High-Throughput Methods to Quantitatively Evaluate TGF-β Signaling in Angiogenesis

A multiscale approach spanning biochemical through 3D cell culture

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Abstract

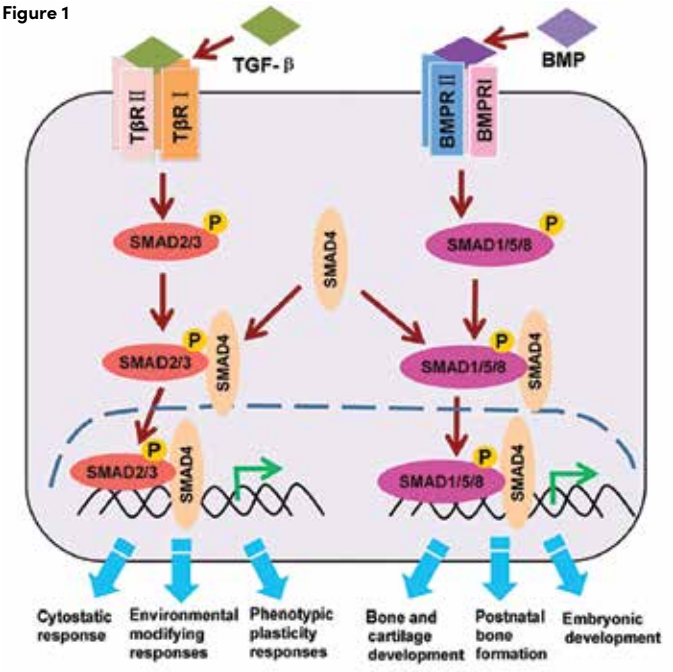
Angiogenesis and cell proliferation are two biological processes that are fundamentally required for growth and homeostasis of tissues. Disruptions to these processes can lead to disease—an observation that is underscored by their identification as foundational hallmarks of cancer. Central to controlling both processes is the TGF-β superfamily of growth factor ligands, which elicit receptormediated responses in cells primarily via the TGF-β and BMP signaling pathways. In both pathways, SMAD proteins serve as the primary downstream effector molecules. Their activities are regulated primarily through receptor-mediated phosphorylation. The magnitude and duration of ligand-induced receptor activation influences the level of SMAD phosphorylation events, which, in turn, influences the magnitude of the downstream cellular response(s). In this study, ligand-induced phosphorylation of receptor-mediated SMAD proteins (R-SMADs) within both TGF-β and BMP signaling axes were quantitatively evaluated. Also, the downstream biological effects of R-SMADs were evaluated at three distinct levels of biological complexity: 1) biochemical; 2) cellular; and 3) tissue, using three-dimensional (3D) spheroid models. Collectively, these three levels of complexity enable a comprehensive evaluation of the SMAD-dependent TGF-β signaling pathway.

Introduction

Angiogenesis and cell proliferation are fundamental biological processes, disruptions to which have been identified as two hallmarks of cancer.[1] Central to controlling both processes are the TGF-β and BMP signaling pathways, for which R-SMAD proteins represent primary downstream effector molecules[2-5]. Ligand–receptor binding elicits the formation of heterotetrameric type I/II receptor complexes, which subsequently undergo site-specific autophosphorylation events. The activated receptor complexes recruit R-SMADs, which are then activated through receptor-kinase-mediated phosphorylation. In the canonical context, TGF-β ligand–receptor interactions result in recruitment and activation of SMAD2/3 proteins, whereas BMP ligand-induced signaling leads to phosphorylation of SMAD1/5/9 (SMAD9 is sometimes referred to as SMAD8). Phosphorylated R-SMADs subsequently form a ternary complex with SMAD4, and this complex is translocated to the nucleus (**Figure 1**), where it functions to modulate the transcriptional activity of target genes.[6,7]

The magnitude of R-SMAD phosphorylation and the resulting functional output (e.g., stimulated angiogenesis or attenuated cell proliferation) depends, in part, on the magnitude and duration of ligand–receptor interaction[8]. This study demonstrates how the use of phosphorylationspecific antibody reagents on the Agilent BioTek Cytation C10 confocal imaging reader can provide a robust and sophisticated method to quantitatively evaluate activation of TGF-β and BMP signaling pathways at multiple levels of biological organization: 1) biochemical; 2) cellular; and 3) tissue, using representative 3D models.

