Original Paper

MALAT1 Modulates TGF- β 1-Induced Endothelial-to-Mesenchymal Transition through Downregulation of miR-145

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Key Words

MALAT1 • miR-145 • Endothelial progenitor cells • Endothelial-to-mesenchymal transition • TGFBR2 • SMAD3

Abstract

Background/Aims: Endothelial-to-mesenchymal transition (EndMT) plays significant roles under various pathological conditions including cardiovascular diseases, fibrosis, and cancer. EndMT of endothelial progenitor cells (EPCs) contributes to neointimal hyperplasia following cell therapy. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long non-coding RNA (lncRNA) that promotes metastasis and cancer. MicroRNA-145 (miR-145) is a tumor suppressor that has been reported to inhibit SMAD3-mediated epithelial-to-mesenchymal transition (EMT) of cancer cells. In the present study, we investigated the role of MALAT1 and miR-145 in EndMT of human circulating EPCs induced by transforming growth factor beta1 (TGF- β 1). **Methods:** Human circulating EPCs were isolated and characterized by fluorescence-activated cell sorting (FACS). Expression levels of EndMT markers were assessed by qRT-PCR and western blotting. Alpha-smooth muscle actin (α -SMA) expression was measured by cell immunofluorescence staining. The regulatory relationship between MALAT1 and miR-145 and its target genes, TGFBR2 (TGF β receptor type II) and SMAD3 (mothers against decapentaplegic homolog 3) was analyzed using the luciferase reporter assay. **Results:** We found that EndMT of EPCs induced by TGF- β 1 is accompanied by increased MALAT1 expression and decreased miR-145 expression, and MALAT1 and miR-145 directly bind and reciprocally repress each other in these cells. Dual-Luciferase Reporter assay indicated that miR-145 inhibits TGF- β 1-induced EndMT by directly targeting TGFBR2 and SMAD3. **Conclusions:** MALAT1 modulates TGF- β 1-induced EndMT of EPCs through regulation of TGFBR2 and SMAD3 via miR-145. Thus, the MALAT1-miR-145-TGFBR2/SMAD3 signaling pathway plays a key role in TGF- β 1-induced EndMT.

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Introduction

Following vascular endothelium injury, circulating endothelial progenitor cells (EPCs), which are positive for CD34 and KDR, can migrate to the injury site, differentiate to mature vascular endothelial cells, and repair the damaged vessel [1]. This discovery has prompted the development of EPC therapy for ischemic cardiovascular diseases [2, 3]. However, EPCs

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can also trans-differentiate into a smooth muscle cell (SMC)-like lineage through a process called endothelial-to-mesenchymal transition (EndMT). In this process, the cells lose their cell-cell adhesion and acquire invasive and migratory properties. Studies have shown that EPCs, by undergoing EndMT, can contribute to intimal hyperplasia at the injury sites during endothelial repair[4]. These detrimental effects raise concerns regarding the safety of the EPC therapy.

During EndMT, the expression of the endothelial markers CD31 and vascular endothelial cadherin (ve-cadherin) is reduced, and the expression of the mesenchymal and smooth muscle markers α -smooth muscle actin (α -SMA), sm22- α , and myocardin is increased [5, 6]. EndMT shares a number of molecular signaling pathways with epithelial-to-mesenchymal transition (EMT). Similar to EMT, EndMT is potently induced by transforming growth factor beta (TGF- β) [7, 8]. SMAD3 (mothers against decapentaplegic homolog 3), a key mediator of TGF- β signaling, plays a critical role in EMT as well as EndMT initiated by TGF- β [9-11]. Specifically, blocking TGF- β signaling by TGF- β neutralizing antibody or short hairpin RNA (shRNA)-mediated SMAD3 knockdown led to reduced EndMT and neointimal formation in a mouse vein graft model [10]. Therefore, the TGF- β -SMAD3 signaling pathway may serve as a potential target to prevent intimal hyperplasia during vascular endothelial repair.

MicroRNAs (miRNAs) are small non-coding RNA molecules (containing about 22 nucleotides) that function in RNA silencing via base-pairing with complementary sequences within mRNA molecules [12]. MiRNAs regulate many cellular functions such as cell proliferation, differentiation, and migration, and are implicated in pathologic conditions including cancer [13, 14]. MicroRNA-145 (miR-145) is a tumor suppressor that inhibits tumor cell growth and metastasis in many cancers [15-18]. Interestingly, a number of recent studies have shown that miR-145 directly downregulates SMAD3 by binding to its three prime untranslated region (3'-UTR) [19-22]. Specifically, miR-145 overexpression sensitized hepatocellular carcinoma (HCC) cells to chemotherapy by suppressing SMAD3-mediated EMT [21]. Furthermore, miR-145 has been reported to directly target TGF- β receptor type 2 (TGFB2) [23]. Downregulation of miR-145 using an antagomir caused an increase in TGFB2 expression and activation of SMAD3 [24]. Based on these findings, we speculated that miR-145 might play a significant regulatory role in EndMT by simultaneously targeting TGFB2 and SMAD3.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts that are longer than 200 nucleotides in length [25]. Of the thousands of lncRNAs discovered, a few have been shown to play crucial roles in different biological processes through chromatin modification, epigenetic regulation, alternative splicing, and translational control [26, 27]. In particular, a number of lncRNAs are emerging as critical regulators of tumor progression and metastasis [28, 29]. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as nuclear-enriched abundant transcript 2 (NEAT2), is a large, ubiquitously expressed lncRNA [30]. Extensive studies have indicated that MALAT1 plays an oncogenic role in various cancers via multiple mechanisms, including triggering EMT in tumor cells and promoting tumor metastasis [28, 31]. Importantly, MALAT1 has been found to be induced by TGF- β 1 and mediate TGF- β 1-triggered EMT in retinal pigment epithelial cells [32]. However, whether MALAT1 contributes to EndMT induced by TGF- β 1 is not clear.

