Chapter 9

Optogenetic Control of TGF-β Signaling

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Abstract

Cells employ signaling pathways to make decisions in response to changes in their immediate environment. The Transforming Growth Factor β (TGF-β) signaling pathway plays pivotal roles in regulating many cellular processes, including cell proliferation, differentiation, and migrations. In order to manipulate and explore the dynamic behavior of TGF-β signaling at high spatiotemporal resolution, we developed an optogenetic system (the optoTGFBRs system), in which light is used to control TGF-β signaling precisely in time and space. Here, we describe about experimental details of how to build the optoTGFBRs system and utilize it to manipulate TGF-β signaling in a single cell or a cell population using microscope or LED array, respectively.

Key words Optogenetics, Signaling transduction, TGF-β, Spatiotemporal precision, Live cell imaging

1 Introduction

As the basic building blocks of life, cell has to perceive, make sense, and respond to its environment, which is implemented by a variety of signaling pathways. Among them, the transforming growth factor beta (TGF-β) signaling pathway plays an important role in various cellular processes including cell proliferation, differentiation, and migration [1]. Accordingly, malfunctions of TGF-β signaling have been connected to a variety of diseases such as atherosclerosis, cancer, developmental defects [2], reproductive disorders, and connective tissue diseases [3]. The canonical TGF-β signaling transduction is relatively simple. Briefly, the TGF-β ligand (the active form) binds two type I receptors and two type II receptors to form a symmetric ligand-receptor-complex [4]. The oligomerization of receptors promotes activation of the type I receptor through transphosphorylation, catalyzed by the constitutively active kinase of the type II receptor [5]. The activated kinase domain of the type I receptor phosphorylates the receptor-regulated Smad proteins (R-Smads, i.e., Smad2 and Smad3 for TGF-β-like signaling pathway; Smad1, Smad5, and Smad8 for

Zhike Zi and Xuedong Liu (eds.), *TGF-Beta Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 2488,
https://doi.org/10.1007/978-1-0716-2277-3_9,

BMP-like signaling pathway) [6]. The R-Smads then bind to the common mediator Smad4 (co-Smad) and translocate into the nucleus [7]. There, these R-Smads/co-Smad complexes bind to DNA in conjunction with other transcription factors/cofactors, regulating the transcription of various target genes [8, 9]. Although the main components of TGF-β signaling have been identified and explored in recent decades, understanding its dynamic behavior is limited by the lack of tools that allow the control of TGF-β signaling at high spatiotemporal resolution.

Several efforts have been made to manipulate the canonical TGF-β signaling [10–13]. But they depend on the preparation of chemicals or antibodies to perturb the cells. The recent development of the field of optogenetics provides us with an alternative strategy to control cellular activities: shining light on cells. At the beginning, optogenetic tools were mainly developed on light-sensitive ion channels for modulating membrane voltage potential, which has transformed the neurosciences [14, 15]. Later on, another set of optogenetic tools have emerged and quickly been developed to allow control of molecular interactions and signaling cascades [16–19]. Among them, CRY2 (cryptochrome 2) of *Arabidopsis thaliana* forms homooligomers, or forms heterodimers with CIB1 (cryptochromes-interacting basic helix-loop-helix 1) upon blue light irradiation [20]. The CRY2-CIB1 system is used as an optogenetic tool in the development of the optoTGFBRs system, which consists of a pair of optoTβRs (optoTβRI: Myr-cytoTβRI-CIBN; optoTβRII: cytoTβRII-CRY2PHR-tdTomato) and a TGF-β signaling readout (iRFP-Smad2). After validating the functionality of the optoTGFBRs system, we have shown that TGF-β signaling can be specifically activated in single cells through modulating light stimulations [21]. Due to the convenience and precision of manipulating light stimulation, optogenetics is a powerful tool to achieve high spatiotemporal resolution, which is not only beneficial for scientific research, but also a potent approach for therapeutic application.

In this chapter, we focus on the description of activating TGF-β signaling in single cells with blue light under a confocal microscope. We attempt to cover as many experimental details as possible, to enable use of the optoTGFBRs system, not only by researchers interested in the TGF-β signaling, but also for broader applications.

2 Materials

2.1 Plasmids

1. pLNCX2-optoTβRs: optoTβRs was constructed by combining optoTβRI (Myr-cytoTβRI-CIBN) and optoTβRII (cytoTβRII-CRY2PHR-tdTomato) using a P2A bicistronic linker sequence (*see* **Note 1**).

2. iRFP682-Smad2: iRFP682 attached to the N-terminus of Smad2 within a retrovirus transfer vector (*see* **Note 2**).
3. Retrovirus packaging vector plasmid: pCL-Ampho.

