A Multiscale Approach to Quantitatively Evaluate the SMAD Signaling Pathway

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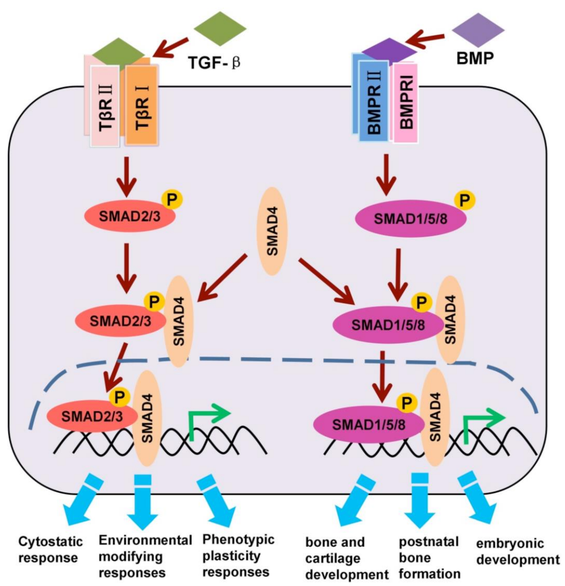
Introduction

Angiogenesis and cell proliferation are fundamental biological processes, and their pathologic states represent two hallmarks of cancer. Central to controlling both processes is the TGF-β/BMP signaling pathway, for which the SMAD proteins serve as major downstream effector molecules. Upon ligand binding, TGF-β or BMP type I/II receptor heterotetramers phosphorylate receptor-regulated SMADs (R-SMADs). TGF-β-receptor binding leads to SMAD2/3 phosphorylation, whereas BMP-receptor binding induces phosphorylation of SMADs 1/5/8. Phosphorylated SMADs (pSMADs) then form a complex with the co-SMAD SMAD4, at which point they undergo nuclear translocation, where they operate as transcriptional modulators of target genes. The magnitude of SMAD phosphorylation and functional output (e.g., stimulated angiogenesis or attenuated cell proliferation) depends on both the ligand concentration and the duration of exposure. In this study, we quantitatively evaluate the phosphorylation states of R-SMADs, as well as their downstream biological effects at three different biological scales: 1) biochemical; 2) cellular; and 3) tissue/3D models. At the biochemical level, the temporal and dose-dependent phosphorylation of R-SMADs in response to growth factor stimulation can be quantified using a quantitative ELISA approach. Growth factor stimulation can be quantified at the cellular level by high-content imaging of SMAD phosphorylation and nuclear translocation, while simultaneously monitoring cell proliferation rates. Lastly, high-resolution confocal imaging can be employed to observe developmental consequences of phospho-SMAD signaling using a HUVEC-based 3D angiogenesis model system.

Canonical TGF-β/SMAD and BMP/SMAD signaling pathway



Cells. 2014, *3* (4), 981–993. doi: 10.3390/cells3040981

Experimental

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). A549 lung carcinoma cells were purchased from ATCC (part number CCL-185; Manassas, VA, USA). CI-huFIBs and A549s cells were cultured in Advanced DMEM (part number 12491; Gibco Thermo Fisher Scientific; Waltham, MA, USA) containing 10% FBS and 1x penicillin/streptomycin/glutamine. HUVECs were maintained in Endothelial Cell Grown Medium-2 BulletKit (EGM-2; part numbers CC-3162; Lonza).

Growth factor treatment

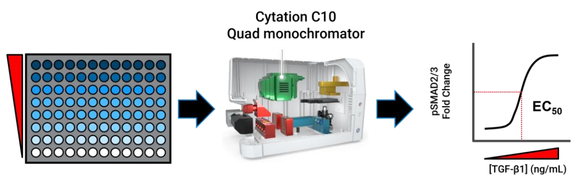
Before exposure with defined concentrations of growth factors, cells seeded in culture plates (12-well for PathScan sandwich ELISA and 96-well for 2D cultures) were subjected to an overnight, 18-hour serum-starvation period using the same basal culture medium as listed above, lacking FBS (A-DMEM) or BulletKit supplements (EBM). Growth factor treatment was conducted by adding hTGF-β1, hBMP-2 (part numbers 75362 and 4697, respectively; Cell Signaling Technology, Danvers, MA, USA), or rhBMP-6 (part number 507-BP; R&D Systems, Minneapolis, MN, USA) diluted in serum-free media to adherent cells at the indicated concentrations and allowed to incubate for 1 hour, followed by 4% paraformaldehyde fixation and immunostaining.

Results and Discussion

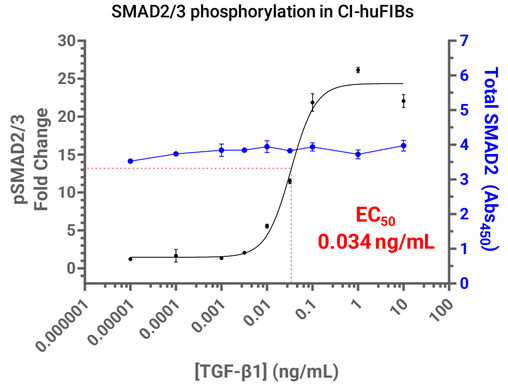
Biochemical analysis: high-throughput ELISA assay

Cell Signaling Technology PathScan sandwich ELISA kits:

- Phospho-SMAD2 (S465/467)/SMAD3 (S423/425), part number #12001
- Total SMAD2/3, part number #12000



