High-Throughput Methods to Quantitatively Evaluate TGF-β Signaling in Epithelial-to Mesenchymal Transitions

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Abstract

The epithelial-to-mesenchymal transition (EMT) is a cellular differentiation process whereby epithelial cells lose many epithelial features, while acquiring mesenchymal, fibroblast-like properties, leading to reduced cell–cell contacts and increased motility. While recognized as a fundamental process required for normal embryonic development, EMT is understood to be co-opted by malignant epithelial tumors to facilitate their metastatic spread. Central to stimulating EMT is the TGF-β superfamily of growth factor ligands, which elicit receptor-mediated responses in cells, primarily via TGF-β/SMAD signaling pathways. In these pathways, receptor-mediated SMAD (R-SMAD) proteins serve as the primary downstream effector molecules, the activities of which are regulated through receptor-mediated phosphorylation. The magnitude and duration of ligand-induced receptor activation influences the level of SMAD phosphorylation, which, in turn, influences the magnitude of downstream cellular response. This study describes high-throughput methods to quantitatively evaluate the biochemical and cellular responses to TGF-β/SMAD pathway activation in a cellular model of TGF-β-induced EMT. Effects of pathway activation are examined at different levels of biological complexity–biochemical, cellular, and multicellular–using two- and three-dimensional (spheroid) models. Collectively, these approaches enable a comprehensive evaluation of TGF-β/SMAD pathway activation that is amenable to high-throughput analysis platforms .

Introduction

EMT is a cellular differentiation process whereby epithelial cells lose epithelial features while acquiring mesenchymal, fibroblast-like properties, such as reduced intercellular adhesion and increased motility. EMT is a critical feature of normal embryonic development, but is also used by malignant epithelial tumors to spread beyond their origin.[13] Central to stimulating EMT is the TGF-β/SMAD signaling axis, for which R-SMAD proteins represent primary downstream effector molecules.[4-7] TGF-β ligand–receptor binding elicits the formation of heterotetrameric TGF-β type I/II receptor complexes, leading to site-specific phosphorylation events that elicit receptor kinase activation. The activated receptor complexes recruit R-SMADs, which are then activated throughreceptor-kinase-mediated phosphorylation.

There are two primary signaling axes within the TGF-β superfamily. TGF-β ligand–receptor interactions result in recruitment and activation of SMAD2 and/or SMAD3 proteins, whereas bone morphogenetic protein (BMP) ligand–receptor interactions lead to recruitment and activation of SMAD1, SMAD5, or SMAD9. In either case, phosphorylated R-SMADs form a ternary complex with SMAD4; this complex then translocates to the nucleus where it regulates the expression of target genes (Figure 1).

Activation of the TGF-β-SMAD2/3 pathway is known to upregulate the expression levels of numerous EMT-associated genes, including HMGA2, ZEB1, Snai1, and Slug, all of which have furthermore been associated with cancer metastasis.[8-13]

The ability to investigate this pathway in detail, at multiple levels of biological complexity, is critical to gain a better understanding of the role of EMT in cancer. This application note demonstrates high-throughput methods—using the Agilent BioTek Cytation C10 confocal imaging reader and Agilent BioTek Gen5 microplate reader and imager software—to quantitatively evaluate the biochemical and cellular responses to TGF-β/SMAD pathway activation in a cellular model of TGF-β-induced EMT. The effects of pathway activation are examined at different levels of biological complexity–biochemical, cellular, and multicellular–using 2D and 3D (spheroid) models. Collectively, these approaches enable a comprehensive evaluation of TGF-β/SMAD pathway activation that is amenable to high-throughput analysis platforms.

