Transforming Growth Factor-β–Induced Endothelial-to-Mesenchymal Transition Is Partly Mediated by MicroRNA-21

Regalla Kumarswamy, Ingo Volkmann, Virginija Jazbutyte, Seema Dangwal, Da-Hee Park, Thomas Thum

Objective—MicroRNAs are a class of small ribonucleotides regulating gene/protein targets by transcript degradation or translational inhibition. Transforming growth factor-β (TGF-β) is involved in cardiac fibrosis partly by stimulation of endothelial-to-mesenchymal transition (EndMT). Here, we investigated whether microRNA (miR)-21, a microRNA enriched in fibroblasts and involved in general fibrosis, has a role in cardiac EndMT.

Methods and Results—TGF-β treatment of endothelial cells significantly increased miR-21 expression and induced EndMT characterized by suppression of endothelial and increase of fibroblast markers. Overexpression of miR-21 alone also stimulated EndMT. Importantly, miR-21 blockade by transfection of specific microRNA inhibitors partly prevented TGF-β-mediated EndMT. Mechanistically, miR-21 silenced phosphatase and tensin homolog in endothelial cells, resulting in activation of the Akt-pathway. Akt inhibition partly restored TGF-β-mediated loss of endothelial markers during EndMT. In vivo, pressure overload of the left ventricle led to increased expression of miR-21 in sorted cardiac endothelial cells, which displayed molecular and phenotypic signs of EndMT. This was attenuated by treatment of mice subjected to left ventricular pressure overload with an antagonir against miR-21.

Conclusion—TGF-β-mediated EndMT is regulated at least in part by miR-21 via the phosphatase and tensin homolog/Akt pathway. In vivo, antifibrotic effects of miR-21 antagonism are partly mediated by blocking EndMT under stress conditions. (Arterioscler Thromb Vasc Biol. 2012;32:361-369.)

Key Words: Akt signaling ■ endothelial mesenchymal transition ■ fibrosis ■ microRNA

MicroRNAs (miRNAs) are small noncoding RNAs that bind to mRNA targets, resulting in repression of target expression by translational inhibition or degradation of target mRNAs. Aberrant expression of selected miRNAs has been linked with various pathological conditions, such as cardiac remodeling.1 Indeed, miRNAs play an essential role during fibrosis of several organs, including heart,2 lung,3 and liver.4 Epithelial-to-mesenchymal transition (EMT) is an essential process during development, in which epithelial cells lose their epithelial markers and start to express fibroblast markers, such as fibroblast-specific protein-1 (FSP1). The process of EMT also plays an important role in various pathological conditions, such as cancer metastasis5 and organ fibrosis.6 Similarly, endothelial-to-mesenchymal transition (EndMT) contributes to fibroblast formation in fibrotic diseases of the heart,7,8 kidney,9–11 lung,12 liver,13 and carcinoma-associated fibrosis.14 Transforming growth factor-β (TGF-β) is the most potent inducer of EndMT and is the primary cytokine driving fibrosis in various organs.15–17 MicroRNA (miR)-21 is highly expressed in fibroblasts,2,18 it is rapidly inducible by TGF-β,19 and its expression can be inhibited by attenuation of TGF-β signaling.20 Phosphatase and tensin homolog (PTEN), which is a well-established target of miR-21,21,22 has been described as a negative regulator of EMT,23,24 as well as Akt activation, which is involved in both EMT25–33 and EndMT.8,34,35 Therefore, we here hypothesized that miR-21 plays an important role in the process of EndMT via targeting the PTEN/Akt pathway.

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Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were maintained in EBM-2 medium supplemented with growth factors and were treated with TGF-β II (R&D Biosystems) at a final concentration of 10 ng/mL. Culture media containing TGF-β or placebo were replaced every 24 hours. For miRNA experiments, cells were transfected with control, pre-miR-21 or anti-miR-21 (precursor or inhibitory molecules of miR-21; Applied Biosystems) at a 100

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nmol/L concentration using Lipofectamine per the manufacturer’s instructions. For Akt inhibition, cells were treated with Akt inhibitor IV (Calbiochem) at a 5 nmol/L final concentration. RNA and protein were isolated at the time points mentioned in Results.

