Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62198R1
Full Title:	TGF-β-mediated endothelial to mesenchymal transition (EndMT) and the functional assessment of EndMT effectors using CRISPR/Cas9 gene editing
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Leiden, the Netherlands
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TITLE

TGF-β-mediated endothelial to mesenchymal transition (EndMT) and the functional assessment of EndMT effectors using CRISPR/Cas9 gene editing

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KEYWORDS

CRISPR/Cas9, knock-out, lentivirus, Immunofluorescence, MS-1, Snail, Slug

SUMMARY

We describe methods to investigate TGF-β2-induced EndMT in endothelial cells by observing cell morphology changes and examining the expression EndMT-related marker changes using immunofluorescence staining. CRISPR/Cas9 gene editing was described and used to deplete the gene encoding Snail to investigate its role in TGF-β2-induced EndMT.

ABSTRACT

In response to specific external cues and the activation of certain transcription factors, endothelial cells can differentiate into a mesenchymal-like phenotype, a process that is termed endothelial to mesenchymal transition (EndMT). Emerging results have suggested that EndMT is causally linked to multiple human diseases, such as fibrosis and cancer. In addition, endothelial-derived mesenchymal cells may be applied in tissue regeneration procedures, as they can be further differentiated into various cell types (e.g., osteoblasts and chondrocytes). Thus, the selective manipulation of EndMT may have clinical potential. Like epithelial-mesenchymal transition (EMT), EndMT can be strongly induced by the secreted cytokine transforming growth factor-beta (TGF-β), which stimulates the expression of so-called EndMT transcription factors (EndMT-TFs), including Snail and Slug. These EndMT-TFs then up- and downregulate the levels of mesenchymal and endothelial proteins, respectively. Here, we describe methods to investigate TGF-β-induced EndMT in vitro, including a protocol to study the role of particular TFs in TGF-β-induced EndMT. Using these techniques, we provide evidence that TGF-β2 stimulates EndMT in murine pancreatic microvascular endothelial cells (MS-1 cells), and that the genetic deletion of *Snail* using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated gene editing, abrogates this phenomenon. This approach may serve as a model to interrogate potential modulators of endothelial biology, and can be used to perform genetic or pharmacological screens in order to identify novel regulators of EndMT, with potential application in human disease.

INTRODUCTION

Endothelial to mesenchymal transition (EndMT) is a multistep and dynamic biological phenomenon that has been linked to diverse physiological and pathological processes[1,2]. Upon EndMT endothelial cells gradually lose their endothelial traits, while acquiring mesenchymal properties[3]; thus, tightly compacted and well organized endothelial cells

differentiate into elongated mesenchymal-like cells. Morphological changes in EndMT coincide with alterations in the expression of certain genes and proteins. In general, the expression of proteins that maintain endothelial characteristics, including vascular endothelial (VE)-cadherin, platelet/EC adhesion molecule-1 (CD31/PECAM-1) declines. Simultaneously, proteins related to mesenchymal functions, such as α-smooth muscle actin (α-SMA) and smooth muscle protein 22α (SM22α) accumulate. Emerging results have demonstrated that postnatal EndMT contributes to the development of human diseases, such as cancer, cardiac fibrosis, pulmonary arterial hypertension (PAH), atherosclerosis (AS), organ fibrosis, etc[2,4-7]. A deeper understanding of the underlying mechanisms of EndMT and how to direct the EndMT process will provide novel therapeutic methods for EndMT-related diseases and regenerative medicine.

TGF-β is one of the main EndMT inducers, and other known involved factors include Wnt/β-catenin, Notch, and some inflammatory cytokines[1]. As the cellular context is key for responses triggered by TGF-β, the interplay of TGF-β with other EndMT promoting signals is relevant for TGF-β to elicit an EndMT response. Upon the activation of TGF-β cell surface type I and type II serine/threonine kinase receptors, the intracellular canonical Smad pathway is activated. TGF-β receptor-mediated phosphorylated Smad2/3 form heteromeric complexes with Smad4 that translocate into the nucleus, where they upregulate the expression of EndMT-related transcription factors. Similar to epithelial-mesenchymal transition (EMT), transcription factors such as Snail, Slug, TWIST, ZEB1 and ZEB2 are induced by TGF-β signaling and contribute to gene reprogramming in EndMT[8].

Snail has been frequently identified as a key factor in EndMT. Snail binds to the promoter of genes encoding cell-cell adhesion proteins and suppresses their transcription, which is counterbalanced by the enhancement of the expression of mesenchymal proteins[9]. Endothelial cells comprise a very heterogeneous population and the relative influence of diverse extracellular stimuli on EndMT may differ among endothelial cellular contexts or cell types[10]. Due to its similarities with EMT, some methodologies are useful to investigate both mechanisms EMT and EndMT[8]. In this regard, the EMT International Association (TEMTIA) strongly emphasizes the need of complementary techniques to ultimately demonstrate the occurrence of EMT/EndMT[11].

Here we describe a method to monitor and visualize the TGF-β-induced EndMT process. Immunofluorescence staining provides the basic information about expression changes in targeted proteins/markers which are used as indicators of whether the EndMT process occurs. Additionally, the immunofluorescence staining can visualize the localization of proteins/markers and cell morphology. To study the potential activity of specific TFs (or other upstream or downstream regulators) involved in TGF-β mediated EndMT, we describe a protocol using clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR-associated protein 9 (Cas9) gene editing to deplete specific genes from cells, using the TF *Snail* as an example. Cas9 is a dual RNA-guided DNA endonuclease that recognizes and cleaves sequences complementary to CRISPR sequences in bacteria[12]. The CRISPR/Cas9 system is currently extensively utilized because it facilitates genetic engineering in vitro and in vivo[13]. Directed by a single guide RNA (sgRNA), ectopically expressed Cas9 generates a double strand break at a preselected targeting sequence in a specific gene locus. Non-homologous end joining (NHEJ) takes place to repair Cas9-induced strand breaks, via random nucleotide

insertions or deletions thereby leading to the disruption and inactivation of the targeted gene. We describe in detail methods for designing selective sgRNAs and generating lentiviral-compatible vectors containing the designed sgRNAs. As a result, stable gene-depleted endothelial cells can be generated in an efficient and reliable manner.

In this study, we used murine pancreatic microvascular endothelial cells (MS-1)[14] as a model system to examine the TGF-β2-induced EndMT process. Our previous study demonstrated that Snail is the main transcription factor increased by TGF-β2, by which EndMT is induced in MS-1 cells[15]. Upon CRISPR/Cas9 gene editing to abrogate Snail expression in MS-1 cells, TGF-β2 failed to mediate EndMT. This workflow can be applied to study other (suspected) EndMT-related genes.

PROTOCOL

1. Induction of EndMT by TGF-β2

1.1. Culture murine pancreatic islet endothelial cells (MS-1) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin in an incubator (5% CO_2, 37 °C). Coat all culture dishes/plates with 0.1% w/v gelatin for 10 min before use.

1.2. Gently wash MS-1 cells with 1x phosphate buffered saline (PBS), add 2 mL of trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA) to a 10 cm dish, and incubate for 2 min at 37 °C to detach them. Subsequently, add 5 mL of complete culture medium to quench the reaction.

1.3. Transfer the cell suspension to a 15 mL tube and centrifuge at 200 x *g* for 3 min at room temperature.

1.4. Discard the supernatant and resuspend the cells in 4 mL of fresh medium containing FBS and penicillin/streptomycin. Count the cells using an automatic cell counter.

