Chapter 25

Measuring TGF-β Ligand Dynamics in Culture Medium

Zipei Feng, Zhike Zi, and Xuedong Liu

Abstract

TGF-β plays an important role in a myriad of cell activities including differentiation, proliferation, and growth arrest. These effects are influenced by the concentration of TGF-β in the surrounding milieu, which is interpreted by mammalian cells and subsequently translated into meaningful signals that guide their proliferation, survival, or death. To predict cellular responses to TGF-ß signaling based on molecular mechanisms, it is important to consider how cells respond to different ligand doses and how variations in ligand exposure impact Smad signaling dynamics and subsequent gene expression. Here we describe methods to measure TGF-β concentration in the environment and approaches to perturb cellular TGF-β exposure to gain a quantitative understanding of signaling dynamics of this pathway.

Key words TGF-β, Signaling, SMAD, Switch-like response

1 Introduction

Transforming growth factor β (TGF-β) is a cytokine that regulates a diverse group of cellular behaviors such as proliferation, differentiation, and growth arrest [1]. These influences in a broader perspective lead to the implication of TGF-β in cancer, cardiac and pulmonary diseases, congenital syndromes such as Marfan and Loeys-Dietz, and diabetes [2]. The classic TGF-β signaling cascade begins as TGF-β ligand binds to TGF-β receptor type 2 (TβRII), bringing it in close proximity to TGF-β receptor type 1 (TβRI) [1]. Next, the constitutively active kinase domain on TβRII transphosphorylates serine and threonine residues of TβRI, which then becomes active and passes the signal downstream to the SMAD proteins [1]. These SMAD proteins include receptor-regulated SMADs or R-SMADs (SMAD2 and SMAD3 in the TGF-β pathway), the common mediator SMAD (Co-SMAD, i.e., SMAD4), and the inhibitory SMAD (I-SMAD, i.e., SMAD6 and SMAD7) [3]. SMAD2 and SMAD3 directly interact with activated TβRI. They are phosphorylated by TβRI at the two distal serines of their C-terminal SSXS motif [4–6]. The phosphorylation event then

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triggers the homo-oligomerization of SMAD2 and SMAD3 and their hetero-oligomerization with SMAD4, resulting in the nuclear accumulation of these complexes followed by transcriptional regulation of various genes [7].

Similar to many other cell processes, TGF-β signaling is highly regulated. The cell is able to sense minor changes in the concentration of signal in the surrounding environment and translate them into largely different corresponding actions. In cell culture studies, exposing cells to different concentrations of TGF-β is able to elicit a switch-like response in transcription and translation of different genes such as PAI-1 [8]. Similar behavior is observed in human and animal models. For example, bone morphogenic protein (BMP), a member of TGF-β superfamily, is crucial in the development of dorsal part of spinal cord and brainstem [9]. The continual release of BMP by non-neural ectodermal cells during development leads to the formation of a concentration gradient in the adjacent dorsal neurotube [9]. The part of the neurotube that receives the highest amount of BMP is signaled to differentiate into somatic sensory neurons, while the cells farther away from the roof plate receiving less BMP are signaled to become visceral afferent neurons [9]. Another well-studied example is Dpp, also a member of the TGF-β superfamily, whose concentration gradient is again absolutely essential in the proper wing development of *Drosophila spp* [7].

The presence of TGF-β ligand is one way that cell response could be affected [8]. When the ligands are withdrawn from the environment, cells lose the stimulation and the phosphorylation of SMAD2 is shut down in a matter of hours [8]. Here we describe a method to efficiently remove most of the TGF-β molecules in the surrounding medium and induce a transient phospho-SMAD2 response, which is lower in amplitude and duration compared to the response elicited when there is sustained TGF-β in the extracellular milieu (Figs. 1 and 2).

Additionally, it seems that cells can also regulate the response to TGF-β signaling through ligand depletion [8, 10]. When the ligand depletion is blocked or slowed down, cells lose the switch-like behavior and respond in a Michaelis-Menten-like fashion to a TGF-β concentration gradient [8]. Ligand depletion can be accomplished experimentally by completely blocking endocytosis, which is often difficult due to off-target effects of certain drugs [8]. Alternatively, one can decrease the rate of ligand depletion simply by increasing the medium volume while keeping the absolute number of ligand molecules constant. This method is effective in slowing down the cellular uptake of TGF-β molecules (Fig. 3) and is able to reduce the switch-like behavior of the TGF-β signaling pathway (Fig. 4).