Figure 1



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Experimental

Materials

Cell lines

Immortalized human fibroblasts (CI-huFIB, part number INS-CI-1010) were a kind gift from InSCREENex GmbH (Braunschweig, Germany). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Bioscience (part number C2519A; Basel, Switzerland). CI-huFIBs were cultured in Advanced DMEM (part number 12491; Gibco Thermo Fisher Scientific; Waltham, MA, U.S.) containing 10% FBS and 1x penicillin/streptomycin/glutamine. HUVECs were maintained inEGM-2 Endothelial Cell Growth Medium-2 BulletKit (part number CC-3162; Lonza Bio science).

Assay reagents

Human TGF-β1 recombinant protein (part number 75362) and human BMP-2 recombinant protein (part number 4697) were from Cell Signaling Technology ("CST", Danvers, MA, U.S.). Recombinant human BMP-6 protein (507-BP) was from R&D Systems (Minneapolis, MN, U.S. Cell lysis buffer (10x) was from CST (part number 9803). PathScan PhosphoSmad2 (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. Phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD9 (Ser465/467) (D5B10) Rabbit mAb (part number 13820, CST) detects SMAD1, SMAD5, and/or SMAD9 proteins, only when phosphorylated at the indicated amino acid residues, and is approved for use in western blot, immunoprecipitation, immunocytochemistry, and flow cytometry. 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.

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) diluted in DPBS for 30 minutes, followed by three washes with DPBS prior to cell seeding.

Cell seeding and growth factor treatment

Before exposure with defined concentrations of growth factors, cells seeded in culture plates (12-well plates for PathScan Sandwich ELISA, and Agilent 96-well plates from Agilent Technologies, Inc. (Santa Clara, CA, U.S.) for 2D cultures (part number 204626-100)) were subjected to an overnight, 18-hour serum-starvation period using the same basal culture medium as listed above, lacking FBS (A-DMEM) or BulletKit supplements (EBM). Cells were treated for 60 minutes at 37 °C with human TGF-β1 recombinant protein or human BMP-2 recombinant protein (both from CST), or recombinant human BMP-6 protein at the indicated concentrations. After treatment, cells were either fixed in 4% paraformaldehyde for 10 minutes for immunostaining analysis, or used for lysate collection using 1x cell lysis buffer supplemented with cOmplete, Mini Protease Inhibitor Cocktail (1186153001; Roche Diagnostics GmbH, Mannheim, Germany).

Three-dimensional sprouting angiogenesis assay

The sprouting angiogenesis assay was conducted in a fibrinogen matrix as previously described[8-10] with the following adaptations: 1,000-cell HUVEC spheroids were preformed in 96-well ULA round-bottom microplates (part number 650979; Greiner Bio-One, Monroe, NC, U.S.). Spheroids were then transferred to an Agilent 96-well imaging microplate, one spheroid per well (part number 204626-100) immediately after initiating fibrinogen matrix gelation. Fibrinogen matrix gelation was set up by mixing 80 µL of a 2 mg/mL fibrinogen + 0.15 U/mL aprotonin premix with 1 µL of 50 U/mL thrombin in the 96-well imaging microplate. Once the spheroids were embedded in the matrix, the plate was allowed to set for 30 minutes at 37 °C in a TC incubator. Normal human lung fibroblast (NHLF)-enriched EGM-2 media was overlaid on top of the fibrinogen gel, and the spheroids were allowed to culture for three to five days.

Sample preparation

For ELISA analysis, 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 then collected and stored at –80 °C, and thawed the day of use. For 2D cellular assays, following growth factor treatment, cells were 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% BSA. Cells were then incubated overnight at 4 °C with the indicated antibody prepared at the recommended dilution range by the manufacturer in "ImmunoWash Buffer" (5% BSA containing 0.1% Tween 20). After three washes with PBS + 0.1% Tween 20, cells were incubated with fluorophore-conjugated goat anti-rabbit polyclonal antibodies (part number 4413, CST; or part number 20131, Biotium) diluted in 5% BSA + 0.1% Tween 20 and the indicated counterstains. For the 3D sprouting angiogenesis 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. Sprouts were then permeabilized with 0.5% Triton X-100 for 1 hour, followed by an overnight block with 5% BSA at 4 °C. The following steps entail an overnight incubation at 4 °C: primary antibody diluted to 0.5 µg/mL in 5% BSA + 0.1% Tween 20, followed by hourly washes with ImmunoWash Buffer with a final overnight wash. Samples were then incubated with secondary antibodies and counterstains diluted in 5% BSA + 0.1% Tween 20, followed by hourly washes with ImmunoWash Buffer with a final overnight wash at 4 °C.