1.5. Seed 1 x 10^3 cells per cm^2 for further culture. For example, seed 9.5 x 10^3 cells/well for 6-well plates, or 1.9 x 10^3 cells/well for 24-well plates.

1.6. Incubate the cells overnight to allow them to adhere and recover, then stimulate MS-1 cells with TGF-β2 for 3 days. Add the TGF-β receptor kinase inhibitor SB431542 (5 µM) 30 min before TGF-β2 stimulation. Treat other cells with vehicle (DMSO).

NOTE: Dissolve TGF-β2 in 4 mM HCl containing 0.1% human bovine serum albumin (BSA). Add the same amount of ligand buffer without TGF-β2 to the control group. The TGF-β2 concentration may be adapted to be 0.1-1 ng/mL for specific assays. See indications in the corresponding figures.

1.7. After 3 days, examine the cell morphology with bright field imaging (with an inverted microscope) and perform immunofluorescence staining (see step 2) to assess EndMT-related marker changes. Perform at least three independent experiments to obtain biological triplicates.

2. Immunofluorescence staining

2.1. Trypsinize cultured MS-1 cells (step 1.2) and then reseed 1.9 x 10^3 cells on a 0.1% w/v gelatin-coated 12 mm round cover glass placed on the bottom of a 24-well plate.

2.2. After culturing the cells overnight, add TGF-β2 (final concentration 1 ng/mL) to the cells for 3 days. Use medium containing ligand buffer as a negative control.

2.3. Perform PECAM-1 and SM22α staining.

2.3.1. After stimulating the cells with TGF-β2 (or Control) for 3 days, remove the medium, and wash the cells with 1x PBS.

2.3.2. Add 300 µL of 4% formaldehyde to each well and incubate for 10 min at room temperature to fix the cells. Wash 3x with 1x PBS after incubation.

2.3.3. Add 300 µL of 0.1% Triton X-100 in 1x PBS to each well and incubate for 10 min at room temperature to permeabilize the cells. After incubation, remove the Triton X-100 solution and wash the cells 3x with 1x PBS.

2.3.4. Block the cells with 3% bovine serum albumin (BSA) in 1x PBS for 45 min at room temperature.

2.3.5. Dilute the primary PECAM-1 and SM22α antibodies that recognize murine proteins 1:500 with 1x PBS. Then incubate the fixed cells with primary antibodies for 45 min at room temperature.

2.3.6. After washing 3x with 1x PBS, incubate the cells with 1000x diluted secondary antibodies, including donkey anti-rat Alexa 488 and goat anti-rabbit Alexa 594, for 45 min at room temperature.

NOTE: Protect the samples from light during staining.

2.3.7. After rinsing 3x with 1x PBS, place the cover glass seeded with cells face down on a drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) on a slide to stain the nuclei.

2.3.8. Fix the periphery of the cover glass with transparent nail polish and store it at 4 °C.

2.3.9. Acquire representative images with a confocal microscope. Set the laser wavelengths at 405 nm, 488 nm and 552 nm to detect DAPI, PECAM-1 and SM22α, respectively. For each channel, all the pictures were taken with the same settings and exposure time. Perform at least three independent experiments to obtain biological triplicates.

3. Knock out of Snail using CRISPR/Cas9 editing

3.1. Design two independent sgRNAs targeting murine *Snail.*

3.1.1. Design sgRNAs using the online tools CHOPCHOP (https://chopchop.cbu.uib.no/) and Cas-OFFinder (http://www.rgenome.net/cas-offinder/) according to the targeted gene name and species.

3.1.2. Predict the off-target activity of the designed sgRNAs targeting to Snail with two independent algorithms, including Cas-OFFinder (http://www.rgenome.net/cas-offinder/) and CHOPCHOP (http://chopchop.cbu.uib.no/).

3.1.3. Choose two sgRNAs with the lowest off-activity. Design two complementary sgRNA oligo DNAs with the *Bve*I cut site. The sense oligo starts with 5'-ACCG-3' and the antisense oligo starts with 5'-AAAC-3'.

3.1.4. Order the oligos to be commercially synthesized for further use.

3.2. Clone the complementary guide RNA oligo DNAs into the *Bve*I-digested AA19 pLKO.1-puro.U6.sgRNA.*Bve*I-stuffer Lentiviral vector plasmid to generate AA19 pLKO.1-Snail-sgRNA[16].

3.2.1. Cut the AA19 pLKO.1-puro.U6.sgRNA.*Bve*I-stuffer plasmid with the *Bve*I enzyme[16]. Mix 2 µg of AA19 pLKO.1-puro.U6.sgRNA.*Bve*I-stuffer plasmid, 5 µL of 10x Buffer O, and 5 µL of *Bve*I enzyme and add sterile water to reach a total volume of 50 µL.

3.2.2. Vortex and briefly spin down the reaction mix. Incubate the reaction at 37 °C for 1 h.

3.2.3. Load the reaction mix on a 1% agarose gel and run in 1x Tris-acetate-EDTA (TAE, 50x TAE stock: 242 g of Tris base dissolved in water, 57.1 mL of glacial acetic acid, 100 mL of 500 mM EDTA (pH 8.0) solution, and add water to a total of 1 L) buffer until a good separation is achieved.

3.2.4. Cut the backbone fragment from the gel, isolate it with a gel extraction kit according to the manufacturer's protocol (see the **Table of Materials**) and elute the backbone in 40 µL elution buffer (EB).

3.2.5. Mix 5 µL of 100 pmol/µL sense oligo and 5 µL of 100 pmol/µL antisense oligo with 1 µL of 1 M Tris-HCl (pH 8.0) and add sterile water to reach a total of 100 µL to anneal the gRNA oligos. Incubate the mixture for 5 min at 100 °C, and then cover the tubes with aluminum foil. After that, slowly cool the solution to room temperature for further use as an insert.

3.2.6. To ligate the complementary gRNA oligo DNAs and the *Bve*I-digested backbone, mix 1 µL of isolated *Bve*I cut AA19 pLKO.1-puro.U6.sgRNA.BveI-stuffer backbone and 2 µL of insert (diluted 1:300) with 2 µL of 10x T4 DNA ligase buffer and 1 µL of T4 DNA ligase, and add sterile water to reach a total of 20 µL. Briefly spin the tube, and incubate it for 4 h at room temperature for further use.

NOTE: When performing the ligation, ensure two control groups are included. For one control group, mix 1 µL of isolated backbone, 2 µL of 10x T4 DNA ligase buffer, and 1 µL of T4 DNA

ligase and add sterile water to reach a total of 20 µL, but without oligo DNA. For the other control group, mix 1 µL of isolated backbone and 2 µL of 10x T4 DNA ligase buffer, and add sterile water to reach a total of 20 µL, but without the oligo DNA and T4 ligase. These two control ligations are used to determine the reaction background in the next transformation step and indicate of how efficient the ligation is.

3.3. Transform the reaction mixture into competent TOP10 *E. coli*.

3.3.1. Take the competent TOP10 *E. coli*. cells from the -80 °C freezer, and thaw them on ice.

3.3.2. Add 2 µL of ligation mixture to 50 µL of competent cells and keep the tube on ice for 30 min.

3.3.3. Heat-shock the tube at 42 °C for 30 s. Put the tube on ice for 2 min.

3.3.4. Add 950 µL of fresh lysogeny broth (LB) medium to the mixture and shake vigorously at 37 °C for 60 min.

3.3.5. Spin down and plate the cells on a warm ampicillin (100 µg/mL) resistance LB plate. Incubate the plate at 37 °C overnight.

