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Chapter 5

Visualizing Dynamic Changes During TGF-β-Induced Epithelial to Mesenchymal Transition

Abhishek Sinha, Pranav Mehta, Chuannan Fan, Jing Zhang, Dieuwke L. Marvin, Maarten van Dinther, Laila Ritsma, Pouyan E. Boukany, and Peter ten Dijke

Abstract

Epithelial to mesenchymal transition (EMT) is crucial during embryonic development, tissue fibrosis, and cancer progression. Epithelial cells that display a cobblestone-like morphology can undergo a switch to mesenchymal-like phenotype, displaying an elongated spindle shape or a fibroblast-like morphology. EMT is characterized by timely and reversible alterations of molecular and cellular processes. The changes include loss of epithelial and gain of mesenchymal marker expression, loss of polarity, increased cell migratory and invasive properties. Epithelial cells can progress unevenly during this transition and attain hybrid E/M states or metastable EMT states, referred to as epithelial cell plasticity. To gain a deeper insight into the mechanism of EMT, understanding the dynamic aspects of this process is essential. One of the most prominent factors to induce EMT is the cytokine transforming growth factor-β (TGF-β). This chapter discusses molecular and cellular techniques to monitor TGF-β-induced signaling and EMT changes in normal and cancer cell lines. These methods include measuring the TGF-β-induced activation of its intracellular SMAD effectors proteins and changes in epithelial/mesenchymal marker expression and localization. Moreover, we describe assays of cell migration and dynamic reorganization of the actin cytoskeleton and stress filaments that are frequently part of the TGF-β-induced EMT cellular response.

Key words EMT, EMP, TGF-β, Mesenchymal, Metastasis, Cell migration

1 Introduction

Epithelial to mesenchymal transition (EMT) is an essential cellular process during mammalian development, whereby tightly coupled cobblestone-shaped epithelial cells display a substantial change in their morphology, polarity, and motility to acquire a more motile fibroblast-like appearance [1]. This change is accompanied by a loss

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of epithelial and gain of mesenchymal marker expression [2] (Fig. 1). EMT plays a pivotal role in physiological processes such as embryogenesis and wound healing and is reactivated in pathological processes such as tissue fibrosis and cancer cell invasion and metastasis. EMT is a dynamic and reversible process, and a reversal of the cell state (from mesenchymal to epithelial) is called mesenchymal to epithelial transition (MET). The dynamic cell behavior and plasticity often result in most cells acquiring hybrid epithelial-mesenchymal (E/M) characteristics. This phenomenon led to The EMT International Association (TEMTIA) proposing the term epithelial-mesenchymal plasticity (EMP) to underscore these E/M mixed phenotypic states [3]. This plasticity emphasizes the occurrence of hybrid E/M states and metastable EMT states [4] leading to express epithelial features while also displaying mesenchymal markers.

The cytokine transforming growth factor-β (TGF-β) is an important driver of EMT [5]. TGF-β elicits its cellular responses by specific binding to cell surface TGF-β type I and type II receptors (i.e., TβRI and TβRII) that are endowed with intrinsic serine/threonine kinase activity. Upon TGF-β-induced heteromeric complex formation the TβRII kinase trans-phosphorylates TβRI which subsequently induces the phosphorylation of receptor-regulated SMAD2 and SMAD3. This phosphorylation occurs at the two most carboxy-terminal serine residues in SMAD2 and SMAD3 [6]. Antibodies recognizing the C-terminally phosphorylated SMAD2 are often used to investigate if cells are responsive to TGF-β [7]. Upon phosphorylation, SMAD2 and SMAD3 form heteromeric complexes with SMAD4, which translocate into the nucleus [8]. These nuclear SMAD complexes act as transcription factors (TFs), and in cooperation with other DNA binding TFs and co-activators and co-repressors regulate target gene expression [9]. SMAD3 and SMAD4 bind directly to DNA to a so-called "CAGA" containing motif. Multimerization of such motif in front of a minimal promoter is sufficient to make the artificial transcriptional reporter construct highly sensitive to and selective for TGF-β/SMAD transcriptional activation [10]. A frequently used method to interrogate the TGF-β-induced transcriptional activation in cultured cells is to transfect or infect them with a $CAGA_{12}$-Luciferase transcriptional reporter construct. Alternatively, the luciferase can be replaced by fluorophores like enhanced Green Fluorescent Protein (eGFP) or Td-Tomato (Marvin DL et al., in preparation) [11]. The intensity of luciferase luminescence or fluorescence signal is used to measure the level of transcriptional activation that occurs.

Activated SMAD complexes stimulate the expression of EMT-inducing TFs (EMT-TFs), such as SNAIL, SLUG, ZEB1/2, and TWIST1/2 [12]. These EMT-TFs mediate the repression of epithelial markers such as E-CADHERIN and Zona Occludens