Interestingly, a recent study has reported that MALAT1 directly interacts with miR-145 in the same RNA-induced silencing complex (RISC), and there is a reciprocal repression between these two non-coding RNAs [33]. In this study, we investigated the functions of MALAT1 and miR-145 in TGF- β 1-induced EndMT of human circulating EPCs and the molecular mechanisms underlying these functions.

Materials and Methods

EPC isolation and characterization

Total human peripheral blood mononuclear cells (hPBMCs) were isolated from human blood and cultured in fibronectin-coated culture dishes as described previously [34]. Cells were seeded in fibronectin-

coated culture dishes and maintained in EC basal medium-2 (EBM-2) (Clonetics, USA), supplemented with microvascular EC growth medium-2 (EGM-2MV) (Lonza, USA), which contains 5% FBS, human vascular endothelial growth factor-1 (hVEGF-1), human fibroblast growth factor-2 (hFGF-2), human epidermal growth factor (hEGF), insulin-like growth factor-1 (IGF-1) and ascorbic acid. After 3 days in culture, nonadherent cells were removed by washing with PBS, and the remaining cells were cultured in fresh media for another 7 days. The expression of the EPC markers CD34 and KDR was assessed by fluorescence-activated cell sorting (FACS) as follows. The differentiating mononuclear cells were detached with 0.25% trypsin and gently washed three times with PBS containing 1 mM EDTA. The cells were subsequently incubated with FITC-conjugated anti-KDR (1:200; ab184903, Abcam, USA) and Phycoerythrin (PE)-conjugated anti-CD34 (1:100; ab157304, Abcam) antibodies, respectively, at 4°C for 30 min. FITC-conjugated mouse monoclonal IgG1 (1:200; ab91356, Abcam) and PE-conjugated mouse monoclonal IgG1 (1:100; ab67435, Abcam) were used as negative control, respectively. After that, the cells were fixed in 1% paraformaldehyde and subjected to analysis on a FACStar flow cytometer (BD Biosciences, USA). To study EndMT induced by TGF- β 1, EPCs isolated as above were treated with TGF- β 1 (5 ng/ml) (Sigma, USA) for 7 days with the medium changed every 2 days during this period.

RNA isolation and Real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR)

Total RNA was extracted for cultured EPCs using Trizol reagent (Invitrogen, Carlsbad, USA) following manufacturer's instructions and RNA molecules smaller than 200 nucleotides in size were purified using the mirVana miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's instructions, as described previously [35]. Complementary DNA (cDNA) was synthesized using the M-MLV Kit (Life Technologies, USA). QRT-PCR was carried out using the Platinum SYBR Green qPCR SuperMix-UDG kit (Life Technologies) on an ABI Prism 7500 PCR system (Applied Biosystems, USA). Data were normalized to β -actin. QRT-PCR analysis of mature miR-145 was performed using the TaqMan MicroRNA Assay Kit (Applied Biosystems), the expression level of mature miR-145 was determined by real-time PCR analysis following stem-loop RT and data were normalized to U6 snRNA according to previous studies [36]. All primers were listed in Table 1. Each test was carried out in triplicate. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [37].

Plasmids construction and cell transfection

For MALAT1 overexpression, the full-length human MALAT1 cDNA was amplified using the following primers: forward, 5'-GCGGTTACC ATGAAACAATTGGAGAAG -3'; reverse, 5'-GCGCTCGAG CTAAGTTTGTA-CATTTTGCC -3'. The cDNA product was subcloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen) at the *KpnI* and *XhoI* sites to generate pcDNA3.1-MALAT1 and stable colonies were screened using G418. The pcDNA3.1(+) vector was used as negative control (pcDNA3.1-NC). For MALAT1 knockdown, two MALAT1-targeting shRNAs (MALAT1-shRNA1, 5'-TCCACTTGATCCCAACTCATC-3'; MALAT1-shRNA2, 5'-TTCCTTAGTTGGCATCAAGGC-3') and a scrambled shRNA (scr, 5'-GACCTGTACGC-CAACACAGTG-3') used as negative control were synthesized at Genechem (Shanghai, China). The shRNAs were subcloned into the psilencer 4.1 CMV neo vector (Invitrogen) followed by selection with puromycin (Gibco, Grand Island, NY, USA) to generate stable cell lines. All plasmids were isolated using the DNA Midiprep kit (Qiagen, Germany). For miR-145 overexpression and knockdown, miR-145 mimics, miR-145 inhibitor, and two scrambled