3.4. Verify the successful insertion of the gRNA oligo DNA in the plasmid.

3.4.1. Pick 3-5 colonies on the plate in 1 mL of ampicillin (100 µg/mL) containing LB medium and shake overnight at 30 °C.

3.4.2. Isolate the plasmid DNA with a plasmid kit according to the manufacturer's protocol (**Table of Materials**) and sequence it with the U6 promoter primer 5'-GAGGGCCTATTTCCCATGATT -3' to verify the successful insertion of the gRNA oligo.

4. Generate Snail knockout MS-1 cells

4.1. Produce lentiviral particles carrying Cas9 or Snail-targeting gRNAs.

4.1.1 Culture HEK 293T cells in advanced Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin in 14.5 cm dishes (or T75 flasks) in an incubator (5% CO_2, 37 °C).

4.1.2. Mix 9.9 µg of targeting gene plasmid, AA19 pLKO.1-Snail-sgRNA or pLV-Cas9[17] plasmid, together with the helper plasmids 3.5 µg of pCMV-VSVG (encoding the G protein of the vesicular stomatitis virus, VSV-G), 6.6 µg of rev-responsive element plasmid pMDLg-RRE (encoding Gag and Pol), and 5.0 µg of pRSV-REV (encoding Rev) in 500 µL of serum free medium. Resuspend 50 µL of polyethylenimine (PEI) (2.5 mg/mL) in 500 µL of serum free medium. Gently mix plasmids and PEI preparations by pipetting up and down. Incubate the mixture for 20 min at room temperature.

4.1.3. Transfect HEK 293T cells by adding the mixture medium from step 4.1.2 to 80% confluent cells in 14.5 cm dishes (or T75 flasks) which contain DMEM medium with 10% FBS and 10% FBS and 100 U/mL penicillin/streptomycin. HEK293T cells are used because they are easily transfected and generate high levels of virus[18].

4.1.4. Transfer transfected HEK 293T cells to a biosafety in microbiological and biomedical laboratory (BMBL) to culture them for 24 h.

4.1.5. In a BMBL laboratory, replace the transfection medium from HEK 293T cells with 12 mL of fresh complete DMEM containing FBS and penicillin/streptomycin. Incubate the cells for 24 h.

4.1.6. Collect and filter the medium with a 20 mL syringe and a 0.45 μm filter. Transfer the conditioned medium into a 15 mL polypropylene tube.

4.1.7. Add 12 mL of fresh complete DMEM containing FBS and penicillin/streptomycin to the HEK 293T culture dish and culture for an additional 24 h.

4.1.8. Collect and filter the medium with a 20 mL syringe and a 0.45 μm filter. Transfer the conditioned medium into a 15 mL polypropylene tube. Store the medium containing lentiviral particles as 1 mL aliquots at -80 °C for further use.

4.2. Infect MS-1 cells with the pLV-Cas9 virus.

4.2.1. Seed 1 x 10^5 MS-1 cells per well in a 6-well plate for 24 h before lentivirus infection.

4.2.2. Thaw the frozen aliquots of pLV-Cas9 virus in a 37 °C water bath.

4.2.3. Mix 1 mL of virus medium with 1 mL of fresh DMEM medium containing FBS and penicillin/streptomycin. Add polybrene to the medium to reach the final concentration of 10 μg/mL.

4.2.4. Remove the medium from the 6-well plate and replace it with the virus/polybrene mix medium and culture the cells in an incubator for 24 h. 24 h post-infection, replace the medium with fresh medium and culture the cells for another 24 h.

NOTE: Always keep an uninfected well as a control group.

4.2.5. Aspirate the medium from the infected group and control group and replace it with DMEM medium with 4 μg/mL blasticidin.

4.2.6. Return the plate to a 37 °C incubator and culture the cells for 1 week. Uninfected cells will die due to the effect of blasticidin. Split the surviving cells when they reach 80% cell confluency and continue with blasticidin selection.

4.2.7. Confirm the expression of Cas9 in MS-1 cells by western blotting using an antibody against Cas9 (molecular weight of Cas9 is approximately 160 kDa).

4.3. Separately infect pLV-Cas9 MS-1 cells with two independent gRNA lentiviruses.

4.3.1. Seed 1 x 10^5 pLV-Cas9 MS-1 cells per well in a 6-well plate for 24 h before infection.

4.3.2. Follow the same protocol as described in 4.2 to separately infect cells with two gRNA lentiviruses.

4.3.3. After 24 h of infection with the gRNA virus, refresh the medium and culture the cells for another 24 h.

4.3.4. Replace the medium with DMEM with 1 μg/mL puromycin. Return the plate to a 37 °C incubator and culture the cells for 1 week. Ensure the uninfected cells are completely dead. Split the cells when they reach 80% confluency and continue puromycin selection.

4.3.5. Confirm the knock-out of Snail in MS-1 cells by western blotting using an antibody against Snail (molecular weight of Snail is approximately 35 kDa).

REPRESENTATIVE RESULTS

TGF-β2 induces EndMT and stimulates Snail expression in MS-1 endothelial cells

TGF-β is one of the cytokines with greatest potential to induce EndMT. After treating MS-1 cells with TGF-β2 (1 ng/mL) for 3 days, endothelial MS-1 cells lose their cobblestone-like structure and differentiate into spindle-shaped mesenchymal-like cells (**Figure 1A**)[15]. To further verify the role of TGF-β2 in inducing cell phenotypic changes, we pre-treated the cells with the small molecule activin receptor-like kinase (ALK)4/ALK5/ALK7 inhibitor SB431542 before TGF-β2 stimulation[19]. SB431542 completely abrogated TGF-β2-induced cell morphology changes (**Figure 1A**). The TGF-β2 induced EndMT process was further investigated by studying changes in the expression of EndMT-related markers. As shown in **Figure 1B**, the endothelial protein PECAM-1 was potently decreased after TGF-β2 stimulation, while the mesenchymal factor SM22α was profoundly upregulated by TGF-β2[15]. These data are consistent with the notion that TGF-β2 triggered EndMT in MS-1 cells. Next, we investigated the effects of TGF-β2 on Snail and Slug expression. As shown in **Figure 1C**, Snail was markedly upregulated by TGF-β2, while Slug expression was not influenced by TGF-β2 in MS-1 cells[15]. The quantification of Snail expression from three independent experiments is shown in **Figure 1D**.

Depletion of *Snail* by CRISPR/Cas9 in MS-1 endothelial cells

As Snail was induced by TGF-β2 and likely involved in TGF-β2-mediated EndMT, we performed CRISPR/Cas9 gene editing to genetically deplete Snail expression in MS-1 cells. We hypothesized that the depletion of Snail would be sufficient to inhibit TGF-β2-induced EndMT. As shown in **Figure 2A**, we generated Snail knockout cells in two steps. Firstly, Cas9 was ectopically expressed by infecting MS-1 cells with a Cas9 expressing lentivirus. Since there is a blasticidin resistance cassette in the pLV-Cas9 construct, we checked the expression of Cas9 by Western blot analysis in blasticidin resistant cells (**Figure 2D**). Subsequently, we introduced sgRNAs that specifically targeted *Snail* to disrupt its protein expression. This procedure was also performed by infection with lentiviral particles carrying the AA19 pLKO.1-Snail-sgRNA construct, which includes a puromycin expression cassette. Cas9-expressing cells were again

infected with gRNA containing lentivirus and further selected with puromycin. Two complementary sgRNA DNA oligos targeting murine *Snail* were designed with a predicted low off-target activity (**Figure 2 B**,**C**). After introducing two independent *Snail* sgRNAs in Cas9 expressing MS-1 cells, Snail protein expression was abrogated (**Figure 2D**)[15].