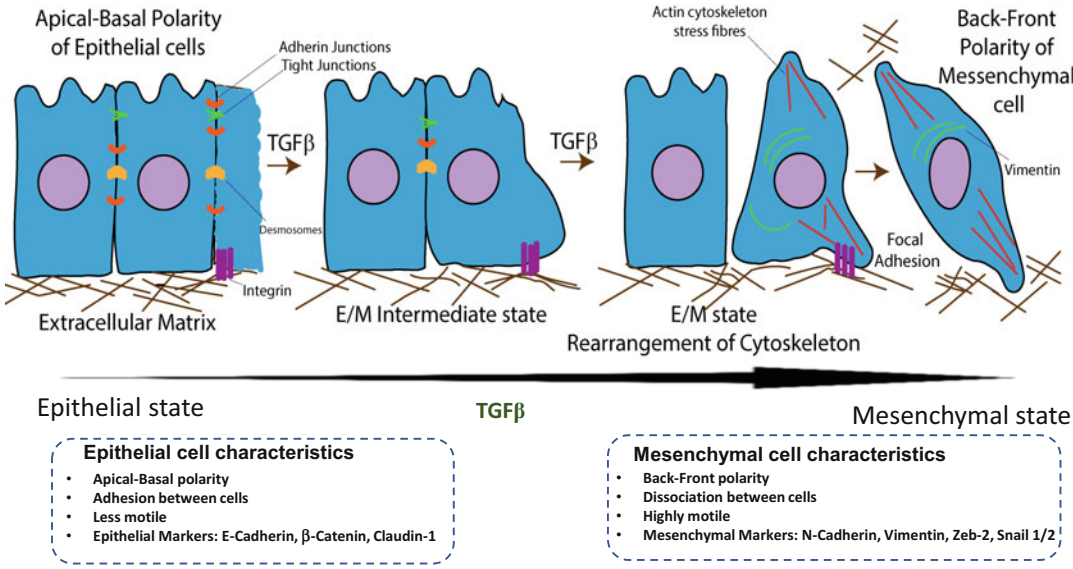

Fig. 1 Schematic diagram of epithelial to mesenchymal transition (EMT). The intermittent and high plasticity of this process is highlighted. Characteristic changes of this transition process, such as change of polarity (from apical-basal to back-front), are shown. The cytokine TGF-β is a main driver of EMT

(ZO) and increase the expression of mesenchymal markers such as N-CADHERIN, α-smooth muscle actin (αSMA), and VIMENTIN [13]. E-CADHERIN, a transmembrane protein that directly interacts with cadherins of neighboring cells, interacts with various regulatory proteins, notably β-CATENIN—which plays a vital role in stabilizing adherens junctions [14]. In the nucleus, β-CATENIN increases the transcription of EMT-inducing factors *SLUG*, *TWIST*, *FIBRONECTIN*, and Matrix Metalloproteinases (MMPs). Canonical TGF-β signaling pathways promote the expression of Fascin, which is subsequently stabilized by the Rho GTPases [15]. Fascin, an actin-bundling protein, is implicated in many cancers such as breast, colon, gastric, and oral squamous cell carcinomas. It promotes metastasis by forming stable F-actin bundles and thus playing a critical role in forming filopodia, which subsequently regulates the invasiveness of cells [16].

TGF-β is also able to activate the non-SMAD pathways that contribute to EMT. These include but are not limited to the phosphatidylinositol 3-kinase (PI3K)/AKT, RHOA GTPase signaling, and p38 MAPK pathways [17–19]. Activation of RHO GTPases RAC and CDC42 are crucial to stimulate cell motility and invasiveness [20]. RHO GTPases can also be indirectly activated in a SMAD-dependent manner by upregulation of guanine exchange factor (GEF) NET1 that subsequently activates RHO [21].

Protrusions like filopodia such as protostomes, invadopodia, and lamellipodia are also a characteristic of EMT and are linked to

invasion [22]. These protrusions are formed and maintained by change in the composition, orientation, and dynamics of cytoskeletal components. Cytoskeletal intermediate filaments (IFs), mediated by the induction of exchange factor activation of RHO kinase, also undergo significant compositional change as epithelial cells undergo EMT. Cells undergoing EMT initiate the expression of VIMENTIN (VIM) IFs, a downstream target of the canonical TGF-β signaling pathway (Fig. 1) [23]. This dramatic change in intermediate filaments composition has led to VIM expression becoming a frequently used mesenchymal marker.

The above described molecular and cellular changes can be observed in two frequently used models for TGF-β-induced EMT, which are NMuMG, a mouse mammary gland epithelial cell line, and A549, a human lung adenocarcinoma cell line [24, 25]. This chapter discusses experimental techniques that can be used to ascertain the dynamic alterations that occur upon TGF-β-induced intracellular SMAD signaling and EMT. Methods to demonstrate different EMT markers' expression changes, changes in cell motility, shape, and cytoskeletal properties will be presented. The mouse mammary gland NMuMG and human lung adenocarcinoma A549 cell lines are used as examples. Whereas the TGF-β-induced SMAD2 phosphorylation peaks in most cells at 45 min to 1 h and thereafter subsides, the TGF-β-induced transcriptional response becomes more prominent after multiple hours of stimulation. TGF-β-induced changes in cell morphology, migration, changes and epithelial and mesenchymal markers become apparent in NMuMG and A549 cells after 1–2 days of treatment. TGF-β and other cytokines have been shown to induce (or synergize in) EMT responses in multiple other cell lines. Moreover, endothelial cells can also have a similar transition, termed endothelial to mesenchymal transition [26]. The procedures detailed here can also be applied (with slight modifications) for these cell types and other EMT stimuli.

2 Materials

2.1 Cell Lines

1. Mouse mammary gland epithelial cells NMuMG cell line (ATCC®-CRL-1636).
2. Human lung adenocarcinoma A549 cell line (ATCC®-CCL-185).
3. A549 cells expressing red fluorescent tagged Vimentin (RFP-VIM) (ATCC®-CCL-185EMT).

2.2 Cell Culture

1. Culture Medium: Dulbecco's Modified Eagle medium supplemented with 10% Fetal bovine serum (FBS), and 100u/ml penicillin and 0.1 mg/ml streptomycin (Pen/Strep).

2. Sterile phosphate buffer saline (PBS): For 1 l, 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4. pH 7.2 adjusted with 1 N HCl.
3. Trypsin and Ethylenediaminetetraacetic acid (EDTA) in PBS: 0.25% Trypsin, 0.02% (0.53 mM) EDTA solution in PBS pH 7.4 solution.
4. Ligand Buffer: 4 mM HCl, 0.1% bovine serum albumin (BSA).
5. TGF-β3 [Gift from Andrew Hinck, University of Pittsburgh] (5 μg/ml) stock prepared in ligand buffer.
6. Cell culture incubator at 37 °C with 5% CO_2 level.