Table 1. The sequences for the primers

gene	Primer direction	Sequence
MALAT1	Forward	5'-AGACCTTGAAATCCAT-3'
	Reverse	5'-CTTCTGCTTCTACTT-3'
CD31	Forward	5'-CCTGCGGTATTCAAAGACAA-3'
	Reverse	5'-TGGGACCAGATCCTTCATTAC-3'
ve-cadherin	Forward	5'-TCGGTTGTCAATGCG-3'
	Reverse	5'-AGCGTCCTGGTAGTCG-3'
α -SMA	Forward	5'-GCGTGGCTATTCCTCGTTA-3'
	Reverse	5'-TGATGCTGTTGTAGGTGTTTC-3'
sm22- α	Forward	5'-GATGAGCAGGTGG-3'
	Reverse	5'-AGGTGCTCCGTAGC-3'
myocardin	Forward	5'-GCCACAGCACATCAG-3'
	Reverse	5'-TTCTCCGCTTTCAATAA-3'
pri-miR-145	Forward	5'-TGGATTGGCTCCTTCCCA-3'
	Reverse	5'-TTGAACCTCATCCTGTGAGCC-3'
Pre-miR-145	Forward	5'-GTCCAGTTTCCAGGAATC-3'
	Reverse	5'-AGAACAGTATTCCAGGAAT-3'
β -actin	Forward	5'-CGTGACATTAAGGAGAAGCTG-3'
	Reverse	5'-CTAGAAGCATTGCGGTGGAC-3'
miR-145	RT primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAACAGT-3'
	Forward	5'-GCCGGTCCAGTTTCCAGG-3'
	Reverse	5'-GTGAGGGTCCGAGGT-3'
U6	RT primer	5'-CGCTTCACGAATTTGCGTGTCTAT-3'
	Forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
	Reverse	5'-CGCTTCACGAATTTGCGTGTCTAT-3'

miRNAs used as negative controls (miR-NC for miR-145 mimics and inhibitor-NC for miR-145 inhibitor, respectively) were purchased from Genechem. The plasmids, shRNAs, and miRNAs were transfected into EPCs using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. At 48 h after transfection, cells were harvested for qRT-PCR analyses or western blotting.

Cell immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, and permeabilized with 0.1% Triton X-100 in PBS. After blocking in 10% goat serum for 1 h at room temperature, the cells were incubated with anti- α -SMA (1:100; ab124964, Abcam) or anti-pSmad3 (Phospho S423+S425) (1:100; ab52903, Abcam) antibody for 16 h at 4°C. After washing, the cells were incubated for 2 h with AlexaFluor® 488-conjugated goat anti-rabbit IgG H&L (1:400; ab150077, Abcam) or goat anti-rabbit (Cy3) preadsorbed secondary antibody (1:100; BA1032, Boster, China) secondary antibody. The cells were subsequently counterstained with DAPI and examined under a confocal microscope.

Bioinformatics

The putative miR-145 binding sites in the 3'-UTR of MALAT1, TGFBR2, and SMAD3 were predicted using TargetScan (<http://www.targetscan.org>). The bioinformatics database at <http://starbase.sysu.edu.cn/mirLncRNA.php> was used in search of lncRNA and miRNA interactions.

Luciferase reporter assay

3'-UTR cDNA fragments of *TGFBR2* and *SMAD3* containing the putative wildtype or mutant miR-145 binding sites were amplified using the following primers: wild type-1 3'-UTR of *SMAD3* (SMAD3-wt1), forward: 5'-CGCCTCGAG GTCTGTCATCTTCCCC-3' and reverse: 5'-CGGGCGGCCGC CTCCATCTCCCTCTAA -3'; mutant-1 3'-UTR of *SMAD3* (SMAD3-mut1), forward: 5'-CGCCTCGAGAGTCTAAATTATTTCTTCTC-3' and reverse: 5'-CGGGCGGCCGCTCTCCATCTCCCTCTA-3'; wild type-2 3'-UTR of *SMAD3* (SMAD3-wt2), forward: 5'-CGCCTCGAGGCATCAGCCGCTTCTC-3' and reverse: 5'-CGGGCGGCCGCCACCTCCACCAACAT-3'; mutant-2 3'-UTR of *SMAD3* (SMAD3-mut2), forward: 5'-CGCCTCGAGAACTTGCCTCATGTATTCTCTT-3' and reverse: 5'-CGGGCGGCCGCCAACTCTTCCAGCCACTGTCCTT-3'; wildtype 3'-UTR of *TGFBR2* (TGFBR2-wt), forward: 5'-CGCCTCGAGTGTTGCCATTGACCT-3' and reverse: 5'-CGGGCGGCCGCAACAGGAGGAGGATG-3'; mutant 3'-UTR of *TGFBR2* (TGFBR2-mut), forward: 5'-CGCCTCGAGGGTTATCAGCATATTCTC-3' and reverse: 5'-CGGGCGGCCGCGCATTCACATTCTTATTT -3'. CDNA fragments of MALAT1 containing the putative wildtype or mutant miR-145 binding sites were amplified using the following primers: wildtype MALAT1 (MALAT1-wt), forward: 5'-CGCCTCGAGTTACTAAACGCAGACG-3' and reverse: 5'-CGGGCGGCCGCGTAAACACCTCATCT-3'; mutant MALAT1 (MALAT1-mut), forward: 5'-CGCCTCGAGAAGTTTGAAGTGGTTTGTAG-3' and reverse: 5'-CGGGCGGCCGCTTCACTGCTATTTTATCCAA-3'. The amplified cDNA fragments were subcloned into the psiCHECK-2 vector (Promega, USA) at the *Xho*I and *Not*I sites downstream of the luciferase gene. HEK293 cells were co-transfected with the luciferase reporter systems and miR-145 mimics/miR-NC as indicated in Figure legends. Luciferase activity was detected 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) following manufacturer's instructions. Data were normalized to Renilla luciferase activity.

