Quantitative evaluation of biomarkers for TGF-β-induced epithelial-mesenchymal transition in biochemical, cellular, and 3D spheroid model systems

Ernest Heimsath[1], Antony Wood[2]

[1] Agilent Technologies, Inc.

[2] Cell Signaling Technology, Inc.

AACR 2024

Poster # 13
Abstract # 204

Introduction

Epithelial-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 the downstream cellular responses. Activation of the TGF-β-SMAD2/3 pathway is known to upregulate the expression levels of numerous EMT-associated genes, including *HMGA2*, *ZEB1*, *Snail1*, and *Slug*, all of which have furthermore been associated with cancer metastasis. In this study, we describe 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 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.

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

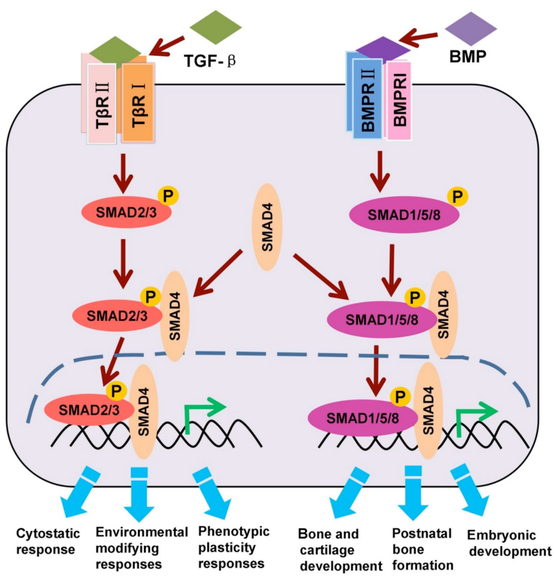


Figure 1. *The canonical TGF-β/BMP/SMAD signaling pathway (adaptation). DOI: 10.3390/cells3040981*

Experimental

Cell lines

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

Growth factor treatment

Before TGF-β1 exposure, adherent cells, or spheroids were subjected to an overnight, 18-hour serum-starvation period using A-DMEM without FBS. Human TGF-β1 recombinant protein (part number 75362; Cell Signaling Technology, Danvers, MA) was diluted in serum-free media and added to cell and spheroid cultures at indicated concentrations. 2D cell cultures were treated with TGF-β1 for 1 hour to measure SMAD protein phosphorylation responses, or 48 hours to measure expression changes in EMT-associated proteins. 3D spheroids were treated with TGF-β1 for 4 days to enable spheroid outgrowth. Cells and spheroids were then fixed in 4% buffered paraformaldehyde for subsequent immunostaining.

Imaging

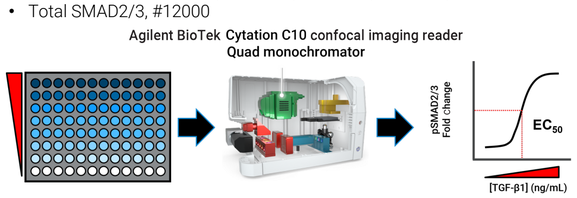
Using the Agilent BioTek Cytation C10 confocal imaging reader equipped with a 60x 1.2 NA *water immersion* objective, a single Z-plane of adherent cells was imaged in three channels: GFP (F-actin; AF488-conjugated phalloidin), CY5 (phosphorylated SMAD2/3; CF633-conjugated goat anti-rabbit), and DAPI (nuclei; Hoechst 34580), with the DAPI channel used to set Z-focus height using laser autofocus. With the same three channels, multichannel Z-stacks of spheroids were imaged in confocal mode using the *deep-sectioning* 60 µm disk and a 20x 0.4 NA objective. Multichannel Z-stacks underwent a background reduction transformation step before generating a maximum intensity project.

Results and Discussion

Biochemical analysis—high-throughput ELISA assay

Cell Signaling Technology PathScan® Sandwich ELISA kits:

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



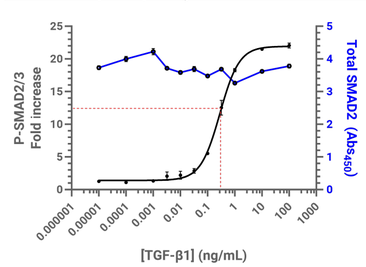


Figure 2. *Dose-dependent SMAD2/3 phosphorylation (black) in response to TGF-β1. The phosphorylated signal is normalized to total SMAD2 (blue).*

Results and Discussion

Cellular assay—high-throughput nuclear translocation assay

Cell Signaling Technology antibodies:

- Phosphorylated SMAD2 (Ser465/Ser467) (E8R3R), #18338
- HMGA2 (D1A7) Rabbit mAb, #8179
- ZEB (E2G6Y) XP Rabbit mAb, #70512
- Slug (C18G7) Rabbit mAb, #9585

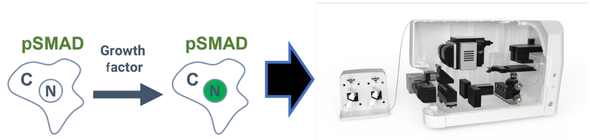


Figure 3A. *TGF-β1-induced SMAD2/3 phosphorylation and nuclear translocation, which is blocked by the TGF-β1 receptor inhibitor SB431542.* The right half of each image illustrates the image analysis carried out in Agilent BioTek Gen5 software, where phosphorylated SMAD2 fluorescence was quantified within each nuclei.