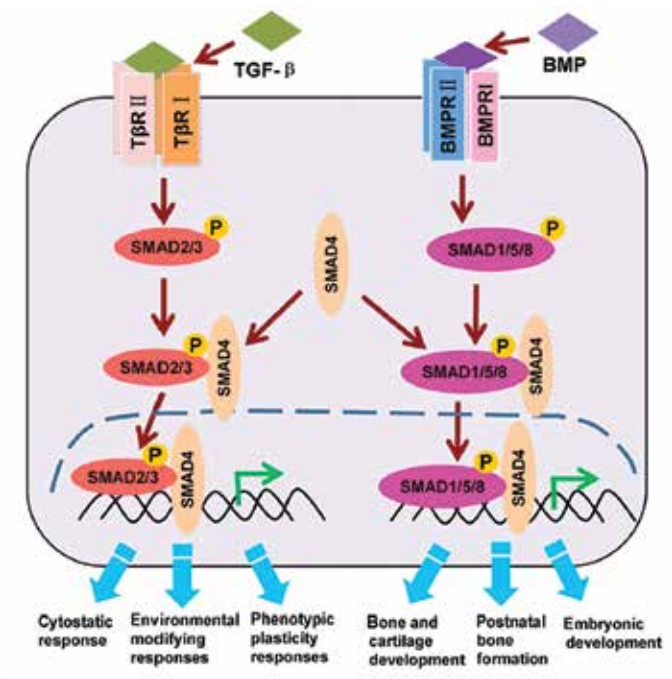

Figure 1. The canonical TGF-β/BMP/SMAD signaling pathway (adaptation).[7]

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Experimental

Materials

Cell lines

A549 lung epithelial carcinoma cells, part number CC-185, were purchased from ATCC (Manassas, VA, U.S.) and were cultured in Advanced DMEM (part number 12491; Gibco Thermo Fisher Scientific; Waltham, MA, U.S.) containing 10% FBS and 1x penicillin/streptomycin/glutamine.

Assay reagents

Human TGF-β1 recombinant protein (part number 75362) was from Cell Signaling Technology (CST, Danvers, MA, U.S.). Cell lysis buffer (10x) was from CST (part number 9803). PathScan Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA kit (part number 12001, CST) detects SMAD2 (Ser465/467) and/or SMAD3 (Ser423/425), only when phosphorylated at the indicated residues. PathScan Total Smad2/3 Sandwich ELISA kit (part number 12000, CST) detects SMAD2 and/or SMAD3 proteins irrespective of phosphorylation status. SMAD4 (D3R4N) XP Rabbit mAb (part number 46535, CST) detects total SMAD4 protein, and is validated for use in western blot, immunoprecipitation, immunocytochemistry, immunohistochemistry, flow cytometry, and chromatin immunoprecipitation. Antibodies targeting HMGA2 (D1A7) Rabbit mAb (part number 8179, CST), ZEB1 (E3G6Y) XP Rabbit mAb (part number 70512, CST), and Slug (C18G7) Rabbit mAb (part number 9585, CST) were used to evaluate TGF-β1-stimulated changes in the expression of EMT-associated target genes.[3]

Fibronectin coating

Agilent 96-well imaging microplates (part number 204626- 100) were treated with 10 µg/mL fibronectin (part number F1141; Sigma-Aldrich, Burlington, MA, U.S.) diluted in DPBS for 30 minutes, followed by three washes with DPBS prior to cell seeding.

Cell seeding and growth factor treatment

Cells seeded in culture plates, or spheroids cultured in round-bottom 96-well plates, were serum starved for 18 hours in basal culture medium (described previously) lacking FBS (A-DMEM) or BulletKit supplements (EBM). Cells were then treated for 60 minutes at 37°C with human TGF-β1 recombinant protein, at the indicated concentrations. After treatment, cells were either fixed in 4% paraformaldehyde (PFA) for 10 minutes for immunostaining analysis, or used for lysate collection, using 1x cell lysis buffer supplemented with cOmplete, Mini Protease Inhibitor Cocktail (part number 1186153001; Roche Diagnostics GmbH, Mannheim, Germany).

Three-dimensional spheroid outgrowth assay

The 3D spheroid outgrowth assay was conducted by preforming 2000-cell A549 spheroids in a 96-well ultra-low attachment (ULA), round-bottom microplate (part number 650979; Greiner Bio-One, Monroe, NC, U.S.). Spheroids were then transferred (one spheroid per well) to an Agilent 96-well imaging microplate (part number 204626-100) containing 40 µL of pregelled type I collagen matrix (part number 254236; Corning, NY, U.S.). Once the spheroids settled on top of the matrix, the plate was returned to a tissue culture (TC) incubator and cultured for three days at 37 °C.