Deficiency of Snail inhibits the TGF-β2-induced EndMT of MS-1 cells

To demonstrate the function of Snail in TGF-β2-mediated EndMT, we performed an EndMT assay in Snail-depleted cells and compared it with parental MS-1 cells. As shown in **Figure 3A**, the knockout of Snail was sufficient to inhibit the fibroblast-like cell morphology driven by TGF-β2 in MS-1 cells[15]. In addition, the TGF-β2-mediated decline in PECAM-1 and enhancement of SM22α were completely blocked in Snail-depleted MS-1 cells. In summary, we demonstrated that Snail is critical for TGF-β2-mediated EndMT in MS-1 cells (**Figure 3B**)[15].

FIGURE LEGENDS

Figure 1. TGF-β2 induces EndMT and Snail expression in MS-1 cells. A. Effects of TGF-β2 and/or SB-431542 on cell morphology. Brightfield images of MS-1 cells upon treatment with TGF-β2 (1 ng/mL) and/or SB-431542 (SB, 5 μM, administered 30 min prior to TGF-β2) for 2 days. Scale bar represents 200 μm. **B.** Immunofluorescence staining of PECAM-1 (green) and SM22α (red) in MS-1 cells cultured in medium containing TGF-β2 (1 ng/mL) for 3 days. Nuclei are visualized in blue (DAPI). Scale bar: 50 μm. **C.** Western blot with whole cell lysate of TGF-β2 stimulated MS-1 cells. The expression of Snail, but not Slug, was enhanced by TGF-β2 stimulation, as previously reported in Ma et al[15]. **D.** Quantification of Snail expression from three independent western blot experiments.

Figure 2. Depletion of *Snail* by CRISPR-Cas9 gene editing. A. Scheme depicting how to generate *Snail* knockout cells. Bsd: Blasticidin. Puro: Puromycin. **B.** Oligonucleotides of two independent sgRNAs targeting *Snail* using CHOPCHOP (http://chopchop.cbu.uib.no/) and Cas-OFFinder (http://www.rgenome.net/cas-offinder/). **C.** The predicted off-target activity of the two gRNAs for *Snail* using Cas-OFFinder (http://www.rgenome.net/cas-offinder/). **D.** Cas9 and Snail expression in wild type (WT) and Cas9-overexpressed MS-1 measured by Western blot analysis. **E.** Knockout of *Snail* with two independent gRNAs in MS-1 cells as measured by Western blot analysis. We reported similar results in Ma et al[15].

Figure 3. Genetic depletion of Snail inhibits TGF-β2-induced EndMT in MS-1 cells. A. Brightfield images of MS-1 cells upon treatment with TGF-β2 (0.1 ng/mL) for 3 days in wildtype (WT, upper panel) and *Snail* knocked out (lower panel) cells. Scale bar represents 200 μm. **B.** Immunofluorescent staining for PECAM-1 (green), SM22α (red) and nuclei (blue) of MS-1 cells cultured in medium containing TGF-β2 (1 ng/mL) for 3 days. Depletion of *Snail* abrogated TGF-β2-induced decrease of PECAM-1 and increase of SM22α expression. Scale bar represents 50 μm. **C.** Schematic representation of the effect of *Snail* knockout on TGF-β-induced EndMT in MS-1 cells. TGF-β stimulates the expression of *Snail* through Smad pathway by phosphorylating Smad2/3 and further drives EndMT. Knocking out *Snail* using CRISPR/Cas9-based gene editing abrogated TGF-β-mediated EndMT.

DISCUSSION

Understanding the mechanism of EndMT is critical for modulating this process and targeting EndMT-related diseases. Here, we described methods to perform a TGF-β-induced EndMT

assay and interrogate the role of the EndMT-TF Snail in TGF-β-triggered EndMT, by performing CRISPR/Cas9-mediated stable gene depletion of Snail from cells. The depletion of Snail using CRISPR/Cas9 approach successfully abrogated TGF-β2 driven EndMT in MS-1 cells (**Figure 3C**). To study the effects of any cytokines, like TGF-β, on EndMT, ECs were exposed to cytokines and then the occurrence of EndMT was assessed according to morphological changes and endothelial and mesenchymal marker expression changes in cells. TGF-β2 strongly induced EndMT in MS-1 cells accompanied by a strong increase in the expression of the transcription factor Snail. The EndMT-TFs induced by TGF-β can differ according to the species or tissue-specific endothelial cell type. For example, we observed that Snail but not Slug was significantly upregulated by TGF-β in MS-1 cells, while in human umbilical vein endothelial cells (HUVECs), both SNAIL and SLUG are increased after exposure to TGF-β[20].

We assessed the extent of the EndMT process in two ways by examining cell morphology changes and then by investigating changes in EndMT-related markers expression. After TGF-β exposure for 3 days, cells underwent EndMT with consistent morphological variations and changes in the expression of EndMT-related markers. In addition to the immunofluorescence staining we performed here, marker variations can also be monitored by western blotting at the protein expression level or by qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) at the gene levels[21]. In addition to these two time- and cost-saving methods that we showed in this protocol, there are other methods to examine EndMT. For example, performing transcriptome analysis (by RNA sequencing or qPCR) to compare the expression levels of endothelial- and mesenchymal-related genes between treated and control cells can precisely assess EndMT[22,23]. In addition, EndMT often involves the stable loss of barrier function, which can be assessed by impedance spectroscopy[24]. Furthermore, additional proof of the acquisition of stem cell-like properties by EndMT-derived cells may be examined. For instance, under specific culture conditions, EndMT mesenchymal-like cells can be further differentiated into osteoblasts, chondrocytes, adipocytes or (myo)fibroblasts. Therefore, additional analysis to confirm the differentiation into different cell types belonging to the mesoderm lineage (i.e., gene expression and matrix staining) is useful for demonstrating the multipotent nature of EndMT-derived cells. Finally, EndMT assessment methods are not limited to in vitro studies, but can be extrapolated to investigate the relation between EndMT and some diseases in vivo or in ex vivo organs. In this sense, the use of endothelial-specific lineage tracing strategies is broadly extended to EndMT-related research[25].

To investigate the role of Snail during EndMT, CRISPR/Cas9 gene editing was used to knock it out this gene in this study. The data showed that TGF-β2 failed to mediate EndMT in Snail deficient MS-1 cells. This observation demonstrated that Snail is essential for TGF-β2 induced EndMT in MS-1 cells. We used an independent U6-driven sgRNA expression cassette to introduce specific sgRNAs for Cas9 to target *Snail.* In addition to this method, Ran et al.[26] described another strategy for cloning the sgRNA oligos sequence into the Cas9 scaffold to generate a construct containing both Cas9 and gRNAs. Emerging novel approaches allow for CRISPR/Cas to incorporate additional functions. For example, double or triple knockouts can be achieved by delivering more sgRNAs into cells expressing Cas9[27]. The engineered Cas13 protein targets and digests RNA molecules without disrupting endogenous DNA[28]. Besides knocking out genes with CRISPR/Cas, short hairpin RNAs (shRNAs) can be used as alternatives to stably knock down targeted gene expression[29]. For all CRISPR/Cas gene editing methods, off-target cleavage should always be taken into consideration. In addition, small interfering

RNAs (siRNAs) transiently silence gene expression and the siRNA concentration is diluted with cell division[30]. Both these methods partially suppress targeted gene expression. In contrast, ectopic gene expression is also used to verify gene function during EndMT/EMT[31]. This approach can determine if the upregulation of a gene is sufficient to elicit an EndMT response. Therefore, currently there are a multitude of technical strategies that can be used to identify and verify potential regulators of EndMT. Besides, transcriptomic analysis can be a good option in the identification and comprehensive analysis of EndMT related regulators. We recommend using different complementary approaches to investigate the modulation of EndMT.