SMAD2/3 phosphorylation in CI-huFIBs

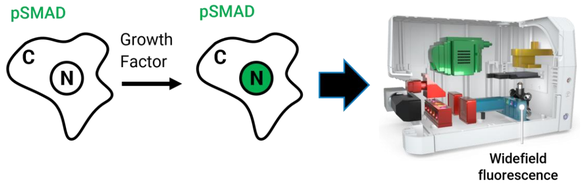


Results and Discussion

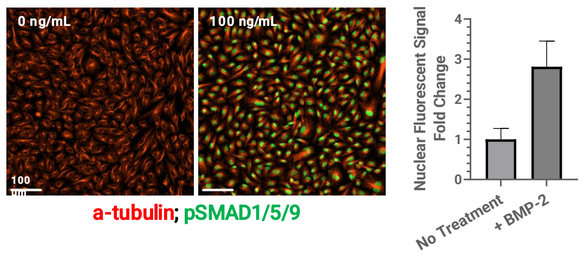
Cellular assay: nuclear translocation

Cell Signaling Technology antibodies:

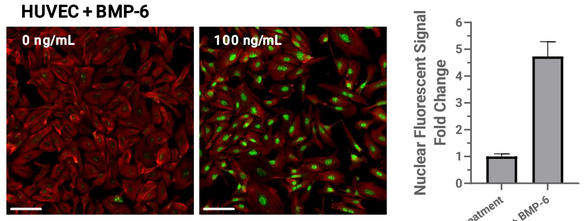
- Phospho-SMAD2 (S465/467) (E8R3R), part number #56532
- Phospho-SMAD1 (S463/465)/SMAD5 (S463/465)/SMAD9 (S465/467) (D3B10), part number 55605



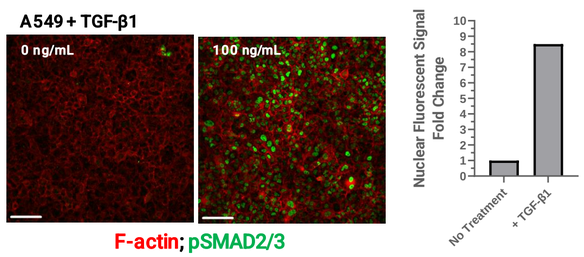
CI-huFIB + BMP-2



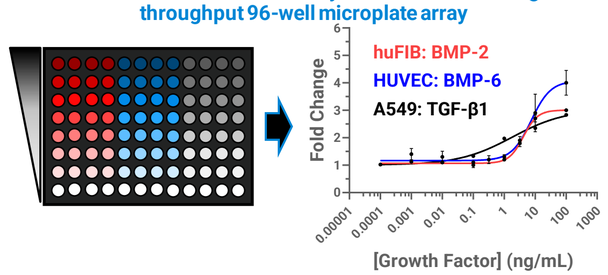
HUVEC + BMP-6



A549 + TGF-β1



The nuclear translocation assay can be scaled to a high-throughput 96-well microplate array

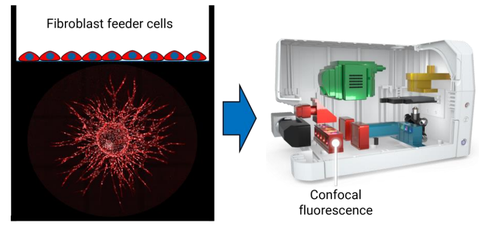


Results and Discussion

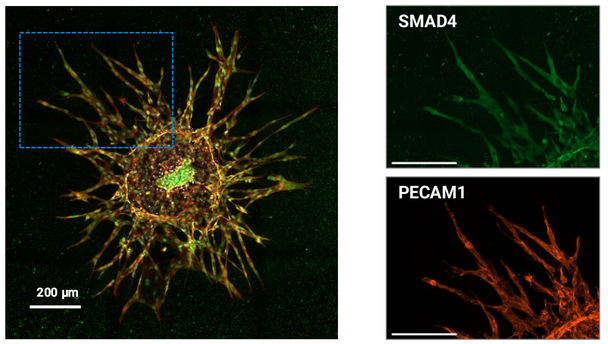
3D assay: spheroid sprouting angiogenesis, and EMT model

Cell Signaling Technology antibodies:

- SMAD4 (D3R4N), part number #24483



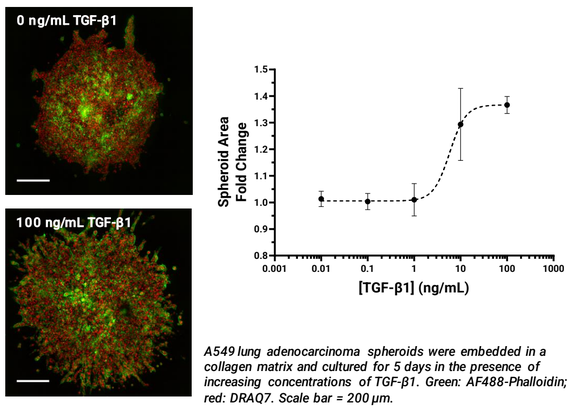
CST reagents can be used for antibody-based detection of SMAD4 expression during in vitro angiogenesis development



Maximum intensity projection of a 187-slice Z-stack spanning 185 µm.
Green: SMAD4; red: PECAM1; gray: DRAQ7.

Enlarged region of the box on the left panel

A549 lung adenocarcinoma spheroids serve as a 3D model for EMT when cultured in the presence of TGF-β1



A549 lung adenocarcinoma spheroids were embedded in a collagen matrix and cultured for 5 days in the presence of increasing concentrations of TGF-β1. Green: AF488-Phalloidin; red: DRAQ7. Scale bar = 200 µm.

Conclusion

The high-quality reagents from Cell Signaling Technology combined with the multifunctional capabilities of the Agilent BioTek Cytation C10 confocal imaging reader enable quantitative evaluation of the TGF-β/BMP pathway activation at different biological scales.

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