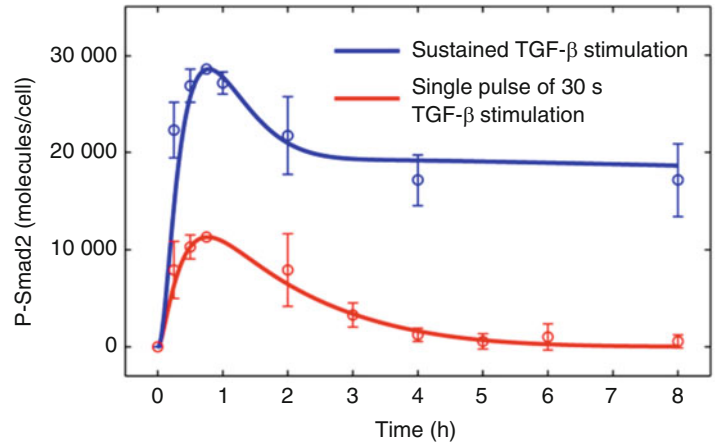

Fig. 1 Phospho-SMAD2 signal following TGF-quantitative analysis. Notice the decrease in amplitude and duration of signal with washout compared to continual stimulation. (Taken from [8])

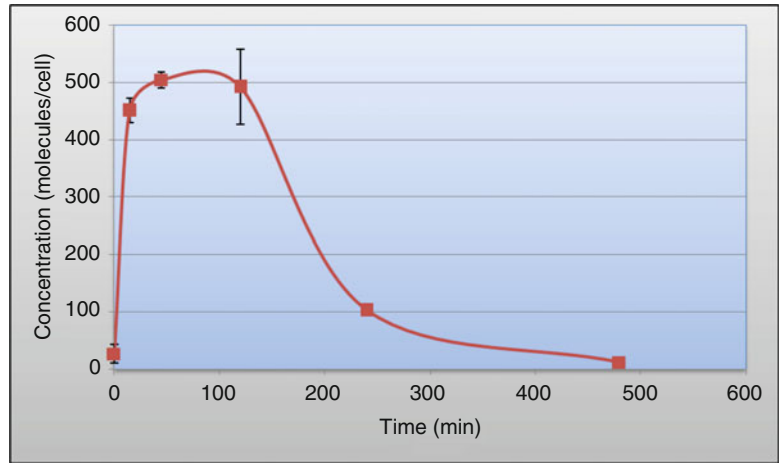

Fig. 2 Numbers of TGF-β molecule per cell after 30 s TGF-β stimulation followed by washout. Minimum amount of TGF-β is left following the washout, and 500 molecules of TGF-β per cell are insufficient to elicit a significant response when exposed to nonstimulated HaCaT cells. (Original data taken from [8])

2 Materials

2.1 Cell Culture

1. Dulbecco's Modified Eagle Medium (DMEM) (GIBCO 12800-082).
2. Fetal Bovine Serum (FBS) (SAFC Biosciences, 12303C).
3. GlutaMAX L-Glutamine Supplement (L-Glu) (GIBCO 35050).
4. Dulbecco's Phosphate Buffered Saline (D-PBS) (GIBCO, Invitrogen 14190-136).

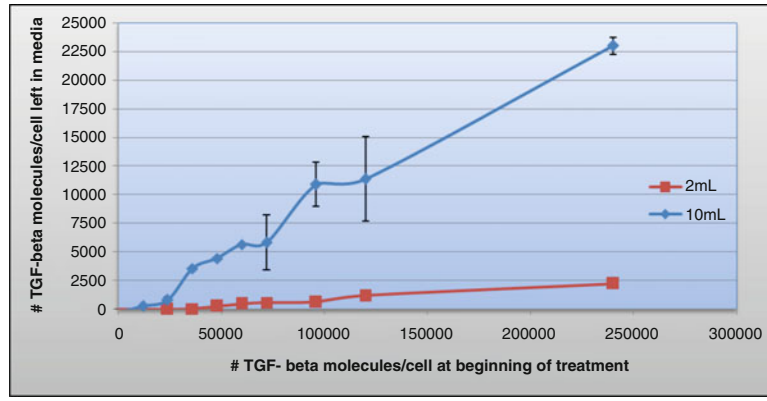

Fig. 3 Number of TGF-β molecules per cell left in media after 24 h treatment. Compared to Fig. 4 we can see that there is a direct relationship between number of TGF-β molecules left in medium and volume of medium. This can be interpreted as slowing down of endocytosis