2.3 Reporter and Constructs

1. pGL3-$CAGA_{12}$ transcriptional reporter vector for CAGA-luciferase assay [10].
2. pLV-$(CAGA)_{12}$-eGFP construct was used to generate lentivirus-mediated stable transfectant of $(CAGA)_{12}$-eGFP containing NMuMG cells (Marvin DL et al. Under preparation),
3. pCMV or SV-40 promotor based β-galactosidase (gal) expression construct [10].

2.4 Western Blotting

1. Lysis buffer: 20 mM Tris-Cl pH 7.4, 75 mM NaCl, 1% NP40, 0.1% Sodium Dodecyl Sulfate, 0.5% sodium deoxycholate containing a protease inhibitor cocktail.
2. Western blotting membrane washing buffer: Tris-buffered saline [20 mM Tris-Cl pH 7.4, 150 mM NaCl] with 0.1% Tween 20 (TBST).
3. Blocking buffer: 5% non-fat dry milk in TBST.
4. Protein estimation: DC Protein Assay Reagent (BioRad).
5. Colorimetry Measurements (protein estimation or other luminescence assays): Victor Multilabel Plate Reader (Perkin Elmer).
6. Polyvinylidene Difluoride (PVDF) membrane.
7. Antibodies: E-Cadherin (BD Bioscience, 610181), N-Cadherin (BD Bioscience, 610920), Vimentin (Cell Signaling, #5741), phospho-SMAD2 antibody (raised antibody against phospho-SMAD2) [7], GAPDH (Merck Millipore, MAB374).
8. Clarity Western Enhanced Chemiluminescence substrate (BioRad).
9. Western Blot Imaging: ChemiDoc Imaging System (BioRad).

2.5 Real-Time Polymerase Chain Reaction (Q-PCR)

1. cDNA synthesis: Deoxynucleotide triphosphates (dNTPs) (Invitrogen), Revert Aid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific).

Table 1
Sequence of primers used in the Q-PCR analysis of genes in mouse NMuMG cells

Sl No.	Primer name	Purpose	Sequence 5′- > 3′
1	*Cdh1* Fwd	Forward primer for *Cdh1* Q-PCR	ACCAAAGTGACGCTGAAGTC
2	*Cdh1* Rvse	Reverse primer for *Cdh1* Q-PCR	GAGGATGTACTTGGCAATGG
3	*Snail* Fwd	Forward primer for *Snail* Q-PCR	CAGCTGGCCAGGCTCTCGGT
4	*Snail* Rvse	Reverse primer for *Snail* Q-PCR	GCGAGGGCCTCCGGAGCA
5	*Zeb2* Fwd	Forward primer for *Zeb2* Q-PCR	TTCTGCAAGCCTCTGTAGCC
6	*Zeb2* Rvse	Reverse primer for *Zeb2* Q-PCR	TTCTGGCCCCATTGCATCAT
7	*Gapdh* Fwd	Forward primer for *Gapdh* Q-PCR	TGGCAAAGTGGAGATTGTTGCC
8	*Gapdh* Rvse	Reverse primer for *Gapdh* Q-PCR	AAGATGGTGATGGGCTTCCCG

2. Nucleic Acid Concentration measurement: NanoDrop 2000/2000c Spectrophotometers (Thermo Scientific).
3. RT-qPCR: GoTaq Q-PCR MasterMix (A600X, PROMEGA). Hard-Shell 384-well Q-PCR plates (BioRad) are used.
4. Primers (Amplification of EMT marker genes): *see* Table 1.
5. CFX connect real-time PCR detection system (BioRad).

2.6 Cell Staining

1. Fixing: Formaldehyde solution, Triton X-100.
2. Staining: 4′6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector Laboratories), Alexa Fluor 555 secondary antibody (Thermo Scientific) and Alexa Fluor 488 Phalloidin (Thermo Scientific).
3. Sterile glass slides and coverslips.
4. Confocal imaging microscope SP8 (Leica).

2.7 Wound Healing/ Scratch Assay

1. PBS: (pH 7.2).
2. 96-well cell culture plates compatible with scratch maker (Sartorius).
3. Automated scratch making system (Sartorius/Essen Bioscience).
4. Imaging: IncuCyte® incubator imaging system (Sartorius/Essen Bioscience).

3 Methods

Before analyzing TGF-β-induced EMT, it is recommended to check if cells respond to TGF-β treatment. To do this, we can perform the following experiments:

(a) TGF-β-induced SMAD2 phosphorylation.

(b) SMAD3/SMAD4-dependent $CAGA_{12}$-luciferase activity.

(c) SMAD3/SMAD4-dependent $CAGA_{12}$-eGFP activity.

A positive result in the above assays only examines the activation of the canonical TGF-β/SMAD signaling pathway. However as discussed above, it is possible for TGF-β to also act via non-SMAD (SMAD-independent) pathways, and this can contribute to the EMT response. Thus, TGF-β activity on activation of non-SMAD signaling pathways can also be tested, before examining EMT. TGF-β-induced EMT can be examined by:

(a) Measuring transcriptional changes of EMT target genes.

(b) Quantifying changes in protein expression of genes encoding for epithelial and mesenchymal markers.

(c) Cell morphology analysis.

(d) Analyzing the cell migratory behavior.

3.1 TGF-β-Induced SMAD Activation

3.1.1 Analysis of Activation of SMAD2 Phosphorylation by Western Blotting

1. Seed 10 mm culture plates containing DMEM medium with NMuMG cells (1×10^6 cells per plate) and allow to attach at the bottom surface by overnight incubation at 37 °C cell culture incubator.
2. Replace the medium the following day with fresh DMEM containing either TGF-β (5 ng/ml) or ligand buffer.
3. Harvest cells at specific time points in accordance following the method detailed below.
4. Transfer plates to ice, takeout the culture medium, and wash with 1 ml of cold PBS.
5. Aspirate the PBS, then add ice-cold lysis buffer. Let the plates sit for 20 min on ice to complete the lysis reaction.
6. Scrape cells off the bottom surface of the dish using a plastic cell scraper, then gently transfer the cell lysate into a 1.5 ml microfuge tube.
7. Centrifuge at $12 \times 10^3 g$ at 4 °C for 15 min and transfer the supernatant to a fresh microfuge tube.
8. Measure the protein concentration of the lysates using a protein quantification assay.
9. Load equal amounts of protein (25 μg of total protein from cell lysate) from each sample into the wells of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and run it at a constant voltage of 100 V.
10. Once the run is complete, transfer the protein from the SDS-PAGE gel to a pre-soaked (methanol) polyvinylidene difluoride (PVDF) membrane at a voltage of 100 V for

1.5 h min at 4 °C. Alternatively, nitrocellulose membranes can also be used.