RNA-binding protein immunoprecipitation (RIP) assay

RNA-binding protein immunoprecipitation (RIP) assay was performed as previously described [33] using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA). Briefly, EPCs were lysed in 100 μ l RIP lysis buffer. The whole cell extract (90 μ l) was incubated with human anti-Ago2 antibody (Abcam, 1:200) or negative control (mouse IgG, Abcam) in RIP buffer containing protein A/G magnetic beads. After washing to remove unbound materials, RNAs were extracted from the magnetic beads bound complexes. The MALAT1 mRNA and miR-145 levels in the precipitates were determined by qRT-PCR. The Ago2 protein levels in the precipitates were determined by western blot analysis.

Western Blot

Cells were lysed in lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, USA). Equal amount of total protein for each sample was separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, USA). After blocking in 1% BSA for 20 min, the membranes were incubated with anti-myocardin

(1:1000; ab203614, Abcam), anti-ve-cadherin (1:1000; ab33168, Abcam), anti-CD31 (1:500; ab28364, Abcam), anti-sm22- α (1:500; ab14106, Abcam), anti- α -SMA (1:2000; ab124964, Abcam) anti-TGFBR2 (1:1000; ab61213, Abcam), anti-pSmad3 (Phospho S423+S425) (1:1500; ab52903, Abcam), anti-Smad3 (1:2000; ab63577, Abcam), anti-Snail (1:500; ab82846, Abcam), anti-Slug (1:1000; ab106077, Abcam), anti-Collagen I (1:5000; ab34710, Abcam), and anti-Collagen III (1:5000; ab7778, Abcam) antibodies, respectively, at 4°C overnight. The membranes were subsequently washed with wash buffer and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:10000; ab6721, Abcam) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence detection reagents (Thermo Scientific, USA) and quantified by densitometric analysis using Image J. Data were normalized to GAPDH or β -tubulin and presented as fold changes over control.

Cell migration and invasion assay

Cell migration was assessed using a Transwell system with 8.0 μ m membrane (Corning, USA). Briefly, cells were suspended in culture medium containing 1% FBS and loaded into the Transwell upper chamber (100 μ l, 4×10^4 cells/well). The lower chamber was loaded with 600 μ l of culture medium containing 20% FBS. Cells were allowed to migrate at 37°C for 36 h. Non-migrating cells on the upper surface of the membrane were gently removed using cotton swabs. The cells that had migrated to the lower surface of the membrane were fixed with methanol, stained with 0.1% crystal violet, and counted in five randomly selected fields under an inverted light microscope. Cell invasion was assessed using a Matrigel-coated Transwell system (Corning) following procedures as above. Each experiment was conducted in triplicate.

Statistical analysis

Results are presented as the mean $\pm$ SD (standard deviation). Data analysis was carried out using SPSS version 13.0 (SPSS, USA). The Student's *t*-test was used to evaluate differences between two groups. Results from multiple groups were compared using an ANOVA followed by Bonferroni's multiple comparison test. *P* values less than 0.05 were considered statistically significant.

Results

MALAT1 is upregulated and miR-145 downregulated during TGF- β 1-induced EndMT

Total hPBMCs were isolated from human blood. After 1 week in culture, the adherent cells displayed morphologic properties of ECs. FACS analysis revealed that about 80-85% of cells exhibited qualitative features of EPCs including increased levels of the stem cell marker CD34 and the endothelial lineage marker KDR (Fig. 1A). After treatment with TGF- β 1 (5 ng/ml) for 7 d, the cells displayed significantly decreased mRNA levels of the endothelial markers CD31 and ve-cadherin along with increased levels of the mesenchymal/smooth muscle markers α -SMA, sm22- α , and myocardin (Fig. 1B, 1C), indicating that the cells underwent EndMT. Previous studies have shown that MALAT1 is highly expressed in endothelial cells [38], and it is induced by TGF- β in cancer and epithelial cells, mediating TGF- β 1-induced EMT [32, 39]. In this study, we found that TGF- β 1 strongly induced MALAT1 mRNA expression in EPCs (Fig. 1D), suggesting that MALAT1 might also play a role in TGF- β -induced EndMT. Interestingly, MALAT1 upregulation by TGF- β 1 was accompanied by pri-miR-145, pre-miR-145 and miR-145 downregulation (Fig. 1E). These results were similar to previous findings that MALAT1 expression inversely correlates with miR-145 expression in cervical cancer cells [33].

MALAT1 is a target of miR-145

The inverse changes in MALAT1 and miR-145 expression in response to TGF- β 1 suggested a regulatory relationship between these two non-coding RNAs. To find out whether endogenous MALAT1 targets and regulates miR-145 expression, we transfected EPCs with MALAT1-shRNA1 or MALAT1-shRNA2 to suppress MALAT1 expression (Fig. 2A). Similar to previous findings in Hela and CaSki cells [33], MALAT1 knockdown by MALAT1-shRNA1 transfection led to significantly increased miR-145 expression in EPCs, but its effects on pri-

Fig. 1. MALAT1 is upregulated and miR-145 is downregulated during TGF- β 1-induced EndMT of human circulating EPCs. Human circulating EPCs were isolated from human blood as described in materials and methods. (A) Cell surface expression of the stem cell maker CD34 and the endothelial lineage marker KDR was determined by FACS analysis. (B-E) Cells were treated with TGF- β 1 (5 ng/ml) for 7 d. The levels of the endothelial markers CD31 and ve-cadherin (B) the mesenchymal/smooth muscle markers α -SMA, sm22- α , and myocardin (C) MALAT1 (D), and pri-miR-145, pre-miR-145 and miR-145 (E) were determined by qRT-PCR. n = 3, * P <0.05, ** P <0.01.