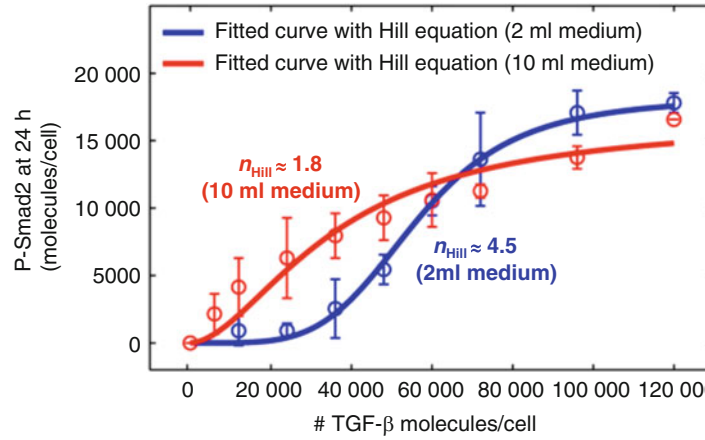

Fig. 4 The effect of increasing volume on phospho-SMAD2 levels. Notice how the switch-like response (described mathematically by Hill's coefficient) is greatly reduced when the volume increased. (Taken from [8])

5. 100× Penicillin G Potassium Solution (ICN Biomedicals, 100548, dissolved in ddH_2O to 10,000 U/mL).
6. 100× Streptomycin Sulfate solution (Sigma S-6501, dissolved in ddH_2O to 10,000 U/mL).
7. Trypsin (0.25 %) (GIBCO, Invitrogen 15050-057).

2.2 Live Cell Treatment

1. Transforming Growth Factor β1 (TGF-β1, stock solution 100 nM) (R&D Systems, 240-B-002).

2.3 Cellular Collection and Analysis

1. RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 % v/v Triton X-100, 0.1 % w/v SDS, 1 % w/v sodium deoxycholate, 1 mM EDTA, 1 mM PMSF (added before use), 25 mM β-glycerophosphate (added before use), and 1 mM sodium orthovanadate (added before use)).
2. Cell Lifter (Costar, Corning 3008).
3. Ponceau S Staining Solution (0.5 g Ponceau S Dye, 5 % Glacial Acetic Acid to 100 mL with deionized water).
4. Protogel 30 % Acrylamide/Bisacrylamide Solution (National Diagnostics, EC-890).
5. Protogel 4× Resolving Gel Buffer (National Diagnostics, EC-892).
6. Protogel Stacking Gel Buffer (National Diagnostics, EC-893).
7. 10 % Sodium Dodecyl Sulfate Solution (10 % w/v SDS in distilled water).
8. Rabbit anti-pSMAD2 antibody (Cell Signaling #3108S).
9. Mouse anti-SMAD1/2/3 antibody (Santa Cruz Biotechnology SC-7960).
10. Mouse anti-β-Actin antibody (Abcam AB-8226100, Cell Signaling #4967).
11. Horse Radish Peroxidase conjugated Sheep anti-mouse antibody (GE Healthcare NA931-1 mL).
12. Horse Radish Peroxidase conjugated Goat anti-rabbit antibody (GE Healthcare RPN4301-1 mL).
13. SuperSignal West Dura Extended Duration Substrate chemiluminescent substrate (Pierce Biotechnology 34076).
14. Protran Nitrocellulose Membrane (Whatman 104024 BA83).
15. Whatman Chromatography Paper (Whatman 3030-917).
16. Semi-dry transfer apparatus (Hoefer TE70).
17. BCA protein assay kit (Thermo Scientific #23225).
18. Spectra broad range multicolor protein ladder (Fermentas SM184).
19. Powerwave X Scanning Spectrophotometer Plate Reader (Bio-Tek).
20. 4× SDS-Gel Loading Buffer (0.08 % w/v Bromophenol Blue, 50 mM EDTA, 40 mM DTT, 40 % v/v glycerol, 8 % w/v SDS, 200 mM Tris–HCl, pH 6.8 in deionized water).
21. Transfer Buffer (5.8 g Glycine, 11.6 g Tris Base, 0.72 g Sodium Dodecyl Sulfate, 400 mL Methanol, to 2 L with deionized water).
22. Tris Buffered Saline supplemented with 0.05 % Tween-20 detergent (TBS-t) (150 mM NaCl, 2 mM KCl, 25 mM Tri-HCl, pH 7.4, 0.05 % Tween 20, in deionized water).

