1 and 2.5 hr after TGF-β stimulation. Western blot analyses on baseline lysates or MEEC that were stimulated for 1 hour with TGF-β demonstrated that silencing of miR-155 increases RhoA protein expression concomitant to the increased activity profile (Figure 3C). The reduction of RhoA activity after 24 and 48 hr most likely involves the TGF-β induced increase in miR-155 levels that may exceed the silencing effect of antagomir-155. To further support the effect of miR-155 silencing on RhoA activity we assessed the phosphorylation of LIM kinase (pLIMK), a downstream effector of RhoA dependent stress fiber formation[24]. MEEC were stimulated with TGF-β in the presence of antagomir-155 or scramblemir and stained for pLIMK at baseline and after 1 hour after stimulation. As shown in figure 3D the expression of pLIMK is increased as a result of miR-155 silencing at both time points again confirming a negative role for miR-155 in RhoA signaling. RhoA has been described to regulate the migration in endothelial cells[26]. We performed a scratch assay to see if cell migration was also affected by the silencing of mir-155. Twenty four hours after making a scratch, we found that the loss of miR-155 activity has a stimulatory effect on Rho-A dependent migration both in unstimulated as well as in TGF-β stimulated cells (Figure 3E). Our data support a rate limiting role for miR-155 in RhoA activity in MEEC.

Overexpression of miR-155 suppresses features of TGF-β induced EndoMT

Following the above we postulated that overexpression of miR-155 could counteract EndoMT in MEEC. To test this hypothesis we treated MEEC for

16 hr with a synthetic pre-miR-155, and we stimulated the cells with TGF-β for 48 hr to induce EndoMT. Increased levels of miR-155 lead to a virtually complete inhibition of TGF-β-induced EndoMT in MEEC, as evidenced by the maintenance of EC morphology (Figure 4A), while Western blot analysis of lysates harvested from MEEC treated with pre-miR-155 revealed that α-SMA expression remained low while CD31 expression remained high, as compared to scramblemiR-treated MEEC (Figure 4B). To confirm that the regulatory role of miR-155 on EndoMT is secondary to inhibition of RhoA expression we also determined RhoA expression at baseline and 1 hr after TGF-β stimulation by Western blot analyses. Indeed, elevation of miR-155 reduces RhoA protein levels compared to the scrambled control pre-miR (Figure 4C). Finally, overexpression of miR-155 was associated with decreased RhoA activity 2.5 hr after TGF-β stimulation (Figure 4D).

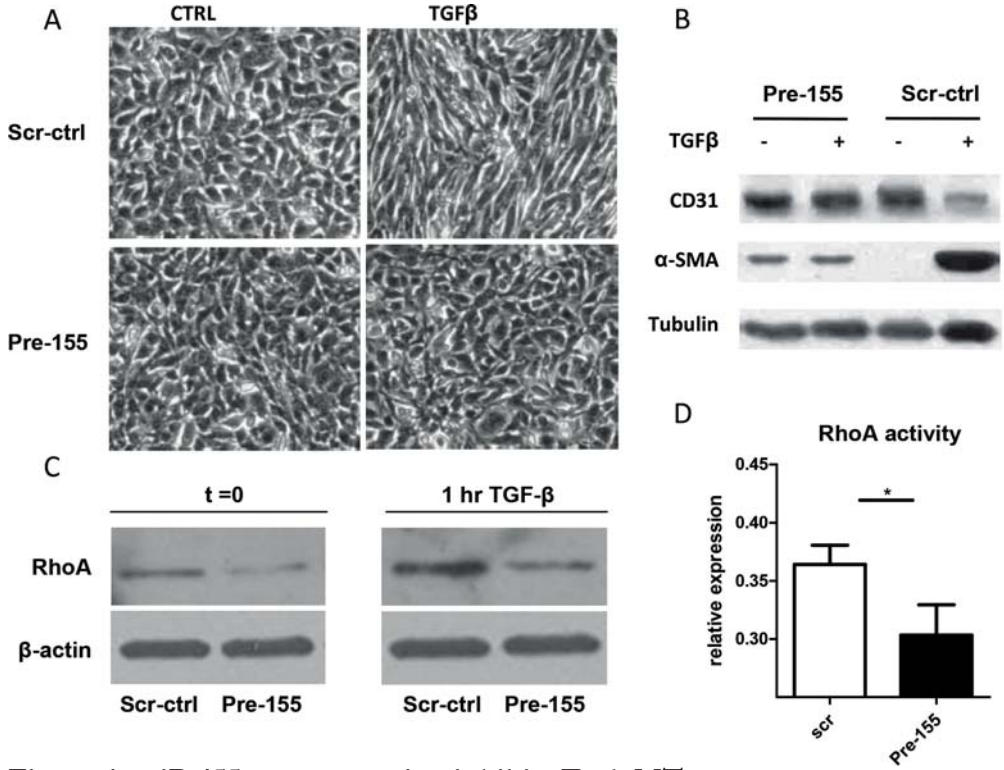

Figure 4. miR-155 overexpression inhibits EndoMT.
A, representative microscopic images of MEEC in the presence or absence of TGF-β, either treated with specific synthetic pre-miR-155 or an unspecific pre-scramblemiR. B, Western blot shows preservation of expression of α-SMA and CD31. Tubulin is used as a loading control. C, RhoA Western blot, pre-miR-155 versus control pre-miR at baseline and 1 hr after TGF-β stimulation. β-actin is used as loading control. D, RhoA activity 2,5 hours after TGF-β stimulation, in the presence of pre-miR-155 or control pre-miR.