Fig. 2. MALAT1 is a target of miR-145. (A) EPCs were transfected with MALAT1-shRNA1, MALAT1-shRNA2, or scr for 48 h. The MALAT1 levels were determined by qRT-PCR. n = 3, ** P <0.01, *** P <0.001. (B) EPCs were transfected with MALAT1-shRNA1 or scr for 48 h. The levels of pri-miR-145, pre-miR-145, and mature miR-145 were determined by qRT-PCR. n = 3, ** P <0.01. (C) The DICER protein levels in EPCs stably transfected with MALAT1-shRNA1 or scr by western blot analysis. Data were normalized to GAPDH. n = 3, ** P <0.01. (D) EPC lysates were incubated with Ago2 antibody-coated magnetic beads. Precipitates were subjected to western blot analysis for Ago2 (upper panel) and qRT-PCR for MALAT1 and miR-145 (lower panel) detection. Preimmune IgG and 10% input from EPC cell extracts were used as controls. n = 3, *** P <0.001. (E) The luciferase reporter constructs containing the wildtype (MALAT1-wt) or mutant MALAT1 sequence (MALAT1-mut). (F) MALAT1-wt or MALAT1-mut was co-transfected into HEK293 cells with miR-145 mimics or miR-NC. Luciferase activity was determined at 48 h after transfection. n = 3, ** P <0.01.

miR-145 and pre-miR-145 levels were insignificant (Fig. 2B). Thus, endogenous MALAT1 likely represses miR-145 mainly through some posttranscriptional mechanism. DICER, also

Fig. 3. MALAT1 and miR-145 reciprocally repress each other. (A) EPCs were transfected with miR-145 mimics, mimics-NC, miR-145 inhibitor, or inhibitor-NC for 48 h. The miR-145 levels were determined by qRT-PCR. $n = 3$, $**P < 0.01$. (B) EPCs were stably transfected with pcDNA3.1-MALAT1 or pcDNA3.1. The MALAT1 levels were determined by qRT-PCR. $n = 3$, $**P < 0.01$. (C) EPCs were transfected with miR-145 mimics/mimics-NC, or miR-145 inhibitor/inhibitor-NC for 48 h. The MALAT1 levels were determined by qRT-PCR. $n = 3$, $**P < 0.01$. (D) EPCs were stable transfected with MALAT1shRNA1/scr or pcDNA3.1-MALAT1/pcDNA3.1-NC. The miR-145 levels were determined by qRT-PCR. $n = 3$, $**P < 0.01$. (E) EPCs were stably transfected with pcDNA3.1-MALAT1/pcDNA3.1-NC or MALAT1-shRNA1/scr and subsequently treated with TGF- β 1 (5 ng/ml) for 7 d. Untransfected cells with or without TGF- β 1 treatment were also included. The miR-145 levels were determined by qRT-PCR. $n = 3$, $**P < 0.01$, $***P < 0.001$. (F) EPCs were transfected with miR-145 mimics/mimics-NC or miR-145 inhibitor/inhibitor-NC for 48 h and subsequently treated with TGF- β 1 (5 ng/ml) for 7 d. Untransfected cells with or without TGF- β 1 treatment were also included. The MALAT1 levels were determined by qRT-PCR. $n = 3$, $**P < 0.01$, $***P < 0.001$.

known as endoribonuclease DICER or helicase with RNase motif, is an enzyme that cleaves pre-miRNAs to form mature miRNAs [40, 41]. We found that MALAT1 knockdown in EPCs led to increased DICER expression (Fig. 2C), suggesting that the endogenous MALAT1 represses miR-145 at least partially by downregulating DICER and thereby inhibiting miR-145 maturation. MiRNAs activate the RNA-induced silencing complex (RISC) to silence complementary target mRNAs [42]. MALAT1 has been reported to have a putative binding site for miR-145 [33], therefore, miR-145 might be able to recruit MALAT1 into the same RISC for degradation. To test this, we conducted a RIP assay using antibody against Ago2, an active component of RISC that cleaves the target mRNA strand [43]. Western blot analysis showed that Ago2 antibody-coated magnetic beads pulled down the Ago2 protein from cell lysates (Fig. 2D, upper panel). QRT-PCR analysis revealed higher MALAT1 and miR-145 levels in the Ago2 precipitates compared with the input control (Fig. 2D, lower panel), supporting co-localization of MALAT1 and miR-145 in the same RISC. These results were similar to previous findings in Hela cells [33]. To confirm that MALAT1 is a *bona fide* miR-145 target, we constructed luciferase reporter plasmids incorporating cDNA sequences of MALAT1 containing the wildtype or mutant miR-145 binding site (Fig. 2E). Co-transfection of the luciferase reporter plasmid containing the wildtype, but not the mutant MALAT1 sequence