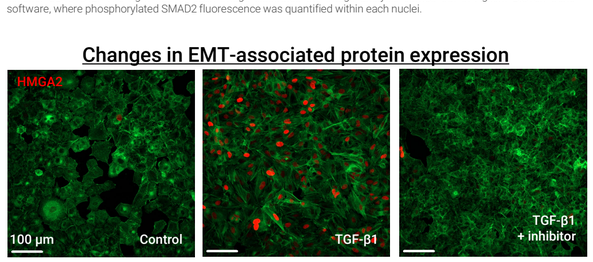


Figure 3B. *Increased HMGA2 expression in response to TGF-β1 treatment (48-h). Increased HMGA2 expression is blocked by the TGF-β1 receptor inhibitor SB431542.*

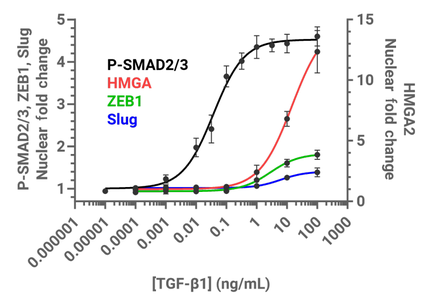


Figure 3C. *Dose-response curves of TGF-β1-induced nuclear expression of phosphorylated SMAD2/3 (P-SMAD2/3), HMGA2, ZEB1, and Slug.*

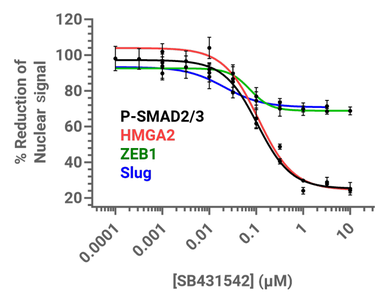


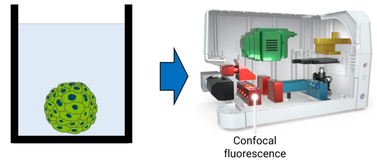
Figure 3D. *Dose-dependent inhibition of TGF-β1-induced SMAD2/3 phosphorylation, and expression of HMGA2, ZEB1, and Slug.*

Results and Discussion

3D assay—spheroid model of EMT

Cell Signaling Technology antibodies:

- Phosphorylated SMAD2 (Ser465/Ser467) (E8R3R) Rabbit mAb, #18338
- SMAD4 (D3R4N) XP Rabbit mAb, #46535



High-throughput imaging and quantification of TGF-β1-induced phosphorylation and nuclear translocation of SMAD2 in 3D spheroids

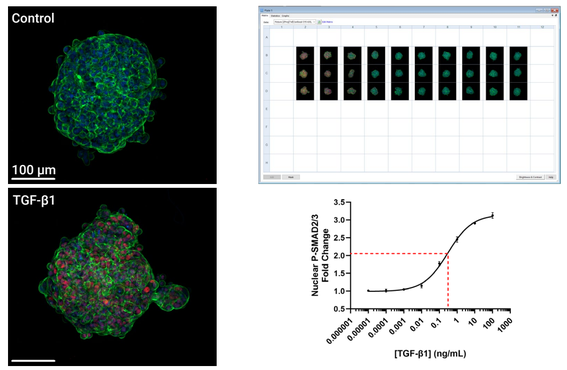


Figure 4. *(Left) Maximum intensity projections of A549 spheroids treated with vehicle control, or 100 ng/mL TGF-β1. Red: SMAD2, Green: F-actin, make unavailable: Nuclei. (Right, top) Plate layout of an experiment where triplicate spheroids were treated with decreasing amounts of TGF-β1 (left to right). (Right, bottom) Quantified nuclear phosphorylated SMAD2 at each concentration assayed, with an EC_{50} of 0.31 ng/mL.*

Quantitative analysis of TGF-β1-induced total SMAD4 protein nuclear translocation in 3D spheroids.

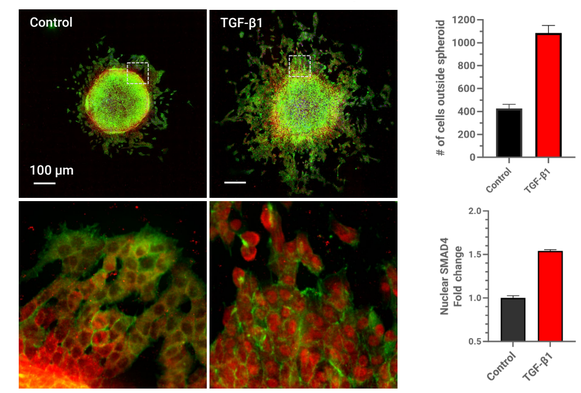


Figure 5. *A549 spheroids were cultured on top of a collagen gel for 4 days in the presence/absence of TGF-β1. Green: F-actin, Red: SMAD4. Bottom panels show an enlarged view of boxed regions in the top panels.*

Conclusion

Recombinant monoclonal antibodies from Cell Signaling Technology combined with the multifunctional capabilities of the Agilent BioTek Cytation C10 confocal imaging reader enables quantitative evaluation of TGF-β1-induced EMT pathway activation at different biological scales.