Protein Expression
Cells were lysed with nondenaturing lysis buffer (Cell Signaling Technology) and separated on a 12% polyacrylamide gel, electroblotted on polyvinylidene fluoride membrane. After the transfer, membranes were blocked and incubated with primary antibodies and then with a horseradish peroxidase–labeled secondary antibody (Cell Signaling Technology) followed by enhanced chemiluminescence–based detection (GE Healthcare). Antibodies for endothelial nitric oxide synthase (eNOS), FSP1, GAPDH, platelet endothelial cell adhesion molecule 1 (PECAM1), PTEN, and vascular endothelial (VE)-cadherin were purchased from Abcam, and phospho-AKT (Ser 473) and total AKT were from Cell Signaling Technology.

Immunofluorescence Microscopy
Expression of VE-cadherin and FSP1 were detected by immunofluorescence microscopy. Briefly, cells were grown on coverslip and treated as desired. At the end of treatment, cells were fixed in 4% paraformaldehyde at room temperature followed by washing 3 times with PBS. Cells were then permeabilized with 0.1% Triton X-100 (in PBS) for 10 minutes at 4°C, washed 3 times with PBS, and blocked with 5% donkey serum (in PBS) for 30 minutes at room temperature followed by incubation with primary antibodies for 2 hours at room temperature. At the end of the incubation, cells were washed 3 times, incubated with fluorescently labeled secondary antibody (1:500) solution containing 4’,6-diamidino-2-phenylindole (5 μL/mL), and then observed under the microscope. For in vivo studies, expression of the fibroblast marker FSP1 by endothelial cells after transverse aortic constriction (TAC) operation was studied by confocal microscopy (Leica Microsystems Heidelberg GmbH) after sections were stained with lectin–fluorescein isothiocyanate (FL-1171, Vector Laboratories Inc) and anti-FSP1. Briefly, 5 μm micro thick paraffin sections were prepared from paraffin blocks, and after deparaffinization, antigen retrieval was performed using Antigen Retrieval Reagent-Basic (R&D Systems). Slides were then blocked with 5% donkey serum for 30 minutes and incubated overnight with primary anti-FSP1 antibody at 4 degrees. Slides were then washed and incubated with Alexa Fluor 594–conjugated secondary antibody, fluorescein isothiocyanate–conjugated lectin, and 4’,6-diamidino-2-phenylindole for 30 minutes, which was followed by washing and mounting. Slides were then examined under a confocal microscope (Leica Microsystems Heidelberg).

Real-Time Polymerase Chain Reaction
RNA was isolated from cells using Trizol reagent per the manufacturer’s instructions. Analysis of primary miR-21 and mature miR-21 expression was carried out by TaqMan assays (Applied Biosystems) specific for primary and mature forms of miR-21 as per the manufacturer’s instructions. Messenger RNA expression was analyzed by SYBR Green method using the primers described in Supplemental Table I, available online at http://atvb.ahajournals.org.

Zymography and ELISAs
Activities of matrix metalloproteinase (MMP) 2 and MMP9 were detected by zymography as described earlier. Briefly, cell culture supernatants were separated in a 10% polyacrylamide gel containing 1 mg/mL gelatin. To allow protein refolding, the gel was incubated in 2.5% Triton X-100 for 1 hour on a shaker. At the end of the incubation period, the gel was kept in enzyme buffer (50 mmol/L Tris, pH 7.5; 200 mmol/L NaCl; 5 mmol/L CaCl2·2H2O; 0.02% Brij-35) overnight at 37°C. Next day the gel was stained with Coomassie Blue solution (0.5% Coomassie Blue G-250, 30% methanol, 10% acetic acid) for 3 hours. Digestion patterns were visualized by destaining procedure (30% methanol, 10% acetic acid). MMP9 secretion was also detected by ELISA (R&D Systems) as per the manufacturer’s instructions.