Discussion

In this study, we have identified that miR-155 plays a central role in regulating EndoMT. We show that TGF-β stimulation of MEEC results in endothelial to mesenchymal transition and that this mechanism involved the activation of RhoA. Endothelial cell markers are downregulated, and myofibroblast markers are upregulated. In addition to that, stress fiber formation is prominent and an increase in production of extracellular matrix is found. When MEEC are cultured under hypoxic conditions, these features are even stronger.

We profiled TGF-β stimulated MEEC versus control MEEC for miRNAs and found several differentially regulated miRNAs. Overall, we found more miRNAs to be downregulated than up regulated, suggesting a general TGF-β effect on miRNA biosynthesis. This could be true as it has been described that TGF-β dependent SMADs have an effect on miRNA maturation[27].
Several papers described endothelial cells to contribute to the development of tissue fibrosis through endothelial to mesenchymal transition[28,29]. The origin of myofibroblasts is controversial though. It has been shown that myofibroblasts in renal fibrotic tissue originate primarily from interstitial, pericyte-like cells[30]. This could mean that the processes of EMT and EndoMT do not contribute as such to the development of tissue fibrosis. However, the loss of capillaries during fibrosis could, in part, be caused by EndoMT. Furthermore, it is generally accepted that EndoMT is important in embryogenesis, e.g. during formation of the heart.

We showed that miR-155 is important in TGF-β induced EndoMT in MEEC through targeting RhoA. We found the effect on RhoA activity and expression to be an early effect as within a few hours after stimulation with TGF-β in the presence of antagomir-155, and vice versa when miR-155 was over expressed, effects were observed. In addition, in retinal epithelial cells it is described that TGF-β signals to activate RhoA and affects cell migration and α-SMA expression that way[31]. Also in endothelial cells TGF-β is described to activate RhoA, thereby inducing endothelial barrier dysfunction[32]. By overexpressing miR-155 we inhibit EndoMT as a result of RhoA inhibition, confirming that RhoA in EndoMT can regulate α-SMA expression.

In concordance with upregulation of RhoA activity due to silencing of miR-155 we found increased expression of phosphorylated LIM-kinase, which is phosphorylated by ROCK, the direct target of RhoA[24]. pLIMK in its turn

phosphorylates cofilin, which then cannot exhibit its actin-depolymerizing activity anymore. So antagomiR-155 contributes to Rho-induced reorganization of the actin cytoskeleton and provides further evidence that miR-155 modulates EndoMT through RhoA regulation. Based on our data and supportive literature we propose a mechanism as illustrated in Figure 5.

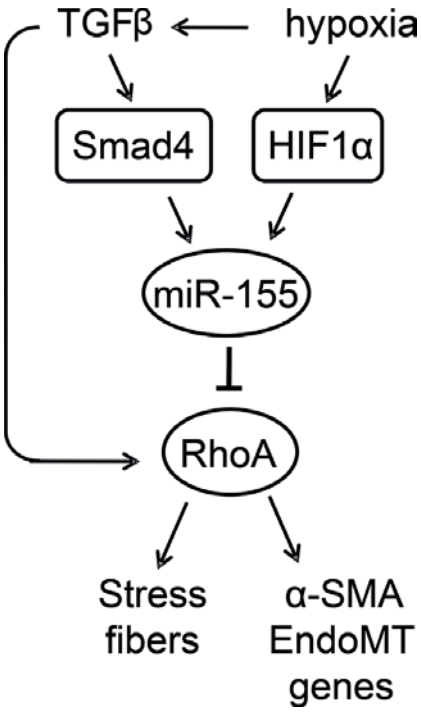

Figure 5. Schematic illustration of miR-155 function in EndoMT.
TGF-β upregulates miR-155 in a SMAD4 dependent manner. In addition, hypoxia upregulates miR-155 through HIF1-α, but also stimulates TGF-β production. TGF-β can stimulate RhoA activity, resulting in stress fiber formation, α-SMA production and induction of other EndoMT related genes. RhoA is suppressed by miR-155, which implies a negative feedback role for miR-155 in RhoA regulation, and therefore EndoMT.

Nevertheless, it is very well possible that miR-155 functions through other targets as well. For example, it has been described that miR-155 targets SMAD5, that inhibits growth-inhibitory effects of TGF-β[33]. This mechanism could also be important in the context of EndoMT as SMAD5 could be counteracting the effects of TGF-β. Another possibly relevant known target includes SMAD2[34], that mediates the effect of TGF-β.

In conclusion, MEEC show to undergo EndoMT after TGF-β stimulation. During this transition miR-155 expression is increased, and is shown to modulate RhoA, and thereby acts as a negative feedback regulator. We showed that over expression of miR-155 can completely inhibit EndoMT and identified miR-155 as a key regulator of EndoMT.

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