2.2 Cell Culture

1. Cell culture vessels: T75 flask, 96-well plate, 6-well plates, 60 mm dishes with TC surface treatment, μ-Slide 8 Well (ibidi, A chambered coverslip with 8 wells for both cell culture and imaging).
2. Cell culture medium: Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 2 mM L-Glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.
3. Cell culture medium for FACS and microscope experiments: phenol-red free DMEM with low glucose, supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.
4. Dulbecco's phosphate-buffered saline (DPBS).
5. 0.05% Trypsin-EDTA.
6. Automated cell counter.

2.3 Transfection, Transduction

1. Transfection: Lipofectamine 3000 Transfection Reagent (Invitrogen, including Lipofectamine 3000 Reagent and P3000TM Enhancer Reagent), Opti-MEM (Gibco, Catalog # 31985088).
2. Transduction: Polybrene.
3. 0.45 μm syringe filter.

2.4 Microscope

1. 2-photon/confocal microscopy: LSM 710 NLO 2-photon/confocal laser scanning microscope (Carl Zeiss).
2. Objective lens. We used the Plan-Apochromat 63x/1.40 Oil DIC M27 objective from Carl Zeiss.
3. Immersion oil.
4. Imaging control software: ZEN 2 Software (Carl Zeiss) to control the microscope, incubation environment, laser scanning, and image acquisition.
5. Incubation chamber for the control of humidity, temperature, and CO2 concentration.
6. Epi-fluorescence microscope (widefield). We use a Zeiss Axio-Observer Z1 fluorescence microscope mounted with a Plan-Neofluar 10 ×/0.3 Ph 1 M27 objective.

2.5 Miscellaneous

1. Microscope slide power meter sensor (THORLABS, Catalog #S170C) and digital power meter (THORLABS, Catalog #PM100D).

2. Fluorescent-assisted cell sorting (FACS) instrument. We use BD FACS Aria II flow cytometer with 561 nm laser and 583/22–25 filter for tdTomato, 633 nm laser and 670/14 filter for iRFP682.
3. Plasmocin Prophylactic (InvivoGen, Catalog #ant-mpp).
4. Red LED light.
5. Image processing software (*see* **Note 3**): an open-source software Fiji.

3 Methods

This section first describes the generation of optoHeLa-TGFBRs cell line stably expressing optoTβRI (Myr-cytoTβRI-CIBN), optoTβRII (cytoTβRII-CRY2PHR-tdTomato), and iRFP682-Smad2 proteins. The optoHeLa-TGFBRs cell line can be generated by two successive rounds of retrovirus transduction. In the first round, HeLa cells stably expressing optoTβRI and optoTβRII are generated. These cells are then transduced with retroviruses harboring the iRFP682-Smad2 DNA fragments following a similar procedure. Then, with the selected functional optoHeLa-TGFBRs cell lines, we show how to measure the dynamics of optoTβRII and iRFP-Smad2 proteins upon 488 nm light activation under a microscope.

3.1 Generation of HeLa Cells Stably Expressing optoTβRI and optoTβRII

1. On day 1, plate 1.5×10^6 packaging HEK293T cells onto a 60 mm cell culture dish, place the dish into a humidified CO_2 incubator (37 °C and 5% CO2) for at least 12 h.
2. On day 2 (the next day following cell plating), perform the following steps. Prepare two microcentrifuge tubes. In tube A, add 6.25 μg pLNCX2-optoTβRs and 6.25 μg pCL-Ampho plasmids to 300 μL prewarmed Opti-MEM medium, then add 25 μL P3000 reagent. Mix by pipetting a few times.
3. In tube B, add 5 μL Lipofectamine 3000 reagent to 300 μL prewarmed Opti-MEM medium. Mix gently by pipetting a few times, avoid formation of bubbles.
4. Add tube A solution to tube B and mix well.
5. Incubate the mixture from **step 4** at room temperature for 10–15 min.
6. Add all the lipid-DNA mixture from **step 5** to the cell culture dish with HEK293T cells drop-wise. Place HEK293T cells in the incubator.
7. Replace the cell culture medium with complete medium 6 h posttransfection.