Animal Experiments
All in vivo experiments were performed according to institutional ethical committee guidelines. Fibrotic response was initiated in a mouse model of cardiac pressure overload due to TAC as previously described. C57/BL6 male mice at 8 to 10 weeks of age were used for this purpose. Sham-operated animals served as controls. Hearts were explanted 5 weeks after TAC and cardiac cells were dissociated using a GentleMACS dissociator (Miltenyi Biotec) as described by the manufacturer. Endothelial cells were isolated by positive selection by magnetic affinity cell sorting (Miltenyi Biotec) using a CD146 antibody and were then used for RNA isolation and subsequent detection of miRNA and gene expression. For studying coexpression of fibroblast markers by endothelial cells, we used cardiac sections from a previous study, in which we performed TAC operation and concomitant placebo or antagomiR-21 treatment. Briefly, for antagomir injection, a jugular vein catheter was inserted before TAC operation, and antagomir-21 (80 mg per kg body weight, provided by Regulus Therapeutics, San Diego, CA) or PBS was injected daily for 3 consecutive days through the catheter. We then performed sectioning of hearts from the different groups (sham, TAC plus placebo, TAC plus antagomir-21; each 3 weeks after TAC or sham) and stained cardiac endothelial and fibroblast cells (see Immunofluorescence Microscopy section).

Statistical Analysis
Statistical analyses were performed by ANOVA followed by the Bonferroni multiple comparison test (for comparison of more than 2 groups) or Student t test (for comparison of 2 groups) (GraphPad Prism). A probability value of <0.05 was considered significant.
TGF-β Upregulates miR-21 in Endothelial Cells, Leading to Enhanced EndMT

Cardiac fibroblasts express high levels of miR-21. We thus first compared expression of miR-21 in human cardiac fibroblasts with different endothelial cell lines, and indeed found miR-21 to be expressed in relatively lower levels in endothelial cells compared with cardiac fibroblasts (Figure 1A). To test whether TGF-β would induce endothelial miR-21 expression in endothelium, HUVECs were treated with TGF-β for different time points, and expression of primary and mature miR-21 was determined. Both primary and mature miR-21 levels increased significantly in endothelial cells after TGF-β treatment (Figure 1B). TGF-β treatment of endothelial cells led to morphological changes resulting in a fibroblast-like spindle-shaped form (Figure 1C). TGF-β mediated morphological changes were not only confined to HUVECs but also observed in murine microvascular endothelial cells, as shown in Supplemental Figure I. Additionally, TGF-β treatment of HUVECs led to a decrease in endothelial marker (VE-cadherin) but an increase in fibroblast marker (FSP1) (Figure 2).

To test whether miR-21 would mimic TGF-β mediated effects, we first performed miR-21 gain-of-function studies in endothelial cells. miR-21 overexpression reduced expression of endothelial markers, such as VE-cadherin, whereas miR-21 blockade partly rescued TGF-β-induced endothelial marker suppression (Figure 2). This indicates that miR-21 is involved in TGF-β induced loss of VE-cadherin expression in endothelial cells. In addition, an increase in the fibroblast marker FSP1 was detected in endothelial cells treated with TGF-β or after miR-21

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Transforming growth factor-β (TGF-β) suppresses vascular endothelial (VE)-cadherin (VE-CDH) and induces fibroblast-specific protein-1 (FSP1) expression via microRNA (miR)-21. Endothelial cells were grown on cover glass and treated with TGF-β (10 ng/mL) for 3 days and stained for VE-cadherin and FSP1. Effects of TGF-β treatment and miR-21 overexpression on expression of VE-cadherin and FSP1 are shown. DAPI indicates 4',6-diamidino-2-phenylindole; Scr, Scrambled microRNA.

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overexpression (Figure 2). The effect of TGF-β-induced EndMT on other endothelial markers, such as PECAM1 and eNOS, was subsequently tested. As shown in Figure 3, both PECAM1 and eNOS expression was downregulated in endothelial cells both after TGF-β treatment and miR-21 overexpression (Figure 3A and 3B). Inhibition of miR-21 by anti-miR-21 transfection partly restored TGF-β-induced loss of PECAM1, whereas it had no significant effect on eNOS expression (Figure 3).

miR-21 Plays an Important Role in TGF-β-Induced MMP Expression and Activity

TGF-β upregulates MMP2 and MMP9, both of which play important roles during EMT. Here, we show that TGF-β treatment as well as miR-21 overexpression upregulates MMP2 and MMP9 mRNA expression in endothelial cells (Figure 4A and 4B). TGF-β-induced MMP expression was significantly attenuated when miR-21 was inhibited, suggesting that miR-21 plays an important role in TGF-β induced MMP2 or MMP9 expression. Similarly, TGF-β treatment resulted in enhanced MMP2 and MMP9 activity in HUVEC culture supernatants based on zymography assays, which also could be attenuated by miR-21 inhibition (Figure 4C). Effects of TGF-β treatment and miR-21 overexpression on MMP9 secretion was also confirmed by ELISA measurements (Supplemental Figure II).