11. Protein transfer to the membrane can be verified (before blocking) using Ponceau S.
12. Block the membrane for 1 h at room temperature or overnight at 4 °C using the blocking buffer.
13. Wash the membrane with TBST for 5 min.
14. Incubate the membrane with primary antibody prepared in blocking buffer for overnight at 4 °C or at room temperature for 1.5–2 h.
15. Wash the membrane thrice for 5 min each in TBST.
16. Incubate the membrane with the appropriate conjugated secondary antibody in the appropriate blocking buffer (follow manufacturer's instructions) at room temperature for 1 h.
17. Wash the membrane three times for 5 min each in TBST.
18. For signal development, incubate 1 ml of western Enhanced Chemiluminescence (ECL) substrate (BioRad) for 1 min.
19. The signals can be detected using the ChemiDoc Imaging System (BioRad). Protein expression varies between cell lines and is also dependent on the treatment. Thus, the exposure time must be optimized for different blots (*see* **Notes 1** and **2**).
20. For statistically significant results, perform three independent repeats. Band intensities can be measured using Image-J software.

3.1.2 TGF-β/SMAD-Induced Activation of CAGA12-Luciferase Transcriptional Reporter Activity

1. Harvest mammalian/mouse cancer cells at 80% confluency using trypsin-EDTA (*see* **Note 3**).
2. Count the harvested cells and resuspend them to create a solution containing 1×10^5 cells/ml of culture medium.
3. Mix the suspension thoroughly and seed 2 ml of the solution in a 6-well plate.
4. Incubate cells, at 37 °C and 5% CO_2, overnight.
5. Transfect the incubated cells in each well with 500 ng of TGF-β/SMAD3 inducible $(CAGA)_{12}$ luciferase transcriptional reporter construct (pGL3-$CAGA_{12}$) and 200 ng of the β-galactosidase (βgal) expression construct (pCMV-βgal) using Lipofectamine 3000 reagent and following manufacturer's instruction.
6. Incubate overnight.
7. Replace the reporter-containing culture medium with culture medium now containing either TGF-β (5 ng/ml) or ligand buffer.
8. Incubate for 24 h.

9. Aspirate culture medium and wash cells twice with PBS.
10. Lyse cells in each well by using 200 μl of lysis buffer (see composition in Subheading 2.4). Keep the 6-well plate on ice for 20 min to complete the lysis reaction.
11. Collect the cell lysates in a 1.5 ml microfuge tube using cell scrapers and spin in a microcentrifuge at $12 \times 10^3 g$ at 4 °C for 20 min.
12. Transfer supernatant to a fresh microfuge tube and measure the protein concentration of the extract.
13. Transfer 50 μl (1 mg/ml concentration of protein) lysate to a 96-well plate, add the luciferin substrate (Sigma, Cat No. L9504) and measure luciferase activity using a plate reader (Victor, Perkin Elmer).
14. In a separate assay, measure β-galactosidase activity similarly.
15. Normalize luciferase activity to β-galactosidase activity to get quantitative change in CAGA-luciferase expression.

3.1.3 TGF-β/SMAD-Induced Activation of CAGA12-eGFP Transcriptional Reporter Activity

1. Seed 1×10^4 NMuMG cells in 100 μl of DMEM, stably expressing CAGA-eGFP, to a 96-well clear bottom black culture plate.
2. Allow the cells to adhere to the bottom surface by incubating in a 37 °C incubator for 8–12 h.
3. Replace media with media containing TGF-β (5 ng/ml) or ligand buffer and transfer the plate to the IncuCyte life cell imager.
4. IncuCyte live-cell imager can acquire images while the cells are maintained in a temperature-controlled CO_2 incubator.
5. Imaging can be performed with various time intervals.
6. Alternatively, after addition of media containing TGF-β (5 ng/ml) or ligand buffer the plates can be incubated in normal incubator and at specific time points the fluorescence intensity can be checked at fluorescence plate reader.
7. A representative result is presented in Fig. 2. CAGA-eGFP signal is examined after addition of different concentrations of TGF-β at 0 h and 24 h in IncuCyte imaging platform and quantified using IncuCyte S3 software. But the same can be assayed for prolonged time in the same platform to understand the dynamics of expression change of eGFP with time using the same platform.

3.2 Real-Time Reverse Transcriptase PCR for TGF-β Target Genes and EMT Markers

The experiment protocol is described with NMuMG cells (*see* **Note 4**).