In summary, we introduced a workflow to identify factors that may play functional roles during TGF-β-induced EndMT. This method can also be used to study whether other stimuli (i.e., cytokines, growth factors, mechanical stimuli, cell-cell interactions) can modulate EndMT, and the interplay of TGF-β with other stimuli. In addition, we highlighted an approach using CRIPSR/Cas gene editing to elucidate whether a certain gene is required for TGF-β-induced EndMT. To illustrate this methodology, we used the strong EndMT inducer TGF-β2 in MS-1 cells, but the protocols can be adapted to other cytokines and other cell types. We expect that this detailed protocol described will serve as a stepping stone for future EndMT-related studies.

DISCLOSURES

The authors have nothing to disclose.

ACKNOWLEDGEMENTS

The research was supported by CGC.NL and the Netherlands Cardio Vascular Research Initiative: the Dutch Heart Foundation, the Dutch Federation of University Medical Centers, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences Grant awarded to the Phaedra-Impact (http://www.phaedraresearch.nl). Jin Ma is supported by the Chinese Scholarship Council. GSD is supported by a Trampoline grant from AFM-Telethon [22379], FOP Italia and a grant from La Fundació La Marató de TV3 (#202038).

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Figure 1

Click here to access/download;Figure;Figure 1 Jove Ma paper.pptx

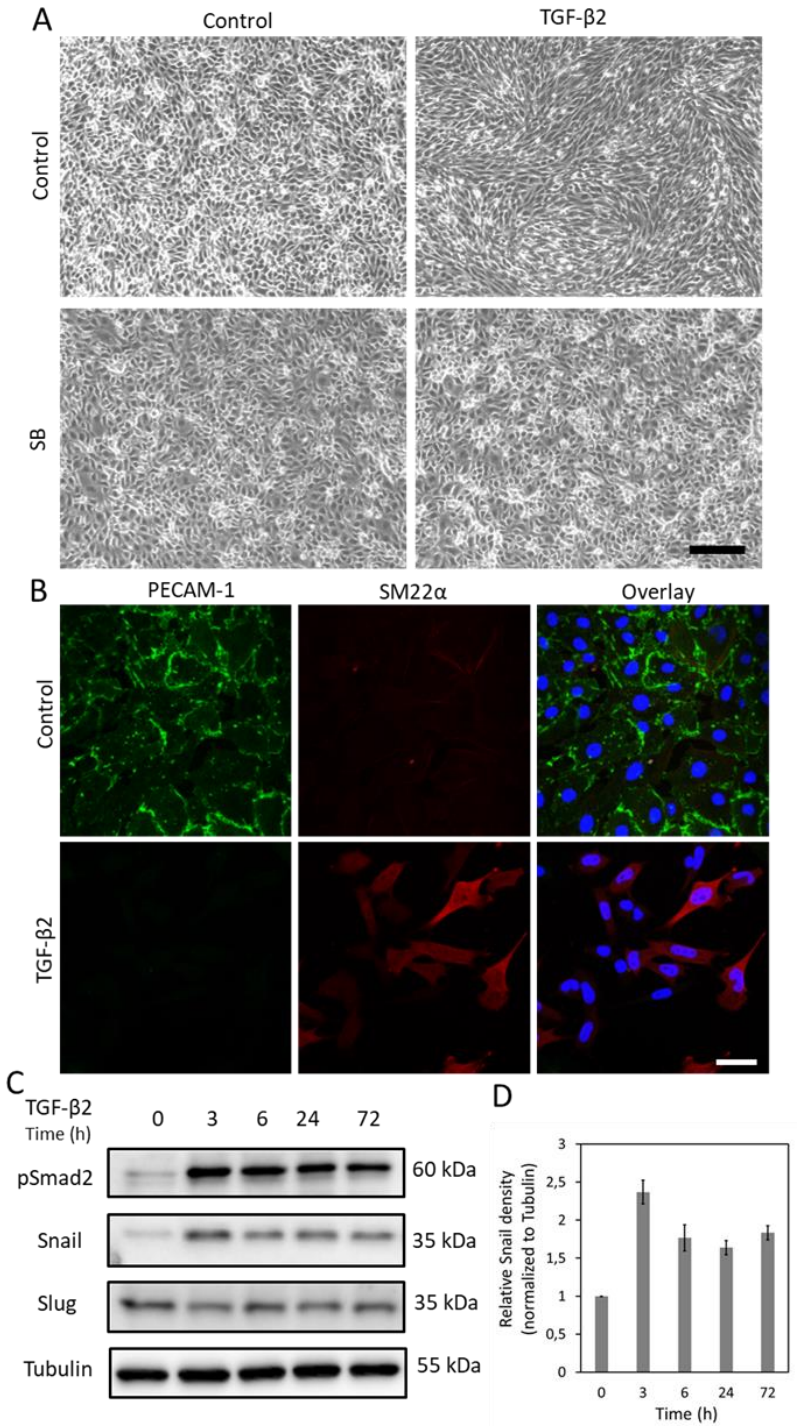
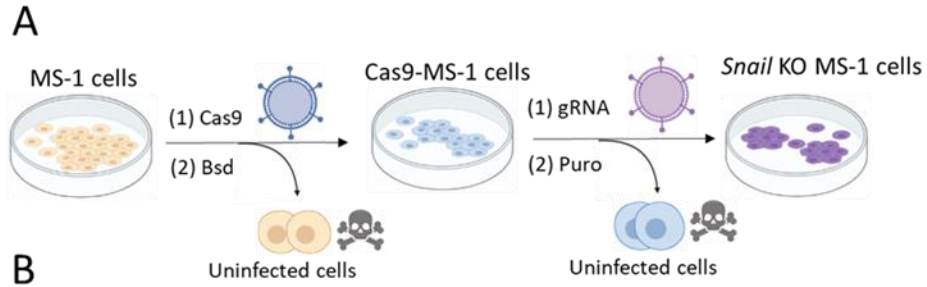


Figure 2



B

Snail gRNA #1 oligos : 5′-ACCGCGCTATAGTTGGGCTTCCGG-3′
3′-GCGATATCAACCCGAAGGCCCAAA-5′

Snail gRNA #2 oligos : 5′-ACCGTATAGTTGGGCTTCCGGCGG-3′
3′-ATATCAACCCGAAGGCCGCCCAAA-5′

C

Predicted off-target activity: low

Target sequence	Mismatch	Number of found targets
CGCTATAGTTGGGCTTCCGGNGG	3	1
TATAGTTGGGCTTCCGGCGGNGG	3	2