AKT Signaling Is Involved TGF-β-Induced EndMT

We then determined miR-21 targets that may be involved in EndMT. PTEN is a negative regulator of Akt and a validated miR-21 target. Therefore, we first tested expression of PTEN in endothelial cells after TGF-β treatment (which leads to an upregulation of miR-21). Indeed, PTEN expression was suppressed by TGF-β in endothelium, but its expression was partly restored by
concomitant miR-21 inhibition (Figure 5A). Similar results were observed in endothelial cells of murine origin (Supplemental Figure III). Akt activation plays dominant roles in both EMT\textsuperscript{25–33} and EndMT\textsuperscript{8,34,35} processes. We thus examined alterations in the expression of endothelial markers after TGF-\(\beta\) treatment alone or during pharmacological Akt inhibition. Consistent with changes in mRNA expression (Figure 3), TGF-\(\beta\) treatment of endothelial cells downregulated expression of PECAM1 and eNOS protein levels. These effects were partly prevented by concomitant Akt inhibition (Figure 5B). Similarly, miR-21 overexpression resulted in a reduction of PECAM1 and eNOS, which could be restored by Akt inhibition (Figure 5B). This identifies TGF-\(\beta\)-stimulated miR-21 to be involved in EndMT via targeting the endothelial PTEN/Akt pathway.

**miR-21 Is Upregulated in Cardiac Endothelial Cells During Pressure Overload–Induced Cardiac Fibrosis**

As EndMT plays an important role in fibrosis development,\textsuperscript{7, 9–12,14} we then tested expression of miR-21 and various endothelial markers in magnetic affinity cell–sorted endothelial cells during the fibrotic response initiated by left ventricular pressure overload due to transaortic constriction (TAC) surgery of mice. TAC-induced cardiac hypertrophic growth was confirmed by increased heart weight/body weight ratios and increased fibrosis (Supplemental Figure IVA and IVB). RNA was isolated from magnetic affinity-sorted endothelial cells of hearts from TAC or sham-operated animals 5 weeks postsurgery. Expression of miR-21 and various endothelial markers was analyzed after confirming that the endothelial cell preparation was free from contaminating cardiomyocytes...
Endothelial microRNA (miR)-21 is upregulated during pressure-overload induced cardiac remodeling and is partly involved in endothelial-to-mesenchymal transition (EndMT). Cardiac remodeling was initiated in mice by pressure overload due to transaortic constriction (TAC).

A, Cardiac endothelial cells were isolated 5 weeks after TAC, and alterations in microRNA/gene expression were studied by real-time polymerase chain reaction. B, In endothelial cells from TAC-operated mice, expression of the endothelial markers endothelial nitric oxide synthase (eNOS), PECAM1, VE-cadherin (VE-CDH), and GATA-2 was studied. C, Expression of a fibroblast marker FSP1 and collagen 1 in endothelial cells derived from TAC animals or sham controls. D, Coexpression of FSP1 in lectin-stained endothelial cells was studied in cardiac sections after TAC or sham-operated animals. TAC-operated mice received placebo or antagonir-21 treatment (as described in Methods). White arrows indicate cells costained with fluorescein isothiocyanate–lectin and fibroblast-specific FSP1 based on confocal microscopy analyses. Data represents mean±SEM of 3 to 5 animals from each group. *P<0.05, **P<0.001, §§P=0.07.
by examining levels of respective markers (Supplemental Figure V). miR-21 was strongly upregulated in sorted endothelial cells (Figure 6A) from pressure-overloaded hearts. However, expression of miR-29b, which has been previously shown to be preferentially expressed in fibroblasts and to be downregulated in total cardiac tissue in infarcted regions,41 was not altered in sorted endothelial cells after TAC. Similarly, miR-216a, which plays important role in diabetic nephropathy,32 was also found to be unaltered in fractionated endothelial cells isolated from pressure-overloaded hearts, suggesting no direct role for those miRNAs for in vivo EndMT processes (Supplemental Figure VI). However, consistent with previous reports, expression of these 2 miRNAs was altered after TGF-β treatment in vitro (Supplemental Figure VI).42.43 Expression of endothelial markers, such as eNOS, PECAM1, VE-cadherin, and GATA-2 (Figure 6B), was decreased, whereas that of CD146 was unchanged (data not shown) in cardiac endothelial cells from TAC operated animals. Under similar conditions, expression of collagen I was significantly increased, whereas fibroblast marker FSP1 showed a trend (P=0.07) in sorted endothelial cells (Figure 6C). As described earlier, a fraction of endothelial cells coexpress fibroblast markers, such as FSP1.7 Indeed, based on confocal microscopy analyses, an increased fraction of endothelial cells in TAC operated animals coexpressed the fibroblast marker FSP1 compared with sham-operated animals. Importantly, this could be strongly inhibited by antagonomiR-21 treatment of mice (Figure 6D). We thus provide further evidence that miR-21 is directly involved, at least in part, in EndMT processes leading to enhanced cardiac fibrosis in a relevant animal model of left ventricular pressure overload.