1. Culture cells as described in Subheading 3.1.1 and allow them to attach overnight.

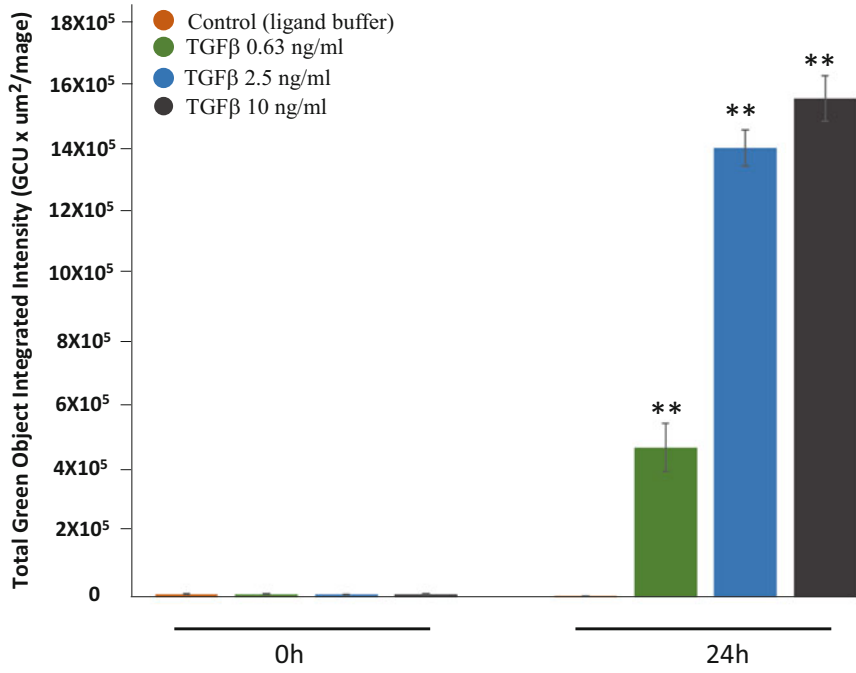

Fig. 2 TGF-β/SMAD-induced transcriptional response in NMuMG cells. TGF-β/SMAD-induced transcriptional response is monitored in NMuMG cells that stably express the eGFP fluorescent maker under transcriptional control of multimerized 5'-CAGA-3' Smad3/4 binding sites coupled to a minimal promoter. Three different concentrations of TGF-β were used, and eGFP levels were measured at 0 h and 24 h timepoints

2. Next morning, treat cells with TGF-β (5 ng/ml) or ligand buffer and incubate for 24 h (*see* **Note 5**).
3. Discard the medium from each well and wash the cell pellet with 1 ml of PBS.
4. Isolate RNA from cultured cells using NucleoSpin RNA isolation kit (Macherey-Nagel). Follow manufacturer's protocol.
5. Measure the RNA concentration using a micro-volume spectrophotometer.
6. Prepare cDNA using first strand cDNA synthesis kit. Follow manufacturer's protocol.
7. Using tenfold diluted cDNA, prepare a reaction mixture containing specific forward and reverse primers of target genes (*see* Table 1 to check primer sequences) and GoTaq QPCR mastermix.
8. With the reaction mixture prepared, set up a Q-PCR.
9. Reaction conditions for the Q-PCR: Initialization at 95 °C for 2 min; denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and, extension at 80 °C for 20 s with 40 cycles to be repeated.