Sample preparation

For ELISA analyses, lysates from cells cultured in 12-well, TC-treated plates were collected, sonicated for 30 minutes at 4°C, then centrifuged at 14,000 rpm for 10 minutes. Supernatants were collected and stored at −80°C, and thawed as needed.
For 2D cellular assays, cells were collected after treatment and fixed with 4% PFA for 10 minutes, followed by three washes with DPBS containing 0.5 M glycine. Cells were permeabilized with 0.5% Triton X-100 for 5 minutes, then blocked for 30 minutes with 5% bovine serum albumin (BSA). Cells were then incubated overnight at 4°C with the indicated antibodies, which were diluted in 5% BSA containing 0.1% Tween 20 (according to CST protocol). After three washes with PBS + 0.1% Tween 20, cells were incubated with a CF633-conjugated goat anti-rabbit polyclonal antibody (part number 20131; Biotium, Fremont, CA, U.S.) diluted to 1:1000 in 5% BSA containing 0.1% Tween 20, 2 µM Hoechst 34580, and 1x Alexa Fluor 488-conjugated phalloidin (part number A12379; Thermo Fisher Scientific). For the 3D EMT assay, samples in 96-well microplates were washed three times with DPBS before fixation in 4% PFA for 1 hour at room temperature, followed by three washes with DPBS containing 0.5 M glycine. Spheroids were then permeabilized with 0.5% Triton X-100 for 1 hour, followed by an overnight block with 5% BSA at 4°C. Fixed, permeabilized spheroids were then incubated overnight at 4°C in primary antibodies, which were diluted in 5% BSA + 0.1% Tween 20 (according to CST protocol), followed by hourly washes with immunowash buffer, concluding with a final overnight wash at 4°C. Samples were then incubated with secondary antibodies and counterstains which were diluted in 5% BSA + 0.1% Tween 20, followed by hourly washes with immunowash buffer with a final overnight wash at 4°C.

Gen5 Imaging, image processing, and cellular analysis

Imaging and image processing

Using the Agilent BioTek Cytation C10 confocal imaging reader and a 20x 0.45 NA objective in widefield mode, a single Z-plane of adherent cell samples was imaged in three channels: GFP (AF488- conjugated phalloidin), CY5 (CF633-conjugated goat anti-rabbit secondary antibody, used to visualize phosphorylated SMAD2/3), and DAPI (Hoechst 34580), with the DAPI channel used to set Z-focus height using laser autofocus. With the same three channels, multichannel, 2 x 2 montage Z-stacks spheroids were imaged in confocal mode (60 µm disk) using a 20x 0.4 NA objective. Following montage stitching, multichannel Z-stacks underwent a background reduction transformation step before generating a maximum intensity project.

Cellular analysis

Primary masks of nuclei were established using Hoechst 34580 signal (DAPI channel; yellow outlines in Figures 3 and 4), and integrated phosphorylated SMAD2/3 or SMAD4 nuclear signals of individual nuclei were quantified using the CY5 channel as a secondary mask. The reported phosphorylated SMAD2/3 signal was derived by first adjusting for the nuclear area using the following custom metric formula: [area-corrected Integral signal] = (Integrated CY5 signal) / (nuclear area). The area-corrected integral phosphorylated SMAD2/3 signal measured at each TGF-β1 concentration was converted to, and reported as, fold change of the signal response relative to vehicle control using the following custom data reduction step: [nuclear P-SMAD2/3 fold change] = (area-corrected integrated P-SMAD2/3 signal) / (mean area-corrected integrated P-SMAD2/3 signal of [4] vehicle control). To quantify the number of cells (nuclei)

that migrated away from the core spheroid (Figure 4D and E), a primary mask identifying the core spheroid was established using the GFP channel (phalloidin stain). Cells outside of the core spheroid were then defined by identifying nuclei using the DAPI channel as a secondary mask, where the primary mask zone is excluded. A ring width for the secondary mask zone was set to 600 µm to ensure all migrated cells were accounted for.

Results and Discussion

Biochemical quantification of TGF-β1-induced SMAD2/3 phosphorylation using PathScan Sandwich ELISA kits coupled with the Cytation C10

ELISA is a powerful and sensitive assay that enables quantitative analysis of biochemical events in biological fluids, including cell lysates. PathScan Sandwich ELISA kits from CST employ a horseradish peroxidase (HRP)-conjugated secondary antibody that provides a colorimetric readout measured by absorbance at 450 nm. Phosphorylated SMAD2/3 values were normalized against total SMAD2/3 protein values to control for protein loading. Using the plate-reader function of the Cytation C10, we demonstrate dose-dependent TGF-β1-induced SMAD2/3 phosphorylation in lysates from A549 lung carcinoma cells with a calculated EC_{50} value of 0.286 ng/mL (Figure 2).