Imaging and image processing

Two-dimensional cell samples were imaged in the Cytation C10 using a 20x 0.4 NA objective in widefield mode. Several 2 µm step-size multichannel Z-stacks were captured with a range that encompassed the nuclei. Multichannel, 2 x 2 montage Z-stacks of angiogenic sprouts were imaged using a 20x 0.4 NA objective, but in confocal mode using the 60 µm disc. Montages were then stitched. Multichannel Z-stacks underwent a background reduction transformation step before generating a maximum intensity project. Cellular analysis steps entailed creating a primary mask for nuclei and a secondary mask in the channel for phosphorylated SMAD signal.

Integrated fluorescence signal from the phosphorylated SMAD secondary mask was converted to fold change relative to the signal of the untreated control samples.

Results and discussion

Materials

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

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 that is measured by absorbance at 450 nm. Phosphorylated SMAD2/3 values were normalized against total SMAD2/3 protein values to control for any variations in the quantity of SMAD proteins in response to treatment. Using the plate-reader function of Cytation C10, we demonstrate dose-dependent TGF-β1-induced SMAD2/3 phosphorylation in lysates from immortalized human fibroblasts (huFIBs), with a calculated EC_{50} value of 0.034 ng/mL (**Figure 2**).

Figure 2

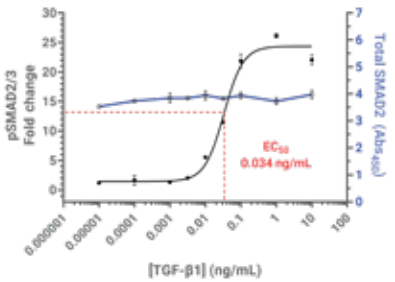

Figure 2. Dose-dependent SMAD2/3 phosphorylation in response to TGF-β1. The PathScan sandwich ELISA kit quantifies TGF-β1-dependent SMAD2/3 phosphorylation in huFIBs. The phosphorylated SMAD2/3 values (pSMAD2/3, black) are reported as fold change above no treatment control, which were normalized for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

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.

As shown in **Figure 3A** and **B**, treatment of huFIB cells with TGF-β1 (1 hour at 100 ng/mL) resulted in a detectable increase in nuclear-localized phosphorylated SMAD2/3 compared with vehicle-treated controls. Likewise, treatment of HUVEC cells with BMP-6 (1 hour at 100 ng/mL) resulted in an increase in nuclear phosphorylated SMAD1/5/9 relative to vehicle-treated controls. Using a high-throughput, 96-well format for analysis, dose–response curves were generated for each cell type in response to growth factor treatment. The EC_{50} for huFIBs treated with TGF-β1 was 0.039 ng/mL (**Figure 3C**), whereas the EC_{50} HUVECs treated with BMP-6 was 6.68 ng/mL (**Figure 3F**).

Visualizing SMAD4 expression in a sprouting angiogenesis assay

In vitro model systems are essential experimental tools that provide foundational insights into more complex in vivo biological processes. The sprouting angiogenesis assay is a well-established in vitro model system that recapitulates many basic mechanisms governing angiogenesis. In this study, we used an in vitro sprouting angiogenesis assay to examine selected aspects of TGF-β signaling during angiogenesis. Preformed HUVEC spheroids were embedded in a fibrin matrix within wells of a 96-well microplate (one spheroid/well). Normal human lung fibroblast-enriched EGM-2 was applied to the matrix-embedded spheroids, which were then cultured for 4 days at 37 °C. During this time, individual HUVEC cells emerge from the spheroid mass as sprouting projections, reminiscent of nascent blood vessels. Being a 3D biological model, the sprouting angiogenesis assay presents optical challenges during both sample preparation and imaging. Samples were therefore imaged with the confocal imaging mode of the Cytation C10, which enables optical resolution of Z-planes in a 3D biological sample. SMAD4 (D3R4N) XP Rabbit mAb was selected for this analysis, due to its validation and approval for use in both immunocytochemistry and immunohistochemistry. A maximum intensity projection of a 187-slice Z-stack (1 µm step size) of an angiogenic sprout was generated after immunostaining the spheroid with SMAD4 (D3R4N) XP® Rabbit mAb and an endothelial-cell-specific antibody, CD31 (PECAM-1) Monoclonal Antibody (Gi18), and counterstained with the nuclear marker DRAQ7 (**Figure 4A**). SMAD4 expression was confirmed within angiogenic sprouts emerging from the core spheroid (**Figure 4B** to **D**) supporting a functional role for TGF-β signaling during sprouting angiogenesis.