Fig. 4. MiR-145 inhibits EndMT induced by TGF- β 1. EPCs were transfected with miR-145 mimics/mimics-NC or miR-145 inhibitor/inhibitor-NC for 48 h and subsequently treated with TGF- β 1 (5 ng/ml) for 7 d. Untransfected cells with or without TGF- β 1 treatment were also included. (A-E) The mRNA levels of the endothelial markers CD31 (A) and ve-cadherin (B) and the mesenchymal/smooth muscle markers α -SMA (C), sm22- α (D), and myocardin (E) were determined by qRT-PCR. $n=3$, * $P<0.05$, ** $P<0.01$. (F, G) The protein levels of CD31, ve-cadherin, α -SMA, sm22- α and myocardin were determined by western blot analysis. $n=3$, * $P<0.5$, ** $P<0.01$. (H) Immunofluorescence staining for α -SMA expression.

with miR-145 mimics in HEK293 cells resulted in decreased reporter activity compared with miR-NC (Fig. 2F). These data strongly suggested that MALAT1 is a target of miR-145. These data also made us believe that, besides DICER downregulation, MALAT1 might repress miR-145 levels by directly binding to it, as the miR-145:MALAT1 complex might not be isolated by the miRVANA kit, which has a cutoff of approximately 200 bp.

MALAT1 and miR-145 reciprocally repress each other

A reciprocal repression between MALAT1 and miR-145 has been reported in cervical cancer cells [33]. To investigate the mechanisms underlying the inverse changes in MALAT1 and miR-145 expression in response to with or without TGF- β 1, we examined the regulatory relationship between MALAT1 and miR-145 using gene overexpression and/or knockdown. We subsequently transfected EPCs with miR-145 mimics or miR-145 inhibitor to overexpress or knockdown miR-145 (Fig. 3A). And increased MALAT1 expression in EPCs transfected with pcDNA3.1-MALAT1 plasmids (Fig. 3B). As shown in Figure 3C, miR-145 overexpression inhibited while miR-145 knockdown increased endogenous MALAT1 expression in EPCs. We next examined the effects of overexpression or knockdown MALAT1 on miR-145 (Fig. 3D). We found that MALAT1 knockdown by MALAT1-shRNA1 transfection was associated

Fig. 5. MiR-145 directly targets TGFBR2 and SMAD3. (A) The luciferase reporter constructs containing the wildtype (TGFBR2-wt) or mutant 3'-UTR of TGFBR2 (TGFBR2-mut). (B) TGFBR2-wt or TGFBR2-mut was co-transfected into HEK293 cells with miR-145 mimics or miR-NC. Luciferase activity was determined at 48 h after transfection. $n=3$, $**P<0.01$. (C, E) The luciferase reporter constructs containing the wildtype (SMAD3-wt1 or SMAD3-wt2) or mutant 3'-UTR of SMAD3 (SMAD3-mut1 or SMAD3-mut2). (D, F) SMAD3-wt1/SMAD3-mut1 or SMAD3-wt2/SMAD3-mut2 was co-transfected into HEK293 cells with miR-145 mimics or miR-NC. Luciferase activity was determined at 48 h after transfection. $n=3$, $**P<0.01$, $***P<0.001$. (G, H) SMAD3-wt2 (G) or TGFBR2-wt (H) was co-transfected into HEK293 cells with miR-145 mimics or miR-NC. Cells were also co-transfected with pcDNA3.1-MALAT1 or pcDNA3.1-NC as indicated. Luciferase activity was determined at 48 h after transfection. $n=3$, $**P<0.01$.

with increased miR-145 expression, while MALAT1 overexpression by pcDNA3.1-MALAT1 transfection was associated with decreased miR-145 expression (Fig. 3D). Moreover, we found that, in TGF- β 1-stimulated EPCs, MALAT1 overexpression by pcDNA3.1-MALAT1 transfection was associated with decreased miR-145 expression, while MALAT1 knockdown by MALAT1-shRNA1 transfection was associated with increased miR-145 expression (Fig. 3E). And in Figure 3F, miR-145 overexpression inhibited MALAT1 expression upregulated by TGF- β 1 while miR-145 knockdown had the opposite effects. These data confirmed that MALAT1 and miR-145 reciprocally repress each other in EPCs under both basal and stimulated conditions.

MiR-145 inhibits TGF- β 1-induced EndMT

MiR-145 has been reported to regulate SMAD3-mediated EMT of HCC cells [21]. Having found that miR-145 is downregulated in response to TGF- β 1 in EPCs, we speculated that it might play a role in TGF- β 1-induced EndMT of these cells. To test this, we examined the effects of miR-145 overexpression and knockdown on the expression levels of EndMT markers using qRT-PCR and western blots. The results showed that miR-145 overexpression restored the endothelial markers CD31 and ve-cadherin downregulated by TGF- β 1 and reduced the mesenchymal/smooth muscle markers α -SMA, sm22- α , and myocardin induced by TGF- β 1 (Fig. 4A-G). Opposite effects were detected with miR-145 knockdown (Fig. 4A-G). Next, we evaluated EndMT by cell immunofluorescence staining for α -SMA expression. Significantly increased α -SMA staining was detected after TGF- β 1 treatment (Fig. 4H). MiR-145 overexpression effectively attenuated while miR-145 knockdown markedly enhanced

Fig. 6. MALAT1 modulates TGF- β 1-induced EndMT through regulation of TGFBR2 and SMAD3 via miR-145. EPCs stably transfected with MALAT1-shRNA1/scr or pcDNA3.1-MALAT1/pcDNA3.1-NC were transfected with miR-NC or miR-145 mimics as indicated for 48 h. Untransfected cells were also included for control purpose. The cells were subsequently treated with TGF- β 1 (5 ng/ml) for 7 d. (A-D) The protein levels of TGFBR2, Smad3, and pSmad3 (A, B) and Collagen I, Collagen III, Snail, and Slug (C, D) were determined by western blot analysis. Data were normalized to GAPDH and β -tubulin, respectively. $n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (E) Immunofluorescence staining for pSmad3 distribution.