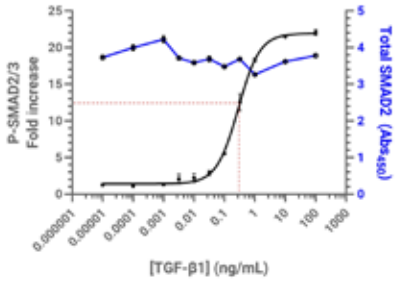

Figure 2. Dose-dependent SMAD2/3 phosphorylation in response to TGF-β1. The PathScan sandwich ELISA kit quantifies TGF-β1-dependent SMAD2/3 phosphorylation in huFIBs. The phosphorylated SMAD2/3 values (pSMAD2/3, black) are reported as fold change above no treatment control, which were normalized for total SMAD2 (blue values). [5]

Quantitative cellular analysis of SMAD phosphorylation and translocation using phospho-specific SMAD antibodies and fluorescence widefield imaging on the Cytation C10

Immunofluorescent image analysis in 2D-cultured cells can provide cellular and subcellular (spatial) insights into signal transduction pathway activity. The Cytation C10 confocal imaging reader enables quantitative analysis of immunostaining data in a high-throughput format. Treatment of A549 cells with TGF-β1 (100 ng/mL) resulted in a detectable increase in nuclear-localized phosphorylated SMAD2/3 compared with vehicle-treated controls (Figure 3A and B). Using a high-throughput, 96-well format for analysis, a dose–response curve was plotted with a calculated EC_{50} for A549 cells treated with TGF-β1 of 0.037 ng/mL (Figure 3D) Similarly, dose-dependent increases in the nuclear expression of EMT-associated transcription factors HMGA2, ZEB1, and Slug were observed in response to TGF-β1 treatment, with EC_{50} values of 14.6, 2.8, and 6.2 ng/mL, respectively (Figure 3D). Inhibition of the TGF-β signaling axis via pretreatment with the TGF-β receptor inhibitor SB431542 (before TGF-β1 treatment) resulted in reduced SMAD2/3 phosphorylation and nuclear translocation (Figure 3C), and a corresponding dose-dependent reduction in the expression of TGF-β1-induced EMT markers (Figure 3E) relative to that observed in cells treated with TGF-β1 alone, further confirming a direct role for the TGF-β pathway in regulating the expression of EMT-associated genes.

Quantifying phosphorylated SMAD2/3 and SMAD4 nuclear translocation in a 3D spheroid outgrowth assay

Acute, 1-hour treatment of A549 spheroids with TGF-β1 (100 ng/mL) resulted in a four-fold increase in nuclear phosphorylated SMAD2/3 (Figure 4A to C). To examine this response in a more complex (multicellular) context, we evaluated the cellular responses to sustained TGF-β1 treatment in a spheroid outgrowth assay. Preformed A549 spheroids were seeded (one spheroid per well) in a 96-well microplate precoated with collagen matrix and cultured for 3 to 4 days in the presence or absence of TGF-β1 (100 ng/mL).

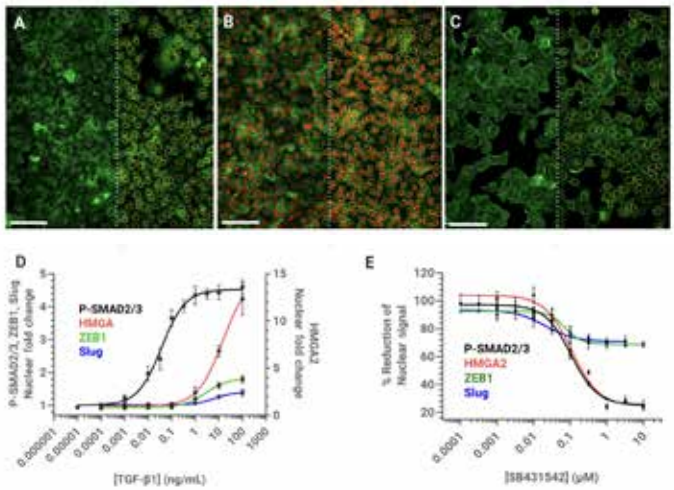

Figure 3. TGF-β1-mediated SMAD phosphorylation and spatial translocation can be quantified in A549 cells. Following a serum starvation period, A549 cells were treated for one hour with (A) vehicle control, (B) 100 ng/mL TGF-β1, or (C) 100 ng/mL TGF-β1 + 10 ng/mL of the TGF-β1 inhibitor SB431542. Cells were then fixed and immunostained for phosphorylated SMAD2/3 or the EMT markers HMGA2, ZEB1, and Slug, and counterstained with Hoechst 34580 and AF488-phalloidin (scale bar = 100 µm). (D) High-throughput imaging in a 96-well plate using nuclear phosphorylated SMAD2/3, HMGA2, ZEB1, or Slug signal as a readout established a respective dose-dependent response to TGF-β1. (E) TGF-β1 inhibition by SB431542 is dose dependent for phosphorylated SMAD2/3, HMGA2, ZEB1, and Slug. The right halves of A, B, and C illustrate the nuclear masking methods used to quantify phosphorylated SMAD2/3 signal, as described in the "Experimental" section.