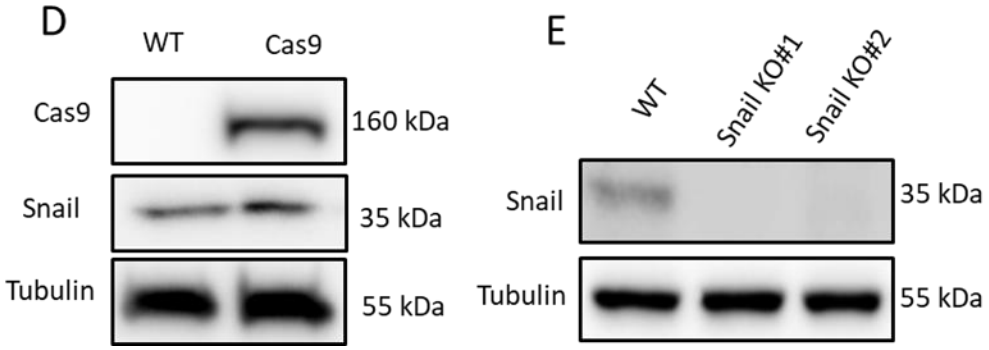


Figure 3

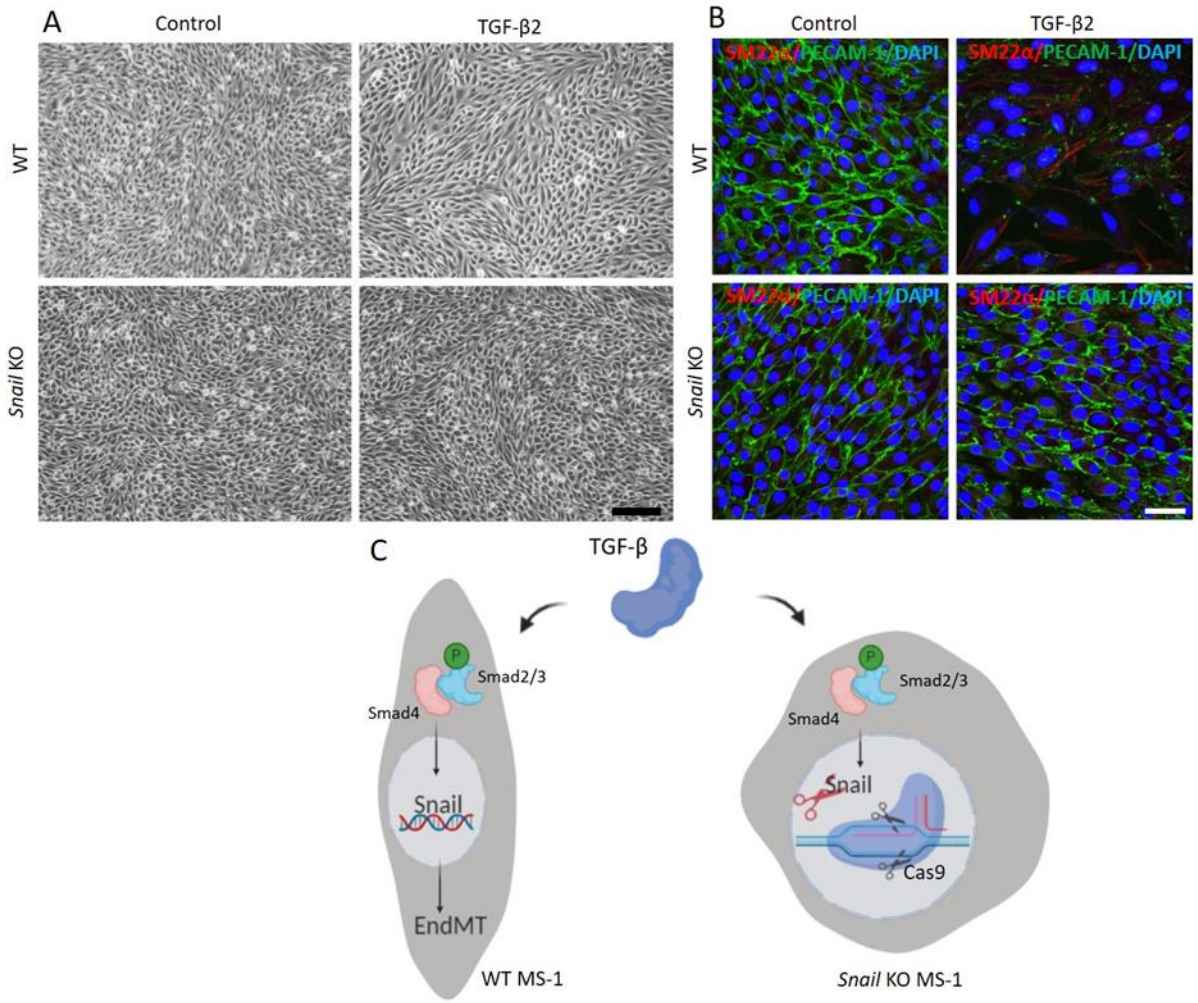


Table of Materials

Name of Material/Equipment	Company	Catalog Number
0.45 µm filter	Pall Corporation, USA	4614
10× T4 DNA ligase buffer	Thermofisher Scientific, USA	B69
12 mm round glass slice	Knittel Glass,Germany	VD10012Y1A.01
20 mL syringe	BD Eclipse, USA	300629
4',6-diamidino-2-phenylindole (DAPI)	Vector Laboratories, USA	H-1200
AA19_PLKO vector	Dr. M Gonçalves, Leiden University Medical Center, Netherlands	
Ampicillin	Serva Electrophoresis, USA	1339903
Anti-Mouse IgG	GE Healthcare, USA	NA931
Anti-Rabbit IgG	Cell signaling, USA	7074
Agarose	Roche, Switzerland	11388991001
Blasticidin	Invitrogen, USA	R21001
Buffer O (10X)	Thermofisher Scientific, USA	BO5
BveI (Bspm1)	Thermofisher Scientific, USA	ER 1741
Confocal microscope	Leica Microsystems, Germany	SP8
DMEM	Thermo Fisher Scientific, USA	11965092
Donkey anti-rat Alexa 488	Invitrogen, USA	A21208
FBS	Thermo Fisher Scientific, USA	16000044
Formaldehyde	Thermo Fisher Scientific, USA	28908
Inverted microscope	Leica Microsystems, Germany	DMi8
Goat anti-rabbit Alexa 594	Invitrogen,USA	A11012
LabNed Plasmid kit	LabNed, USA	LN2400004
MS-1 cell line	American Type Culture Collection (ATCC)	
Nail polish	HEMA, Netherlands	
PECAM-1 antibody	Becton Dickinson,USA	553370
PEI	Polysciences, USA	23966-1
PLV-Cas9 plasmid	Sigma-Aldrich, USA	Cas9BST-1EA
Puromycin	Sigma-Aldrich, USA	P9620
QIAquick gel extraction kit	Qiagen, Germany	28706
SM22α antibody	Abcam, UK	ab14106
Snail	Cell signaling, USA	3879
T4 DNA ligase	Thermofisher Scientific, USA	EL 0014

TC20 automated Cell Counter	Bio-Rad, USA	1450102
Human TGF-β2	Joachim Nickel, University of Wurzburg	Gift
Triton X-100	Merck, USA	1086031000
SB431542	Tocris Bioscience, UK	1614
Tris	Roche, Switzerland	11814273001

polybrene	Sigma-Aldrich, USA	107689

Comments/Description
VECTASHIELD Antifade Mounting Media
Gift
Transparent

Other commercial recommendation: 302-B2, R&D systems

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: Many thanks for your valuable comments. We have thoroughly proofread the manuscript using Nature Editing services (see certificate enclosed) and corrected the errors. We have carefully check the manuscript and make sure all abbreviations at first use.