23. 3 % BSA in TBST, filtered with 45 μm filter.
24. Western Blot Film (ISC BioExpress F-9023-8X10).

2.4 Luciferase Assay

1. Promega Dual Luciferase Kit (#E1910).
2. Beckman Coulter Multimode Detector DTX880 (Beckman).

3 Methods

3.1 Cell Culture and Reducing TGF-β Ligand Depletion

1. Working DMEM medium consisting of 10 % FBS, 2 mM L-Glu, 100 U/mL Penicillin G, and 100 U/mL Streptomycin is prepared and stored at 4 °C until use.
2. One 15 cm tissue culture plate is seeded with 8×10^6 adherent human keratinocytes (HaCaT) cells in 20 mL of the working DMEM medium. The cells are allowed to grow for 48 h at 37 °C in a 100 % humidified environment with 5 % CO_2. Medium is changed every 24 h (*see* **Note 1**).
3. Four 6-well plates are labeled as in Fig. 5. Cells are subsequently counted and reseeded into the 6-well plates at a density of 8.5×10^5 cells in 1.5 mL medium per well. Plates are then incubated overnight at 37 °C in a 100 % humidified environment with 5 % CO_2.
4. Next morning, twenty 15-mL conical tubes are prepared. 3 mL of pre-warmed medium is transferred into tubes 1–10 while 11 mL is transferred into tubes 11–20. Cells in the well

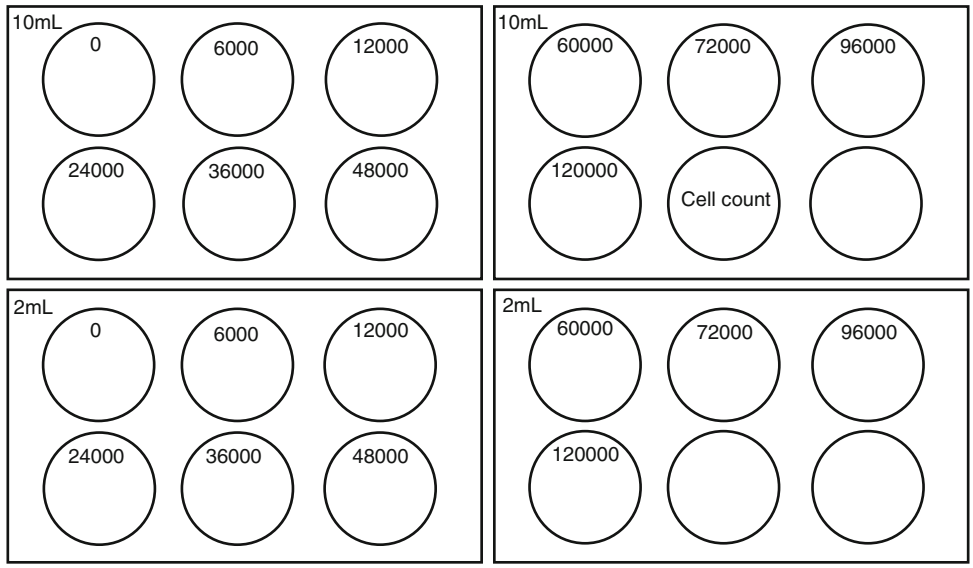

Fig. 5 Plate schematic. The labeled numbers correspond to different number of TGF-β molecules per cell