α -SMA staining induced by TGF- β 1 (Fig. 4H). Collectively, these results supported an inhibitory role of miR-145 in TGF- β 1-induced EndMT.

MiR-145 directly regulates TGFBR2 and SMAD3 by targeting their 3'-UTRs

MiR-145 has been reported to negatively regulate TGF- β signaling by directly targeting SMAD3 and TGFBR2 [19-22, 24]. We thought that this might be the mechanisms by which miR-145 inhibits TGF- β 1-induced EndMT. To confirm this, we prepared luciferase reporter expression systems containing the wildtype or mutant 3'-UTR of TGFBR2 and SMAD3 (Fig. 5A, 5C, 5E). Co-transfection of the luciferase reporter system containing the wildtype, but not the corresponding mutant 3'-UTR of TGFBR2 or SMAD3 with miR-145 mimics in HEK293 cells resulted in significant loss of reporter activity compared with miR-NC (Fig. 5B, 5D, 5F). These results confirmed that miR-145 downregulates TGFBR2 and SMAD3 by directly targeting their 3'-UTRs. Further, in HEK293 cells co-transfected with the luciferase reporter plasmid containing the wildtype 3'-UTR of SMAD3 or TGFBR2 and miR-145 mimics, MALAT1 overexpression restored reporter activity downregulated by miR-145 mimics (Fig. 5G, 5H). These data were in alignment with miR-145 repression by MALAT1.

Fig. 7. MALAT1 modulates TGF- β 1-induced EndMT via miR-145. EPCs stably transfected with MALAT1-shRNA1/scr or pcDNA3.1-MALAT1/pcDNA3.1-NC were transfected with miR-NC or miR-145 mimics as indicated for 48 h. Untransfected cells were also included for control purpose. The cells were subsequently treated with TGF- β 1 (5 ng/ml) for 7 d. (A, B) The protein levels of CD31, ve-cadherin, α -SMA, sm22- α , and myocardin determined by western blot analysis. Data were normalized to β -tubulin. $n = 3$, * $P < 0.05$, ** $P < 0.01$. (C) Immunofluorescence staining for α -SMA expression.

MALAT1 modulates TGF- β 1-induced EndMT through regulation of TGFBR2 and SMAD3 via miR-145

Increased protein levels of TGFBR2, Smad3, and phospho-Smad3 (pSmad3) were detected in EPCs following TGF- β 1 stimulation (Fig 6A, 6B). A plausible explanation is that TGF- β 1 treatment induced MALAT1 expression, leading to downregulation of miR-145 and upregulation of its targets TGFBR2 and Smad3. TGF- β 1 also induced the expression of the mesenchymal cell markers Collagen I and Collagen III [44], and the EndMT-related transcription factors Snail and Slug [45] (Fig. 6C, 6D), demonstrating that EPCs acquired mesenchymal phenotype through an EndMT process. MiR-145 mimics inhibited TGF- β 1-induced Collagen I, Collagen III, Snail, and Slug expression, and these changes were accompanied by reduced TGFBR2, Smad3 and pSmad3 levels (Fig. 6A-D). These data supported the inhibitory role of miR-145 in TGF- β 1-induced EndMT through downregulation of TGFBR2 and Smad3. MALAT1 knockdown further reduced while MALAT1 overexpression restored the levels of TGFBR2, Smad3 and pSmad3, as well as the EndMT-related proteins Collagen I, Collagen III, Snail, and Slug downregulated by miR-145 mimics (Fig. 6A-D). These effects of MALAT1 knockdown and overexpression were presumably mediated by up and downregulation of endogenous miR-145, respectively. In addition, our immunofluorescence staining revealed increased nuclear translocation of pSmad3 in response to TGF- β 1 stimulation (Fig. 6E). MALAT1 knockdown further reduced while MALAT1 overexpression restored pSmad3 nuclear translocation downregulated by miR-145 mimics in TGF- β 1-treated EPCs (Fig. 6E). These data further supported the involvement of the Smad3 signaling in TGF- β 1-induced EndMT as well as its regulation by MALAT1 and miR-145. Finally, we assessed the endothelial and mesenchymal/smooth muscle markers (CD31, ve-cadherin, α -SMA, sm22- α and myocardin)

Fig. 8. MALAT1 modulates TGF- β 1-induced EPC migration and invasion via miR-145. EPCs stably transfected with MALAT1-shRNA1/scr or pcDNA3.1-MALAT1/pcDNA3.1-NC were transfected with miR-NC or miR-145 mimics as indicated for 48 h. Untransfected cells were also included for control purpose. The cells were subsequently treated with TGF- β 1 (5 ng/ml) for 7 d. Cell migration (A) and invasion (B) were determined by Transwell assays. n = 3, * P < 0.05, ** P < 0.01.

by western blot analysis and α -SMA expression by immunofluorescence staining. The results further supported that MALAT1 modulates TGF- β 1-induced EndMT via miR-145 (Fig. 7A-C).