2. Please provide an email address for each author.

Response: We have provided the email addresses of all the authors in the submission system. Also the email addresses are listed here:

Jin Ma: J.Ma@lumc.nl

Gerard van der Zon: G.C.M.van_der_Zon@lumc.nl

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Peter ten Dijke: P.ten_Dijke@lumc.nl

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Falcon tube etc

Response: We have removed all the commercial language and put the commercial products in the Table of Materials and Reagents.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have removed personal pronouns unless necessary (“our previous study” at page 4) in the manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We have rephrased the first sentence of the “and should be used at a final concentration of 5 µM. Other cells should be treated with vehicle” to “and was used at a final concentration of 5 µM. Other cells were treated with vehicle” at page 4. The sentence at page 6 “all the pictures should be taken with the same exposure time” was rephrased to “all the pictures were taken with the same exposure time”. At page 6, “The sense oligo should start with 5’-ACCG-3’ and the antisense oligo should start with 5’-AAAC-3’” was rephrased to “The sense oligo starts with 5’-ACCG-3’ and the antisense oligo starts with 5’-AAAC-3’. At page 7, “When performing the ligation, two control groups should be included” was rephrased to “When performing the ligation, ensure two control groups are included”. At page 9,

“Uninfected cells should die due to the effect of blasticidin” was rephrased to “Uninfected cells died due to the effect of blasticidin”.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: We have checked all the steps in the protocol based on the request and added multiple details, such as the incubation time, temperature, to make the steps as clear as possible. We have included the sentences in the imperative tense, for example, the sentence of “At least three independent experiments are needed to obtain biological triplicates” in protocol 1.8 and 2.3.11.

7. Lines 315-316: Which medium do you use to culture HEK 293T cells? Do you mean culture them according to guidelines of BMBL?
Response: We have rephrased the sentence “Transfect HEK 293T cells by adding the above mixture medium to 80% confluent cells in 14.5 cm dishes” to “Transfect HEK 293T cells by adding the mixture medium from step 4.1.2 to 80% confluent cells in 14.5 cm dishes which contain 15 mL DMEM medium with 10% FBS and 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin.”.

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Response: We have highlighted the step 1 and step 2 in the manuscript.

9. Please include a section “Figure and Table Legends” to follow the representative results section (before the discussion section) and include the figure legends in this legends section.
Response: we have included the “Figure and Table Legends” section at the end of the manuscript.

10. As we are a methods journal, please move a lot of background information to the Introduction, and revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: Every step in our protocol is important and need to be performed accurately.

In the 2nd paragraph of the discussion section, we have discussed the outlined methods and their limitations, for example, “In addition to the immunofluorescence staining we performed here, marker variations can also be monitored by western blotting at the protein expression level or by qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) at the gene expression level”. We also introduced other existing methods, such as “there are other methods to examine EndMT. For example,…”.
Also, the limitation of CRISPR/Cas9 system and the alternative techniques and their future possible applications have been discussed in the 3rd paragraph of the discussion section.

11. Please sort the Materials Table alphabetically by the name of the material.
Response: We have alphabetically sorted the Materials Table.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this manuscript, Ma et al. use the CRISPR-Cas9 as a model in MS-1 endothelial cells to show that EndMT is dependent on TGF-beta2 induced Snail upregulation, which has been reported previously. The main relevance of this work is the method in which the authors use CRISPR-Cas9 lentiviral approach to knock out Snail in MS-1 cells to show it relevance in EndMT.
The manuscript is well written and focused. I have only minor concerns with this work

Minor Concerns:
Line 42. Swap S (capital letter) to s: " then up- and downregulate the levelS of mesenchymal"
Response: Many thanks for your valuable comments. We have revised this as “the levels of”.

Line 155. Delete in: " added 30 min in before TGF-β2 stimulation"
Response: The “in” in this sentence has been deleted.

Line 195. " Dilute the primary PECAM-1 and SM22α antibodies that recognize murine proteins 1:1000 with 1x PBS"
Where the antibodies really diluted 1:1000 for IF?
Please, give more information on the antibodies, either here or in the summary table of materials below.
Response: We made a mistake about the dilution of primary antibody and the secondary antibody. We have corrected this mistake: 500 times dilution was used for the primary antibody and 1000 times dilution was used for the secondary antibody. The information about the antibodies is now listed in the Table of materials.

Line 223. Delete most and swap activity for target " 3.1.3. Choose two sgRNAs with the most lowest off-activityactivity"

Response: We have deleted “most” and the extra “activity” and included target in 3.1.3: " 3.1.3. Choose two sgRNAs with the lowest off-target activity"

Line 307. Delete one e in " µL polyethyleneimine (PEI) (2.5 mg/mL)"
Response: We have deleted the “e” to “polyethylenimine (PEI) (2.5 mg/mL)”.

Line 312. " in 14.5 cm dishes" (what size of flasks?)
Response: We have included the size of flask here as “14.5 cm dishes (or T75 flasks)”

Line 338. " 4.2.3. Mix 1 mL virus medium with 1 mL fresh medium"
Please explain here the what the fresh medium is
Response: We have rephrased this sentence as “Mix 1 mL virus medium with 1 mL fresh DMEM medium containing FBS and penicillin/streptomycin”.

Line 352. Delete s in reaches " should die due to the effect of blasticidin. Split the surviving cells when they reaches80% cell"
Response: We have deleted “es” in this sentence.

Line 371. Delete e in reach " uninfected cells are completely dead. Split the cells when they reache 80% confluency"
Response: The improper “e” was deleted.

Line 355. Explain what Cas9 antibody is being used. " 4.2.8. Using an antibody against Cas9 to check for successful Cas9 expression in MS-1 cells by western"
Response: We have included the Cas9 antibody information in the Table of materials.

Line 374. Explain what Snail antibody is being used " 4.3.6. Using an antibody against Snail to check the Snail knockout"
Response: We have included the Snail antibody information in the Table of materials.

Line 383. „with the small molecule TGF-β type I receptor kinase inhibitor SB431542"
Please, be more specific the SB431542 is not only a TGF-β type I receptor kinase inhibitor but rather an ALK4/5/7 inhibitor.
Response: We have rephrased this sentence as “pre-treated the cells with the small molecule activin receptor-like kinase (ALK)4/ALK5/ALK7 kinase inhibitor SB431542 before TGF-β2 stimulation”.

Line 396. Replace with with in " As shown in Figure 2A, we generated the Snail knockout cells with two steps" .
Response: We have rephrased this sentence as “we generated the Snail knockout cells in two steps”.

Table of materials: all the antibodies in Western blotting are missing here.
Response: We have included the Snail and Cas9 primary antibodies and the secondary antibodies information in the Table of materials.

Fig1: Please, quantify the WB bands in Fig1C.

Response: We quantified the Snail expression changes from three independent western blot experiments. The result is shown in Figure 1D and is described in the results section as “The quantification of Snail expression changes from three independent experiments are shown in Figure 1D”.

Fig2: The knock-out of Snail is nicely demonstrated in Fig2E. However, could you please, explain what the control stands for? In Fig1C, you showed that Snail expression is dependent on TGF-beta2 stimulation. In Fig2E it seems as if Control means untreated wt cell, i.e. Snail expression is present here without TGFbeta2 stimulation…

Response: The control cells in Figure 2E are wild type MS-1 cells. This information has been included in the revised figure. Snail is already expressed in the wild type MS-1 cells; Snail is detected in the control group of both Fig 1C and Fig 2E. The stimulation of MS-1 cells with TGF-β2 strongly increased the expression of Snail.

Reviewer #2:

Manuscript Summary:

The authors propose a protocol that could be the bases for studying the mechanisms of Endmt-related studies. They refer to their previous studies on TGFb-2 as a strong inducer of EndMT through the upregulation of TF as Snail and Slug which control the regulation of different markers.

The authors describe a protocol based on CRIPR/Cas9 gene editing technology, which could be adapted to different cell types and to the study of the function of different cytokines involved in these processes.

The methods are clearly described. Easy to follow. All necessary times, concentrations and material characteristics are specified. In addition, the authors provide clear notes and examples, which help to better understand the protocol.