labeled as "cell count" in Fig. 5 are trypsinized and counted. The cell number is assumed to be the same in all the wells and is used to calculate the amount of TGF-β to be added into each conical tube. The final concentration of TGF-β in each tube is 0, 6000, 12,000, 24,000, 36,000, 48,000, 60,000, 72,000, 96,000, and 120,000 molecules of TGF-β per cell, respectively (Fig. 5). Medium in the remaining 20 wells is aspirated off and replaced with media from the conical tubes containing the corresponding volume and concentration of TGF-β. The amount of media will be 10 mL or 2 mL according to the labels in Fig. 5. Plates are then incubated for 24 h at 37 °C, 100 % humidity and 5 % CO_2 (*see* **Note 2**).

5. Plates are collected from incubator; each well containing roughly 8.5×10^5 adherent HaCaT cells are rinsed three times with 2 mL of pre-chilled D-PBS. D-PBS is subsequently removed by titling the plate vertically at an 80° angle in a bucket full with ice for 30 s and aspirating all liquid from the bottom corner of the plate using a P-200 pipette. 75 μL of RIPA buffer is added to each well, and the cells are scraped with a cell scraper and transferred into pre-chilled 1.7 mL Eppendorf microcentrifuge tubes. The tubes are rotated for 1 h at 4 °C (*see* **Note 3**).
6. Fractions are spun for 10 min at 13,200 × *g* and 4 °C. Supernatants are transferred to new labeled tubes and stored on ice.

3.2 Determining Protein Concentration and Performing Western Blot Analysis

1. Protein concentration is determined according to manufacturer's instructions using a BCA assay kit. In short, serial dilutions of bovine serum albumin (BSA) stock solution (2 mg/mL) are made in distilled water to produce final concentrations of 1.0, 0.5, and 0.25 mg/mL. A blank is also prepared from distilled water alone (*see* **Note 4**).
2. 1:10 Dilutions of cell lysates are made in separate tubes for a total of 20 μL.
3. In a 96-well plate, 5 μL of each unknown or standard is mixed with 100 μL of 1× BCA solution (50:1 mixture of solutions A and B, from the BCA kit) in triplicates. Plates are then completely sealed with Parafilm and incubated at 37 °C for 30 min.
4. The 96-well plate is read for absorbance at 562 nm using a 96-well plate scanning spectrophotometer. The raw data is plotted on Excel and the unknown is calculated using standard BSA concentrations. The yield is roughly 200 μg of protein per 75 μL of the lysate.
5. Cell lysate containing 60 μg of total protein is mixed with 4× SDS loading buffer and incubated at 95 °C for 5 min. Tubes are inverted and liquid contents are briefly spun at 13,200 × *g* for 10 s.

6. Two 1.5 mm, 12 % polyacrylamide gels (SDS-PAGE Protogel reagents from National Diagnostics) are polymerized with a 10-well comb according to manufacturer's instructions. All lanes are loaded with samples and 5 μL of Spectra protein ladder is added to the same lane as cell lysate without TGF-β treatment. Each gel is run at 30 mA until the bromophenol blue dye reaches the bottom of the gel (*see* **Note 5**).
7. Each gel is transferred in a semi-dry western blot horizontal transfer unit, using a sandwich from cathode (bottom piece) to anode (top piece) with the following scheme: three pieces of 0.5-cm (thick) chromatography paper, one piece of nitrocellulose membrane, SDS-PAGE gel containing samples, three pieces of thick chromatography paper. This sandwich is assembled in 100 mL of transfer buffer with gel above the nitrocellulose membrane, and transferred to the transfer apparatus in the same configuration. The sandwich is then lightly ironed with a test tube to ensure no air bubbles are trapped between the membrane and the gel. For each sandwich containing one gel, 45 mA (corresponding to 6 V) of current is applied to the apparatus for 2 h.
8. After the transfer is finished, the nitrocellulose membrane is removed from the apparatus and stained with 10 mL of Ponceau S staining solution at room temperature for 30 s. The staining solution is removed and the membrane is rinsed with distilled water until nonspecific background of Ponceau S stain is removed. Each lane should have even and bright red staining bands.
9. Each membrane is blocked with 5 mL of 3 % (w/v) BSA in TBS-t at room temperature for 30 min.
10. Blocking buffer is completely removed and both membranes are rinsed 1× with TBS-t prior to addition of 3.5 mL of 1:500 diluted rabbit anti-pSMAD2 in 3 % (w/v) BSA in TBS-t.
11. Both blots are incubated for 3 h at room temperature on a tabletop rocker.
12. Membranes are rinsed twice and washed 1× for 5 min with 10 mL of TBS-t.
13. Each membrane is then incubated with 3.5 mL of 1:2000 diluted anti-rabbit HRP conjugated secondary antibody solution in 3 % (w/v) non-fat dry milk in TBS-t for 50 min at room temperature on a tabletop rocker.
14. Each membrane is rinsed once, and washed 3× with 10 mL of TBS-t for 5, 10, and 10 min, respectively.
15. Membranes are removed from wash buffer and allowed to drip for 10 s before being placed protein side up on a piece of clear plastic(cut three-ringed binder sheet protector). 200 μL of