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Results and discussion

Figure 3

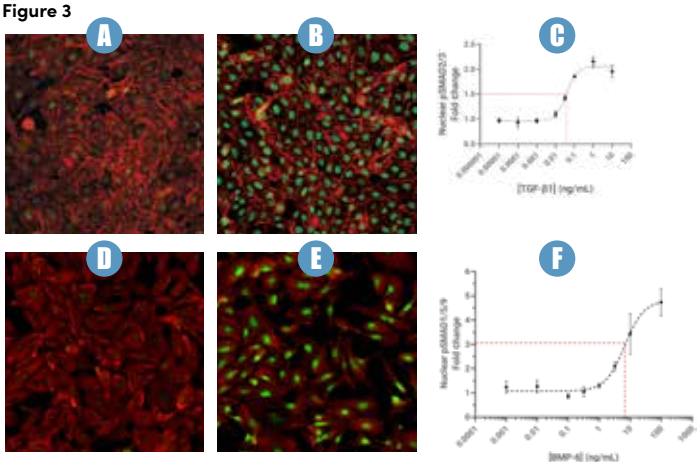

Figure 3. SMAD phosphorylation and subcellular localization can be quantified in 2D cell cultures. Following serum-starvation, huFIBs were treated with either vehicle control (**A**) or 100 ng/mL TGF-β1 (**B**) for 1 hour, whereas HUVECs were treated with vehicle control (**D**) or 100 ng/mL BMP-6 (**E**) for 1 hour. Cells were then fixed and immunostained for either phosphorylated SMAD2/3 (pSMAD2/3) (**A**, **B**) or phosphorylated SMAD1/5/9 (pSMAD1/5/9) (**D**, **E**) and counterstained with Hoechst 34580 and AF488-phalloidin.

Figure 4

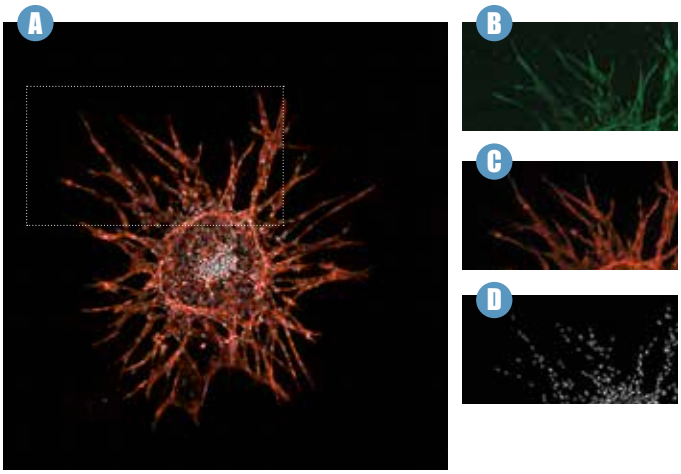

Figure 4. SMAD4 expression can be imaged in a 3D sprouting angiogenesis model. After culturing for 4 days, a HUVEC spheroid embedded in a fibrinogen matrix that developed angiogenic sprouts was fixed and immuno-stained with an anti-SMAD4 and an anti-PECAM1 antibody, and counterstained with DRAQ7. (**A**) Maximum projection from a 3 x 3 montage Z-stack of a HUVEC spheroid with angiogenic sprouts. (**B**) to (**D**) shows the inset region in (**A**) with the signal for (**B**) SMAD4, (**C**) PECAM-1, and (**D**) DRAQ7.

Conclusion

Rigorously validated recombinant monoclonal antibody reagents from Cell Signaling Technology, combined with the multifunctional capabilities of the Agilent BioTek Cytation C10 confocal imaging reader enable robust quantitative analysis of TGF-β and BMP pathway activation at different biological scales.

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