Minor Concerns:

1. Suggestion for figure 1A. The differences are well observed, but perhaps the area of the photograph could be amplified or perhaps images where there is not so much confluence of cells in order to better visualize the morphological changes of the cells.

Response: Many thanks for your valuable comments. We have increased the size of the pictures of each group to make the morphological changes more clearly observable.

2. There is no catalog number for TGF-B2. Perhaps the authors could suggest a commercial house or a product where, according to their experience, they know that this cytokine works. In this paper TGF-B2 is the main cytokine. Perhaps it would be helpful for scientists who may be starting to study this cytokine.

Response: We have included a commercial TGF-β2 product and related information in the Table of materials.

Reviewer #3:

In this workflow, the authors used CRISPR/Cas9-mediated gene editing to study TGF-β-

induced endothelial-mesenchymal transition. The protocol is highly feasible and most of the steps are detailed and clear, and if the questions listed below areamended, the whole work will be somewhat improved.
Question 1:
Different from the below manuscript (that has not been officially published), all the pictures in the manuscript do not seem clear, and can they be replaced with high-resolution pictures?
Response: Many thanks for your valuable suggestions. We will take this point in consideration in our another manuscript (not been officially published one).

Question 2:
(Page 4, Line 138-139):The conventional trypsin concentration we use to digest anchorage-dependent cells is 0.25 %, will the 0.5 % concentration you used affect the cell state?
Response: We double checked with our technician who is responsible for preparing trypsin and EDTA. The trypsin-EDTA solution we used is 0.25% trypsin and 0.02% EDTA. We have re-phrased this accordingly in step 1.2. We do not appreciate that these conditions compromise cell behavior, when the trypsin is properly deactivated.

Question 3:
(Page 5, Line 198, 2.3.8): Do you choose to avoid light before incubating the secondary antibody? Are your immunofluorescence results based on experimental conditions that are not avoided from light?
Response: The secondary antibody incubation should be performed without light. We included this very important note after 2.3.8. protocol as “NOTE: Protect the samples from light during staining”.

Reviewer #4:
Manuscript Summary:
In this manuscript, Jin Ma and co-authors propose a workflow to identify factors involved in the endothelial-to-mesenchymal transition. The authors use a murine endothelial cell line exposed to TGFb-2 and characterize EndoMT by a) morphological changes using brightfield microscopy b) by change in EndoMT markers visualized by fluorescence confocal microscopy (endothelial marker CD31/PECAM decrease vs. mesenchymal/myofibrobastic SM22a increase). Authors also provide a genetic deletion by CRISPR-Cas9 approach to invalidate TGF B-regulated transcription factor Snail, in order to validate its implication in promoting EndoMT.

Major Concerns:
This methods article is well written and easy to follow. The potential of this workflow/protocol is important, as it can be very adaptable: adaptable to other stimuli (soluble factors, mechano-stimulation, oxidative stress), to other transcription factors (with the limit that these factors must be previously identified) and to other cell types and cell species. The discussion is fair and address main issues.
Steps listed in the procedure are overall clearly explained, controls are clearly mentioned but critical steps are not that much highlighted. This could be improved.
I would require some additional information listed below.
-More details about the MS-1 cell line used (ATCC ref if this is the case)

Response: Many thanks for your valuable comments. The MS-1 cell line was obtained from ATCC. We have included a reference at line 122, page 4. In addition, the information about the MS-1 cell line we used was included in the Table of materials.

-More information about the source of TGFB2 used (Purified, recombinant, home made? why not using commercial available TGFB2?)
Response: The TGF-β2 ligand we used in our study is human TGF-β2. We have included the source of the ligand in the Table of materials. The human recombinant purified TGF-β2 we used was a kind gift from Joachim Nickel, University of Wurzburg. We also recommended a commercial available source of human recombinant purified TGF-β2. This information was provided in the Table of materials.

- Step 2.3.9: This step is the mounting of the coverslip on a slide. Authors should clarify " a drop of DAPI" as according to the reference H-1200, this is the mounting medium Vectashield containing DAPI.
Response: we have rephrased “on a drop of DAPI” to “on a drop of mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI)”.

- Step 2.3.11 Authors mentioned that all pictures should be taken at the same exposure time. This is true also for channel settings.
Response: In order to make the fluorescent intensity of all the images comparable, the images need to be acquired with the same settings. We have now included a reference to the channel settings in this sentence as “all the pictures were taken with the same channel settings and exposure time” at step 2.3.11.

- Step 4.1.5. Use of antibiotics in medium of cell upon DNA transfection is not usually recommended. Do authors confirm using DMEM plus Peni/Strep at this stage?
Response: When performing DNA transfection in step 4.1.2, we used serum and Peni/Strep free DMEM medium to resuspend the plasmids and reagents. But we used the normal culture medium (DMEM containing FBS and penicillin/streptomycin) when producing the lentivirus in step 4.1.5.

-Step 4.2: Is there a step of viral titration at stage 4.1.8? What is the MOI used for transduction of MS-1 endothelial cells? This is an important point. Authors have to add these information or comment why not included.
Response: Many thanks for your valuable comments. Determination of viral titer(which enables the expression of a dominant selection marker in the transduced cells) is recommended. We didn’t perform a viral titration here, so the precise multiplicity of infection (MOI) that we used is unknown. We included a negative control group, i.e. cells without virus infection. When antibiotics are added to these cells, they should all die. If the cells that have been virally infected survive, this is indicative for a successful infection.

- Figure 2E: Expression of Snail in Cas9- MS-1 cells should be included.
Response: The Snail expression in WT and Cas9 expressed cells are included in Fig2.

-Figure 3: It has to be mentioned in the legend or in the text that MS-1 wt are MS-1 stably transduced with Cas9.
Response: The control cells are wild type MS-1 cells (not stably transduced with Cas9). This information has been included in Figure 2D and Figure 2E and their figure legends.

Question : Is the expression of Cas9 not affecting at all the phenotype of MS-1?
Response: We haven't observed obvious morphology changes after overexpressing Cas9 in the MS-1 cells.

In the discussion, I would point more that the genetic deletion approach would require an initial identification of relevant transcription factors regulating EndMT in the system explored, and this may require a larger analysis of factors, for which transcriptomic analysis would be more suitable than IF and WB.
Response: We included the transcriptomic analysis method in the Discussion 3rd paragraph as "Besides, transcriptomic analysis can be a good option in the identification and comprehensive analysis of EndMT related regulators".

2 references should be added:
As EndMT is becoming an important issue in cancer biology, the review from Clere et al (Front Cell Dev Biol. 2020 Aug 14;8:747) should be included to the list of references (2,4-6) line 82 in the introduction

With the same logic, in the discussion, an excellent review on endothelial-specific lineage tracing strategies may be included line 449 (Li Y, Lui KO, Zhou B. Nat Rev Cardiol. 2018 Aug;15(8):445-456)
Response: The references were included in the revised manuscript and cited at appropriate places.

Minor Concerns:
-As a general comment, once an abbreviation is defined, it has to be used (cf FBS, DMEM, BSA …)
Response: We have carefully checked the manuscript text and made sure that all abbreviations are used after first definition.

-line 70 : on a gelatin-coated 12 mm (re-precise 0,1% w/v gelatin)
Response: We have corrected this description as "0.1% w/v gelatin-coated 12 mm" at step 2.1.

- Minor typing errors
Response: We have thoroughly proofread the manuscript using Nature Editing services.

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TGF-β-mediated endothelial to mesenchymal transition (EndMT) and functional assessment of EndMT effector using CRISPR/Cas9 gene editing

prepared by the authors

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