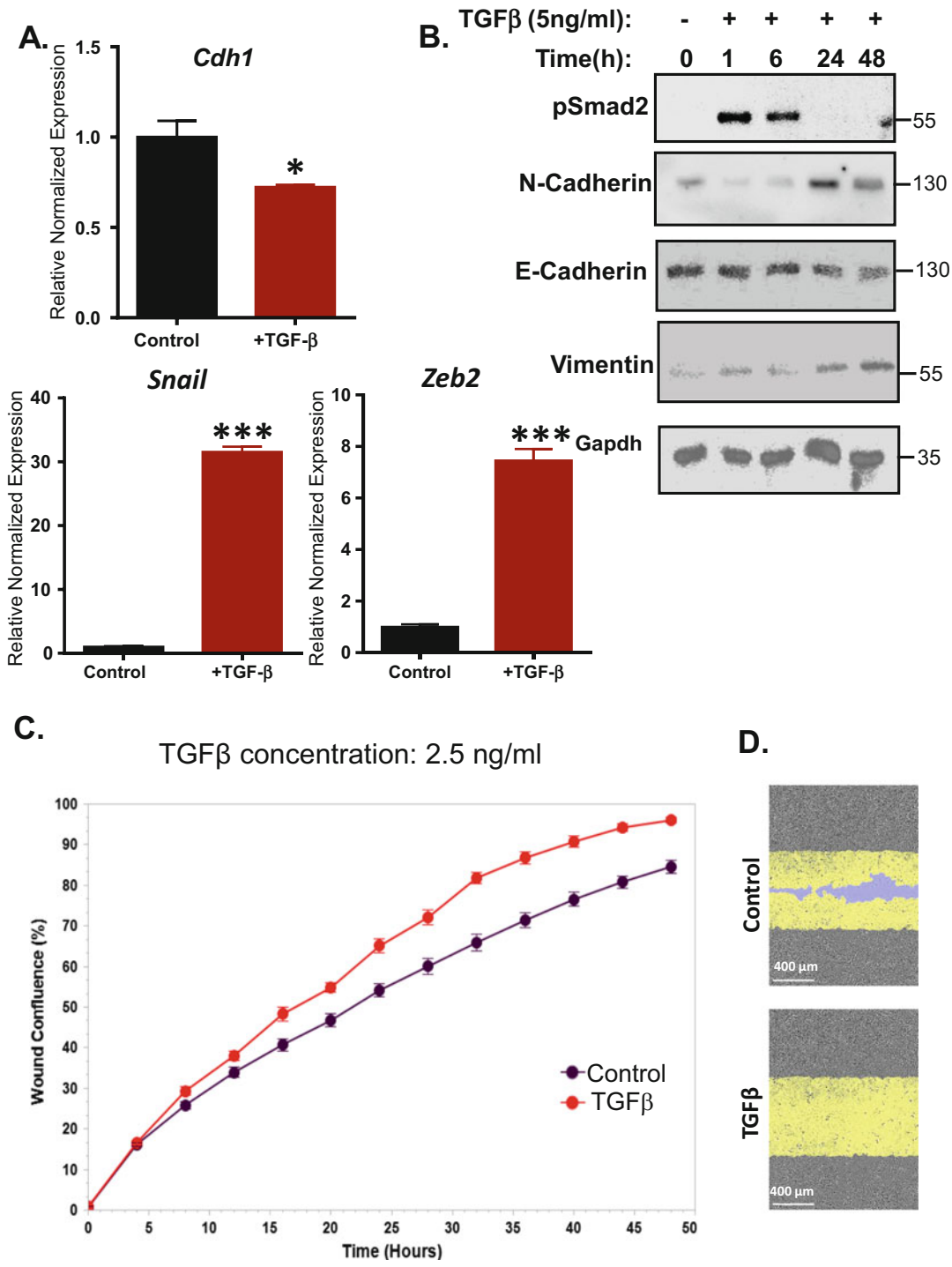

Fig. 3 TGF-β-induced changes in Smad2 phosphorylation, epithelial and mesenchymal marker expression, and cell migration of NMuMG cells. (**a**) Q-PCR analysis of expression levels of *Cdh1* (E-Cadherin), *Snail*, and *Zeb2* mRNA in NMuMG cells upon stimulation with TGF-β for 24 h. Figure adopted from Zhang et al. 2020 (https:/www.jove.com/v/61830/studying-tgf-signaling-tgf-induced-epithelial-to-mesenchymal). The results are expressed as the mean ± s.d., $n = 3$. Student's t-test, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (**b**) Western blot analysis of expression levels of phospho-Smad2, N-Cadherin, E-Cadherin, and Vimentin in response to

10. For statistically significant results, perform three independent repeats as described in Fig. 3a.
11. The results can be analyzed using the Gene Set Enrichment Analysis (GSEA) 4.0.3 software (Broad Institute).

3.3 TGF-β-Induced Changes in EMT Markers Using Western Blot Analysis

1. Culture cells as described in Subheading 3.1.1, and isolate protein extracts as mentioned in the same section followed by western blotting.
2. For EMT marker protein expression analysis, use anti-E-cadherin, anti-N-cadherin, anti-Vimentin, and anti-GAPDH primary antibodies.
3. Subsequent steps are also similar as Subheading 3.1.1.

Changes in the expression of EMT marker genes in response to TGF-β are shown in Fig. 3b.

3.4 TGF-β-Induced Cell Migration Analyzed Using Wound Healing/Scratch Assay

1. For this experiment special types of scratch resistant 96-well plates are used, and real-time imaging is conducted using IncuCyte® live-cell imaging system. Images are taken every 2 h for 2.5 days.
2. Seed 3×10^4 A549 cells in 96-well scratch resistant plates and add 100 μl of culture medium to each well (*see* **Note 6**).
3. Incubate overnight (37 °C and 5% CO_2), to allow the cells to adhere to the surface.
4. Aspirate medium and add 100 μl of fresh culture medium containing 0.5% serum and antibiotic mix in each well.
5. Create a wound in the wells using an automated scratch maker.
6. After wound creation, aspirate culture medium and wash the wells with 50 μl of prewarmed sterile PBS (*see* **Note 7**).
7. Aspirate the PBS (taking away all suspended cells).
8. Mix TGF-β (5 ng/ml) or ligand buffer with fresh culture medium (DMEM containing 0.5% FBS and antibiotic mix) and add 100 μl to each well.
9. Place the plate in the IncuCyte imaging system, maintaining growth conditions at 37 °C with 5% CO_2 level inside the system. Set up a time-series, taking images in either brightfield or fluorescence based on the requirement.
10. The width of the wound can be calculated with the help of in-built software (IncuCyte S3) and the wound healing pattern

Fig. 3 (continued) TGF-β (5 ng/ml) stimulation of NNuMG cells. GAPDH was included as the protein loading control. (**c**) Migration of A549 cells treated with either ligand buffer (Control) or TGF-β (2.5 ng/ml). (**d**) Representative image of scratch at 48 h time points of the same experiment as described in panel C. Gray: Cells, Yellow: scratch healed; Purple: final wound remaining

can be graphed across different time points as shown in Fig. 3c. The wound healing kinetics can also be presented in form of videos. A representative video taken using the IncuCyte live-cell imaging system, showing change in cell shape and size upon TGF-β treatment is presented as Video S1 (Electronic Supplementary Material). Images of wound healing are shown at Fig. 3d for 48 h time point.

3.5 TGF-β-Induced Induction of Vimentin in A549 VIM-RFP Cells

A549 cells expressing Red Fluorescent Protein (RFP) labeled Vimentin are used for this experiment. This assay can be applied for other cell lines expressing different IF markers labeled with fluorescent tags.

1. Seed 1×10^4 cells to 96-well culture plates containing fresh culture medium (*see* **Notes 8** and **9**).
2. Incubate overnight to allow the cells to adhere to the surface.
3. Following incubation, stimulate cells with TGF-β (5 ng/ml) or ligand control and place the 96-well plate in the IncuCyte.
4. Create a time-series imaging sequence to capture images every 2 h for 3 days with brightfield and in red fluorescence (TRITC) filters.
5. Analyze the results looking for changes in red fluorescence protein (RFP)-Vimentin expression for cells with and without TGF-β stimulant (Fig. 4).
6. Time-dependent changes of RFP-Vimentin expression can also be presented in the form of videos as shown in Videos S2 and S3 (Electronic Supplementary Material).

3.6 TGF-β-Induced Changes in EMT Marker Localization Using Indirect Immunofluorescence and Cytoskeleton Reorganization by Direct Fluorescence Staining

For this experiment, NMuMG cells treated with either TGF-β or ligand buffer are stained at specific time points. The experimental protocol is as follows.

1. Place sterile 18 × 18 mm glass slips in 6-well plates (One coverslip in each well, *see* **Note 10**).
2. Seed 5 × 104 cells in each well of the plate containing 2 ml culture medium.
3. Incubate overnight to ensure cells adhere to the coverslip.
4. Following incubation, remove the coverslips and place them in a fresh 6-well plate.
5. Stimulate cells with a fresh culture medium containing either TGF-β (5 ng/ml) or ligand buffer.
6. Incubate cells for 2 days.
7. Aspirate culture medium after 2 days and wash twice with prewarmed PBS.