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Figure 4

Being a 3D, multicellular model, spheroids present technical challenges in sample preparation, immunostaining, and image analysis. SMAD4 (D3R4N) XP Rabbit mAb was selected as the immunostaining reagent, due to its validation and approval for use in complex biological samples (e.g., FFPE). Spheroids were imaged using the confocal imaging mode of the Cytation C10, which enables optical resolution of Z-planes in a 3D biological sample. After immunostaining, spheroids were counterstained with the Hoechst 34580 and CF633- conjugated phalloidin, and a maximum-intensity projection was generated and analyzed, which consisted of a stitched 3 x 3 montage comprised of a 101-slice Z-stack (2 µm step size) spanning 200 µm (Figure 4D to F). In contrast to vehicle-treated controls, spheroids cultured in the presence of TGF-β1 exhibited dramatic out-migration of cells from the initial (core) spheroid mass, consistent with the increased migratory capacity of cells undergoing EMT (Figure 4F). The extent of cell extrusion from the spheroid mass was furthermore positively correlated with increased nuclear SMAD4 signal (Figure 4G to I), clearly visible in cells outside of the core spheroid (Figure 4H). These observations confirm activation of the TGF-β pathway activation in TGF-β1-treated spheroids exhibiting phenotypic changes consistent with EMT.

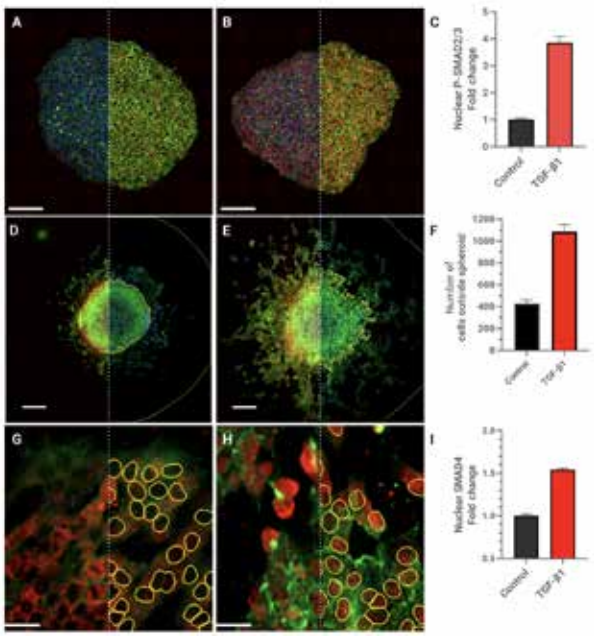

Figure 4. Phosphorylated SMAD2/3 and SMAD4 nuclear translocation can be quantified in a 3D spheroid outgrowth model. (A-C) A maximum projection of an A549 spheroid cultured in suspension, treated with (A) vehicle, or (B) TGF-β1, immunostained for phosphorylated SMAD2/3 (red), and counterstained with phalloidin (green) and Hoechst 34580 (blue). (C) Quantification of nuclear phosphorylated SMAD2/3 using DAPI signal as a primary mask. Scale bar = 100 µm. (D to F) A maximum projection from a 3 x 3 montage Z-stack of an A549 spheroid demonstrates increased out-migration of cells from the core spheroid mass in the presence of (E) 100 ng/mL TGF-β1 compared to (D) vehicle-treated controls. Scale bar = 100 µm. (F) Quantitative analysis of spheroid cell out-migration in response to sustained treatment with TGF- β1 (100 ng/mL). (G-I) Insets indicated in panels D and E. Increased nuclear SMAD4 observed after sustained treatment with (G) 100 ng/mL TGF-β1 versus (H) control; (I) quantification of nuclear SMAD4 signal in spheroid cells in response to sustained treatment with TGF- β1 (100 ng/mL). Scale bar = 50 µm. The right halves of A, B, D, E, G, and H illustrate the nuclear masking methods used to quantify nuclear SMAD signal, as described in the "Experimental" section.

Conclusion

Technology, combined with the multifunctional capabilities of the Agilent BioTek Cytation C10 confocal imaging reader and the Agilent BioTek Gen5 microplate reader and imager software, together enable high-throughput, quantitative analysis of TGF-β pathway activation in 2D and 3D cellular models.

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