Discussion

MicroRNAs have been shown to be involved in EMT,44 but their potential role in EndMT is currently unknown. Results of the present study indicate that miR-21 plays an important role in TGF-β-induced EndMT via a PTEN/Akt-dependent pathway. We found TGF-β to increase endothelial expression of both primary and mature miR-21. The miR-21 promoter includes a consensus sequences for AP1.45 AP1 can be activated by TGF-β,46 which could explain the increase in primary miR-21 expression after TGF-β treatment. However, the increase in mature miR-21 expression was much higher compared with the primary transcript, which could be due to enhanced Smad-mediated processing of primary miR-21 to mature miR-21 following TGF-β treatment as described earlier.19

Akt plays a crucial role in endothelial homeostasis and eNOS activation.47 However, prolonged Akt activation results in reduced vascular endothelial growth factor and Ang-2 expression,48 impaired coronary angiogenesis and fatal vascular malformations.49 Endothelial specific deletion of PTEN resulted in embryonic lethality due to cardiovascular defects,50 suggesting fine-tuning of Akt signaling to be very important. Indeed, Akt activation plays an important role in both EMT29,31 and EndMT.8,34,35 Expression of PTEN, which is one of the best validated targets of miR-21,21,51 decreases after treatment with TGF-β.52 Loss of PTEN expression has been linked with Akt activation53 and EMT.23 Consistent with this, TGF-β treatment has resulted in loss of PTEN expression, which could be restored partly by miR-21 inhibition. In line with the negative regulatory effect of PTEN on Akt activation, miR-21 overexpression resulted in PTEN repression and Akt activation in endothelial cells.

MMP2 and MMP9 are known to mediate EMT during developmental and pathological processes.39,40 Although direct evidence for an involvement of MMPs in EndMT has not yet reported, several parallelisms to their established role in EMT exist in EndMT. For example, elevated levels of MMP2 and MMP9 were observed in myocardium of plasminogen activator inhibitor-1–deficient mice, whose endothelial cells are more susceptible to EndMT,54 and VE-cadherin was found to be degraded by MMP2 and MMP9.55 Therefore, our observation that miR-21 is involved in endothelial MMP activation is in line with such previous hypotheses. Reck56–58 and Timp39 are endogenous inhibitors of MMP2 and MMP9. At the transcriptional level, MMPs are induced by transcriptional factors, including AP1.60,61 which is inhibited by PDCD4.62 All these negative regulators of MMPs (Reck, Timp3, and PDCD4) are validated targets of miR-21,63,64 suggesting that alterations in miR-21 expression would have a profound influence on MMP2 and MMP9 activities. miR-21 inhibition has been shown to suppress MMP activity in vitro and in vivo.63 Consistent with this, both TGF-β treatment and miR-21 overexpression resulted in enhancement of MMP2 and MMP9 expression and activity. These results suggest that MMPs are upregulated and activated during TGF-β-induced EndMT. However, it should be noted that so far no causal relationship between MMP activation and EndMT has been established in the presentstudy. Snail, which is a pivotal transcriptional activator/repressor inducing EMT or EndMT, can upregulate MMP2 and MMP9 expression.65,66 As TGF-β and miR-21 treatment of endothelial cells induced Akt activation (Figure 5B), which is known to stabilize Snail by Gsk3 phosphorylation,67 it is tempting to speculate that alterations in MMP expression are, at least in part, mediated by Snail. Furthermore, Akt-mediated Snail stabilization has already been noted during EndMT.8,35