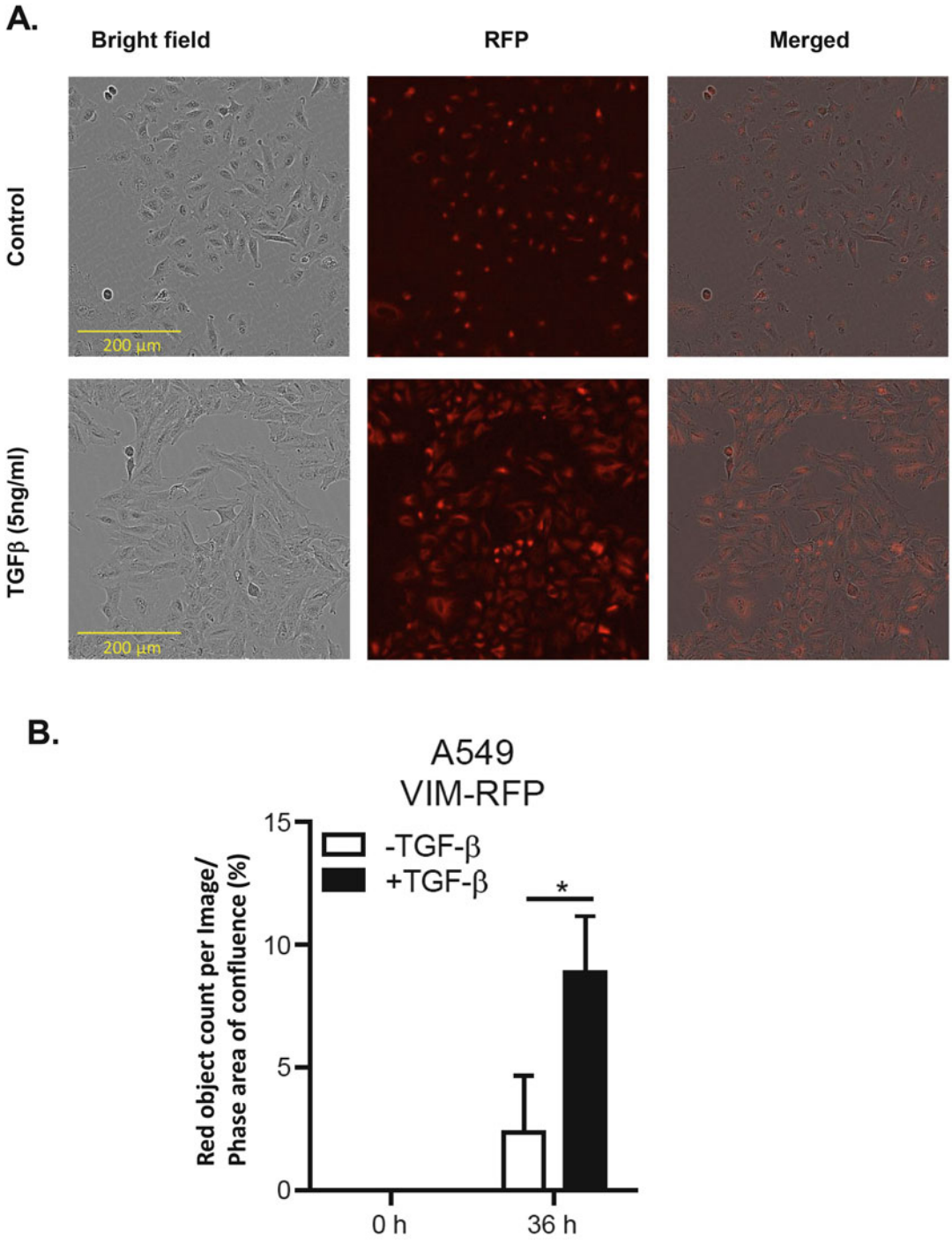

Fig. 4 TGF-β-induced morphological change and expression of VIMENTIN in A549 lung adenocarcinoma cells in which RFP is inserted into the endogenous locus of *VIMENTIN*. (**a**) A549 cells were stimulated with TGF-β (5 ng/ml) and changes in cell morphology were visualized by bright field images and appearance of RFP signal, indicative of Vimentin expression, is measured using a fluorescent microscope. Imaging was performed in an automated IncuCyte platform and the changes in cell morphologies and vimentin expression was evident from fluorescence images. (**b**) The expression of VIMENTIN-RFP was quantified using IncuCyte S3 software and signal levels of VIM-RFP were plotted at 0 h and 36 h time points. Results were analyzed using one-way ANOVA followed by Tukey's HSD test ($n = 4$) * represents $p < 0.1$

8. Add 1 ml of formaldehyde and incubate for 20 min at room temperature to fix cells.
9. Wash the cells twice with PBS.
10. Permeabilize cells using 0.1% Triton X-100 solution for 10 min at room temperature.
11. Aspirate the Triton solution, wash the cells once with PBS and block with 3% BSA solution in PBS for 45 min to 1 h at room temperature.
12. Wash the cells twice with PBS.
13. Incubate the cells with anti-E-Cadherin antibody (1:1000 in PBS) by placing 100 μl of antibody solution on top of coverslip.
14. Incubate for 1 h at room temperature.
15. Remove the primary antibody solution and wash the slides three times with PBS (5 min each wash).
16. Add Alexa Fluor 555 tagged secondary antibody solution in PBS (1:500 dilution) on the coverslips.
17. During the same time, stain the cells by adding Alexa Fluor 488 Phalloidin (1: 1000 dilution in PBS) on the coverslips and incubate the coverslips at room temperature for 1 h in a dark cabinet in presence of both the fluorophore tags.
18. Wash the cells thrice with PBS.
19. Remove the coverslip from the 6-well plate and mount them onto fresh slides using a mounting medium.
20. Use a confocal microscope to image and compare the fluorescence intensity of the cells.
21. Actin reorganization was evident when 48 h TGF-β-treated cells were compared with ligand buffer-treated cells (Fig. 5).