MALAT1 modulates TGF- β 1-induced EPC migration and invasion via miR-145

A hallmark of EndMT is the loss of cell-cell adhesion and gain of migratory and invasive properties. Thus, we examined EPC migration and invasion behavior using Transwell assays. Our results showed that, in alignment with increased mesenchymal marker expression, TGF- β 1-treated cells exhibited increased cell migration and invasion (Fig. 8A, 8B). MiR-145 mimics inhibited cell migration and invasion induced by TGF- β 1 (Fig. 8A, 8B). Moreover, MALAT1 knockdown further reduced while MALAT1 overexpression restored cell migration and invasion inhibited by miR-145 mimics (Fig. 8A, 8B). These results were consistent with changes in the expression of EndMT-related proteins detected in these cells. Taken together, results from our study indicated that the MALAT1-miR-145-TGFBR2/SMAD3 signaling pathway plays a key role in TGF- β 1-induced EndMT of EPCs.

Discussion

Circulating EPCs can differentiate into vascular endothelial cells and thus are being investigated as a potential therapeutic modality for cardiovascular diseases [46]. However, EPCs can undergo TGF- β 1-dependent EndMT [6], which is believed to contribute to neointimal hyperplasia inside EPC-capturing stents [47-49]. The mechanisms underlying TGF- β 1-dependent EndMT of EPCs are not fully understood. In this study, we found that MALAT1 is upregulated and miR-145 is downregulated in TGF- β 1-treated EPCs undergoing EndMT. MALAT1 and miR-145 directly bind and reciprocally repress each other in these cells, and MALAT1 mediates TGF- β 1-induced EndMT by regulating TGFBR2 and SMAD3 through miR-145.

Many miRNAs have been reported to regulate EMT of cancer cells [50-56]. Recent evidence has revealed that miRNAs can also mediate or inhibit TGF- β 1-dependent EndMT

[57]. For example, MiR-21 mediates TGF- β 1-dependent EndMT by targeting PTEN [58]. MiR-126 and miR-155 inhibit TGF- β 1-dependent EndMT by targeting the PI3K subunit p85 and Rho A, respectively [59, 60]. In this study, we found that miR-145, a well characterized tumor suppressor, inhibits TGF- β 1-induced EndMT of human circulating EPCs by directly targeting TGFBR2 and SMAD3. We also found that MALAT1, a lncRNA that promotes metastasis and cancer, is induced by TGF- β 1 in EPCs. MALAT1 downregulates miR-145 by suppressing the expression of endoribonuclease DICER. Conversely, miR-145 suppresses MALAT1 expression by recruiting MALAT1 to RISC for degradation. MALAT1 modulates TGF- β 1-induced EndMT through regulation of miR-145 and its target genes TGFBR2 and SMAD3. These findings identified the MALAT1-miR-145-TGFBR2/SMAD3 signaling axis as a key regulator of TGF- β 1-induced EndMT.

EndMT was originally observed during heart development, and later studies have revealed its important roles in various pathological processes such as fibrosis and cancer [61]. EndMT plays a critical role in generation of fibroblasts in renal, pulmonary, and cardiac fibrosis [62-64]. EndMT in cancer is an important source of cancer-associated fibroblasts (CAFs), which have been shown to contribute to tumor growth and metastasis [65]. Although EndMT has traditionally been considered as a negative contributor to diseases, its ability to generate various connective tissues may be harnessed to repair damaged tissues in degenerative diseases [57]. Thus, modulation of EndMT may have potential therapeutic value in treatment of EndMT-related diseases or regenerative medicine.

Understanding the mechanisms of EndMT regulation is key to the development of EndMT modulators. The TGF- β superfamily of proteins are the most common cytokines that regulate EndMT [8]. Other signaling pathways such as Wnt/ β -catenin and Notch can also activate EndMT [66, 67]. Our findings identified the MALAT1-miR-145-TGFBR2/SMAD3 signaling axis as a key player in EndMT of circulating EPCs triggered by TGF- β . Whether this signaling axis is involved in the regulation of EndMT under other developmental or pathological conditions awaits further investigation.

Abbreviations

EndMT (Endothelial-to-mesenchymal transition); EPCs (EndMT of endothelial progenitor cells); MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1) ; lncRNA (long non-coding RNA); miR-145 (MicroRNA-145); EMT (epithelial-to-mesenchymal transition); TGF- β 1 (transforming growth factor- β 1); FACS (Fluorescence-activated cell sorting); TGFBR2 (TGF- β receptor type 2); SMC (smooth muscle cell); ve-cadherin (vascular endothelial cadherin); α -SMA (alpha-smooth muscle actin); SMAD3 (Sma and Mothers against decapentaplegic homolog 3); shRNA (short hairpin RNA); miRNAs (MicroRNAs); 3'-UTR (three prime untranslated region); HCC (hepatocellular carcinoma); NEAT2 (nuclear-enriched abundant transcript 2); RISC (RNA-induced silencing complex); hPBMcs (human peripheral blood mononuclear cells); RIP (RNA-binding protein immunoprecipitation); HRP (horseradish peroxidase); pSMAD3 (phospho-SMAD3); CAFs (cancer-associated fibroblasts).

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Disclosure Statement

The authors declare no conflicts of interest.

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