West Dura solution (a mixture of 100 μL solution A and 100 μL of solution B) is added to each membrane and the membranes are lightly tilted to ensure even spread. Another clear plastic sheet is then covered over the membranes and the sandwich is allowed to sit for 30 s prior to ironing out excess liquid from inside the sandwich with a paper towel.

16. The sandwich is then taped to the inside of an imaging cassette, and exposed with X-ray developing film in a dark room for 5 s, 15 s, 30 s and 1 min to produce various exposures. The film is then developed using an automatic film developer.
17. The membranes are taken out of the sandwich, rinsed with TBS-t, and placed in box with TBS-t.
18. **Steps 9–17** are repeated with mouse β-actin and mouse SMAD1/2/3 (primary antibody is incubated overnight instead of 3 h at room temperature).

3.3 Cell Culture and TGF-β Ligand Washout

1. Working DMEM media consists of 10 % FBS, 2 mM L-Glu, 100 U/mL Penicillin G, and 100 U/mL Streptomycin is prepared and stored at 4 °C until use.
2. Cells are seeded to 11 wells of 6-well plates at a density of 8.5×10^5 cells in 1.5 mL medium. Plates are then incubated overnight at 37 °C, 100 % humidity and 5 % CO_2.
3. Next morning, two 50 mL conical tubes each with 25 mL of pre-warmed DMEM are prepared. Cells in 1 well of the 11 wells are trypsinized and counted. The cell number is assumed to be the same in every well and is used to calculate the amount of TGF-β needed to make a final concentration of 60,000 molecules per cell. TGF-β is added to one of the 50 mL conical tubes. Medium in the remaining 10 wells is aspirated off and replaced with 2 mL DMEM containing 60,000 molecules per cell of TGF-β. Plates are then incubated for 30 s at room temperature and the medium is immediately aspirated off. The plates are rinsed 3× with D-PBS and fresh pre-warmed DMEM media is added to each well. Plates are incubated for 0, 15, 30, 45, 60, 120, 180, 240, 300, 360, 420, and 480 min (*see* **Note 6**).
4. Plates are individually collected from incubator, rinsed three times with 2 mL pre-chilled D-PBS. D-PBS is subsequently removed by titling the plate vertically at an 80° angle in a bucket full with ice for 30 s and aspirating all liquid from the bottom corner of the plate using a P-200 pipette. Plates are then snap-frozen with liquid nitrogen and placed in –80 °C incubator until all plates are collected.
5. Plates are allowed to thaw on ice. Seventy-five microliters of RIPA buffer is added to each well. Plates are scraped with a cell scraper and the mixture is transferred into corresponding

pre-chilled 1.7 mL Eppendorf microcentrifuge tubes. The tubes are rotated for 1 h at 4 °C.

6. Fractions are spun for 10 min at 13,200 × *g* and 4 °C. Supernatants are transferred to new labeled tubes and stored on ice.
7. Protein concentration, western blotting, is done using the same protocol described above.