4 Notes

1. As a positive control during PAGE running, cell lysate from cells transfected with constitutively active ALK5 (pcDNA3 with $ALK5^{T204D}$) and SMAD2 can be used [27]. These cells will express high pSMAD2 levels. By taking the same aliquot along, this can serve as an internal standard to confirm that transfer of proteins from gel to PVDF membrane has occurred properly, compare the results from different experiments, and understand where the pSMAD2 signal runs on the western blot.
2. Depending on the gel thickness (0.8 mm/1 mm/1.5 mm), expression levels of the target proteins, the sensitivity of an antibody, and the number of wells in the PAGE, one can vary

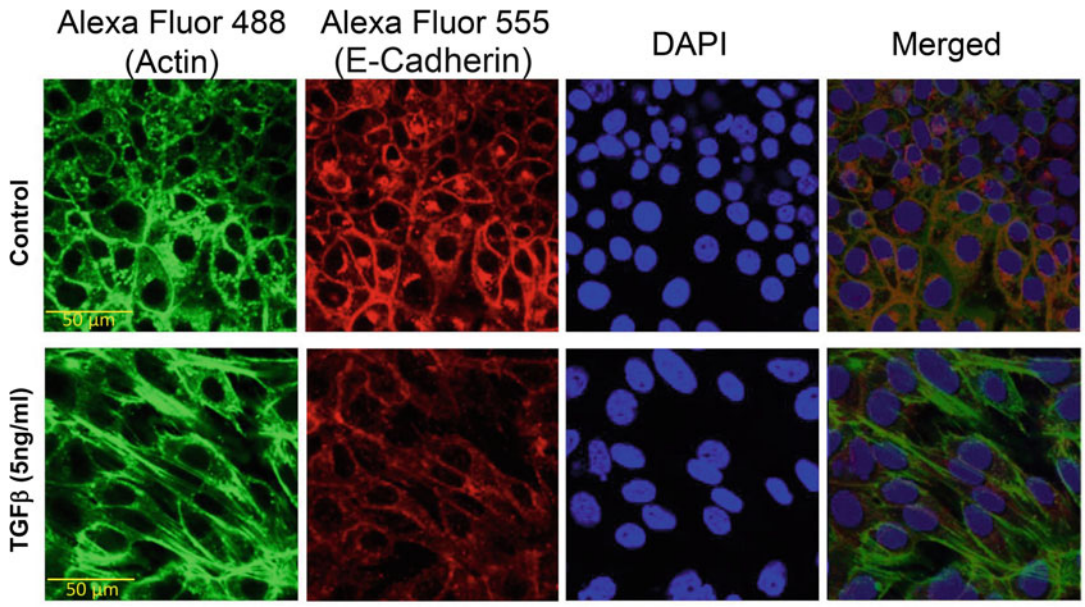

Fig. 5 Changes in F-actin cytoskeleton arrangements and E-Cadherin expression in NMuMG cells treated with TGF-β. Confocal imaging of NMuMG cells treated with ligand buffer or TGF-β (5 ng/ml) for 48 h and stained with Phalloidin (Alexa Fluor 488), anti-E-cadherin antibody (Alexa Fluor 555 tagged secondary antibody), and DAPI (nuclear staining). Actin cytoskeletons are stained in green, nucleus in blue, and E-Cadherin in red colour

the amount of protein to be loaded. The protein weight (25 μg) stated here is used for the ten-well page with a thickness of 1.5 mm. Although we ran the PAGE at constant voltage, in some cases, it is preferable to run at much lower voltage till the dye font crosses the stacking gel and then increase the voltage to 100 volts.

3. Different immortalized cell lines vary in their growth and cell division kinetics. Similarly, their response to TGF-β also varies. For gene induction activity, usually serum-starved cells are used to obtain strong response. If cells need to be monitored for extended periods, serum can be added; it can also promote the TGF-β-induced EMT response. For growth inhibition or stimulation also different serum concentrations are required. Very high serum concentration may compromise the observation of growth arrest/apoptotic or proliferation response. Similarly, the robustness and duration of TGF-β response are also time-dependent. In some cell lines, the reporter activity is induced rapidly (within 6 h) and long lasting, but in others it can take longer or may be transient.
4. As discussed previously (*see* **Note 3**) TGF-β response for certain cell lines requires low concentration of serum into the medium. Similarly, optimum TGF-β concentration also varies for different cell lines. For some cell lines transcriptional response can be

observed at much lower concentration of TGF-β (1–2.5 ng/ml).

5. For some cell line the change in transcriptional response can start very fast within an hour, compared to 24 h as we shown in our representative result with NMuMG cells. Hence standardization of optimum time points is necessary before conducting large-scale transcriptional response studies.
6. The cells must be close to 100% confluency to ensure optimal wound creation and examination of wound healing dynamics. Depending on the cell line, the number of cells seeded will vary, necessitating standardization before proceeding with the experiment.
7. Post wound creation, it is imperative to clear the cell debris for accurate measurements and imaging. To ensure this occurs, wash the 96-well plate two times with PBS.
8. 96-well plates in an IncuCyte live-cell imager are used in the protocol to visualize RFP-Vimentin expression changes at differing time points. Alternatively, this experiment can be conducted with various tissue culture plates in any microscope with a red fluorescence filter.
9. For microscopy experiments, it is recommended to seed fewer cells and begin observations at 50–60% confluency. Overcrowding can compromise the experiment as it affects cell shapes and sizes, making EMT visualization difficult.
10. It is preferable to use gelatin-coated coverslips for cell attachments, as cells do not attach well on normal glass surface. Otherwise normal glass coverslips need to be placed on 6-well plates, coat surface with 600 μl of gelatin solution, incubate them for 1 h at 37 °C, aspirate the unattached solution and dry the coverslips by keeping the plates open in a hood. It will take 15–25 min for the coverslips to dry. After that the coverslips can be used immediately or can be kept for future experiments for 4 months.

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