In this study, we found that Akt activation following TGF-β treatment or miR-21 overexpression resulted in loss of endothelial markers resembling an EndMT process. TGF-β or miR-21 efficiently suppressed expression of endothelial markers, such as VE-cadherin, PECAM1, and eNOS, and upregulated expression of the fibroblast markers. Furthermore, both TGF-β and miR-21 increased MMP2 and MMP9 transcription and activity, and the effects of TGF-β could be effectively blocked by miR-21 inhibition. This suggests that miR-21 plays an important role in TGF-β-mediated EndMT. However, it is pertinent to note that the efficiency of anti-miR-21 in restoring the endothelial markers downregulated by TGF-β was not uniform, and some endothelial markers, such as the von Willebrand factor, were unchanged after treatment with TGF-β or miR-21 (data not shown), suggesting that involvement of other factors may be essential to fully complete the EndMT process.

miR-21 is upregulated in cardiac fibroblasts after cardiac stress and contributes to fibrosis development.2 miR-21...
inhibition led to inhibition of fibrosis in heart, \(^2\) kidney, \(^6\) and lung, \(^3\) suggesting a general effect on fibrosis and thus therapeutic potential in many chronic diseases. Here, we showed that miR-21 is additionally upregulated in endothelial cells isolated from fibrotic hearts; this upregulation is accompanied by a decrease in endothelial and increase in fibroblast markers. Thus, the results of the present study indicate that miR-21 plays an important role in EndMT both in vitro and in vivo (see Supplemental Figure VII for a mechanistic scheme).

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**Disclosures**

Dr Thum has filed and licensed patents on cardiovascular microRNAs.

**References**

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TGF-beta-induced endothelial-to-mesenchymal transition is partly mediated by microRNA-21

Regalla Kumarswamy, Ingo Volkmann, Virginija Jazbutyte, Seema Dangwal, Da-Hee Park and Thomas Thum

Supplemental Figures I-VII

Supplemental Table I
Supplemental Figure I. TGF-beta (10ng/ml) induced morphological alterations in murine microvascular endothelial cells.
Supplemental Figure II. TGF-beta and miR-21 enhance MMP9 secretion. Data represents mean ± SEM of 3 experiments. **p<0.001.
Supplemental Figure III. TGF-beta suppresses expression of PTEN and PECAM1, which could be restored by inhibition of miR-21 in mice microvascular endothelial cells.
Supplemental Figure IV. A) Heart weight / body weight ratio in mice after TAC or Sham operation. B) Picosirius red staining of sham and TAC-operated animals. Data represents mean ± SEM of 5 animals from each group. **p<0.001
Supplemental Figure V Detection of cardiomyocyte (alpha-MHC, beta-MHC) and endothelial markers (eNOS, VE-cadherin) in MACS-sorted endothelial cells isolated from murine hearts.
Supplemental Figure VI

Expression of miR-29b and miR-216a in endothelial cells after TGF-beta treatment (A, B) or in sorted cardiac endothelial cells from mice after Sham or TAC-operation (C, D). n=3-5 experiments/animals per group. *, P<0.05; **, P<0.001.
Supplemental Figure VII. Hypothetic scheme: TGF-beta up-regulates miR-21 which led to suppression of PTEN (endogenous Akt inhibitor) and subsequent activation of Akt. Sustained activation of Akt has been shown to induce EMT/EndMT processes. MiR-21 over-expression is also known to suppress PDCD4 (programmed cell death-4, negative regulator of AP1), TIMP3 (tissue inhibitor of metalloproteinases-3) and RECK (Reversion-inducing-cysteine-rich protein with kazal motifs), that are known to suppress MMP2/9 secretion or activity through various mechanisms, leading to elevated MMP2/9 levels finally eventually resulting in EMT/EndMT. Our data together with existing literature suggests that miR-21 is partly responsible for TGF-beta induced EndMT processes.
## Supplemental Table I

### Primer sequences

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