3.4 Luciferase Assay on Washout Experiment

1. The assay is performed according to manufacturer's protocol (Promega E1910). Briefly, HaCaT cells with TGF-β responsive CAGAx12 luciferase reporter is seeded in 30 wells of 12-well plates at a density of 200,000 cell/well in 750 μL of medium. The cells are allowed to settle down for 12 h in 37 °C in a 100 % humidified environment with 5 % CO_2.
2. A TGF-β standard is prepared at concentrations of 0, 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 pM (corresponding to 0, 282, 565, 1130, 2261, 4523, 9047, 18,094, 36,190, 72,380 molecules/cell, with cell number being estimated at 832,000).
3. The medium (750 μL) is aspirated off from each well and replaced with either 2 mL of standard, or 2.0 mL saved conditioned medium for each of the conditions in the 10 mL versus 2 mL TGF-β ligand depletion experiment (2.0 mL conditioned media from "10 mL" set and 2.0 mL conditioned media from the "2 mL" set). The plates are incubated for 10 h at 37 °C, 100 % humidity and 5 % CO_2.
4. The plates are taken out of incubator, rinsed 2× with pre-chilled D-PBS. D-PBS is subsequently removed by titling the plate vertically at an 80° angle in a bucket full with ice for 30 s and aspirating all liquid from the bottom corner of the plate using a P-200 pipetor.
5. 200 μL of 1× cell lysis buffer is added to each well, and the cells are scraped off the plate with a mini cell scraper and transferred to corresponding pre-chilled 1.5 mL Eppendorf microcentrifuge tubes. The tubes are then rotated for 45 min at 4 °C and spun down for 10 min at 13,200 × *g*.
6. 50 μL of the supernatant is added to each well of a white opaque 96-well plate in duplicates.
7. With a timer in hand, the luciferase reagent is added to each well in sequence at exactly 10 s from the time at which reagent is added to the previous well. When all the wells are finished, the plate is placed in the Beckman Coulter Multimode Detector DTX880 and each well is read at 10 s intervals.
8. The standard is fitted with a curve and is used to extrapolate the amount of TGF-β from the depletion experiment.

4 Notes

1. Cells reached roughly 80 % confluency by day 2.
2. Actual cell number is counted for more accurate calculation of number of TGF-β molecules per cell.
3. The term "rinse" is used here to refer to the brief adding, mixing, and removal of solutions.
4. As long as no more than 2 mM of diethylthreitol (DTT) or a similar reducing equivalent is added to RIPA buffer, there is minimal effect on the final absorbance read. Therefore diluting BSA standards in RIPA buffer is not necessary.
5. No effect on signal of p-SMAD2, SMAD1/2/3, and β-actin is observed with addition of Spectra protein ladder.
6. The media added are pre-warmed to 37 °C, based on the assumption that there is minimal change of temperature in 30 s even when plates are placed at room temperature. No significant differences in pSMAD2 signal are observed when the plate is incubated at 37 °C incubator with 100 % humidity and 5 % CO_2.

References

1. Massague J (1998) TGF-β signal transduction. Annu Rev Biochem 67:753–791
2. Akhurst RJ, Hata A (2012) Targeting the TGFβ signalling pathway in disease.Nat Rev Drug Discov. 11:790-811
3. Heldin CH, Miyazono K, ten Dijke P (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390(6659):465–471
4. Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL (1997) TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem 272(44): 27678–27685
5. Liu X, Sun Y, Constantinescu SN, Karam E, Weinberg RA, Lodish HF (1997) Transforming growth factor β-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. Proc Natl Acad Sci U S A 94(20): 10669–10674
6. Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P, Heldin CH (1997) Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-β signaling. J Biol Chem 272(44): 28107–28115
7. Shi Y, Massague J (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113(6):685–700
8. Zi Z, Feng Z, Chapnick DA et al (2011) Quantitative analysis of transient and sustained transforming growth factor-β signaling dynamics. Mol Syst Biol 7:492
9. Hornbruch A, Ma G, Ballermann MA, Tumova K, Liu D, Cairine Logan C (2005) A BMP-mediated transcriptional cascade involving Cash1 and tlx-3 specifies first-order relay sensory neurons in the developing hindbrain. Mech Dev 122(7–8):900–913
10. Clarke DC, Brown ML, Erickson RA, Shi Y, Liu X (2009) Transforming growth factor β depletion is the primary determinant of smad signaling kinetics. Mol Cell Biol 29(9): 2443–2455