8. Place the dish in the incubator and let HEK293T cells to grow for around 40 h.
9. On day 3, plate 2.5×10^6 target HeLa cells onto a T75 flask. Place the flask in the incubator for at least 24 h.
10. On day 4, collect the retrovirus supernatant. Add medium from the transfected HEK293T cell culture (containing packaged retrovirus) from **step 8** into a tube, remove HEK293T cell debris and aggregated virus by low speed ($500 \times g$) centrifugation for 5 min at room temperature.
11. Filter the retrovirus supernatant through a 0.45 μm syringe filter.
12. Prepare an infection cocktail consisting of 2 mL retroviral-containing supernatant, 8 μL of 10 mg/mL Polybrene stock, and 8 mL fresh complete medium.
13. Aspirate the medium of HeLa cells (plated one day before) and add the prepared infection cocktail to the HeLa cells (*see* **Note 4**) in the T75 flask. Wrap the T75 flask with aluminum foil (*see* **Note 5**) and place it in the incubator.
14. Let the HeLa cells be infected for 3 h in the incubator, replace the infection medium with 10 mL fresh complete medium. Keep the HeLa cells in the incubator overnight.
15. On day 5, remove the culture medium from the infected HeLa cells and add fresh cell culture medium containing G418 antibiotics at the final concentration of 800 μg/mL for screening optoTβRs genome integration (*see* **Note 6**). Keep the infected HeLa cells in the incubator for a week or more (*see* **Note 7**). Change medium or subculture the cells when necessary.

3.2 Single Colony Isolation of the Infected HeLa Cells

1. Collect infected HeLa cells through trypsinization and resuspend them in phenol-red free DMEM (*see* **Note 8**), count cells with a cell counter. Prepare cell solution at a concentration of ~one million cells per 1 mL.
2. Prepare a 96-well plate with 200 μL cell culture medium per each well. In addition, plasmocin prophylactic was supplied at a final concentration of 5 μg/mL for the prevention of mycoplasma contamination.
3. Use FACS to sort 1 cell/well (with optoTβRII-tdTomato signal) into the 96-well plates prepared in **step 2**.
4. Place the 96-well plates into the incubator for around 2 weeks depending on the growth of the colonies. Check the growth of the colonies under a microscope (*see* **Note 9**).
5. Place the plate under an epifluorescence microscope, find focus with the tdTomato channel, using a 10× objective. Find colonies with only cytoplasmic tdTomato signal.

6. Expand the positive colonies from the 96-well plates to 6-well plates. Let the cells grow for around 1 week.
7. Split the cells, aliquot about 1×10^4 cells to a well on a 96-well-plate for each colony.
8. Plate the plate under an epifluorescence microscope, find focus with the tdTomato channel, using a 10× objective.
9. Take an image in tdTomato channel for each colony.
10. Use blue light to stimulate the optogenetic system. Specifically, take an image of the GFP channel with 10% excitation intensity and 100 ms exposure time (*see* **Note 10**).
11. Immediately after blue light exposure, take an image per minute for 10 min using the tdTomato channel.
12. Select and label the colonies showing tdTomato signal plasma-membrane localization immediately after blue light stimulation, as well as recovery of tdTomato signal into the cytosol in the dark**.** Expand these colonies and cryopreserve them.

3.3 Generation of optoHeLa-TGFBRs Cells with the Expression of iRFP-Smad2 Protein

1. To express iRFP-Smad2 in the selected HeLa cell colonies expressing functional optoTβRI and optoTβRII, follow the steps in Subheading 3.1 by replacing HeLa cells with the selected HeLa cell colonies with optoTβRs, changing pLNCX2-optoTβRs plasmid to pREX-iRFP682-Smad2 plasmid in **step 2**, and substituting G418 antibiotics with 10 μg/mL Blasticidin in **step 15** described in Subheading 3.1.
2. Isolate the optoHeLa-TGFBRs single cell colonies following the **steps 1**–7 described in Subheading 3.2.
3. Select and label the functional optoHeLa-TGFBRs colonies that show both the recruitment of cytoTβRII-CRY2PHR-tdTomato proteins at plasma membrane and nuclear translocation of iRFP-Smad2 protein following blue light stimulation.
4. Expand the functional optoHeLa-TGFBRs colonies and cryopreserve them.

3.4 Measuring Light-Induced TGF-β Signaling Dynamics in optoHeLa-TGFBRs Cells

1. Trypsinize and plate 2×10^4 optoHeLa-TGFBRs cells per well onto an 8-well μ-Slide. Wrap the slide with aluminum foil and place it into the incubator for at least 18 h.
2. Start up the microscope following the manufacturer's protocols. Switch on the incubation chamber (37 °C and in 5% CO_2) to warm up the lens and the hardware at least 30 min before the experiments (*see* **Note 11**). Clean the lens with ethanol. Turn off the room lights (*see* **Note 12**).
3. Drop the immersion oil onto the 63× lens, place the slide into the slide chamber. Cover the chamber with its lid.

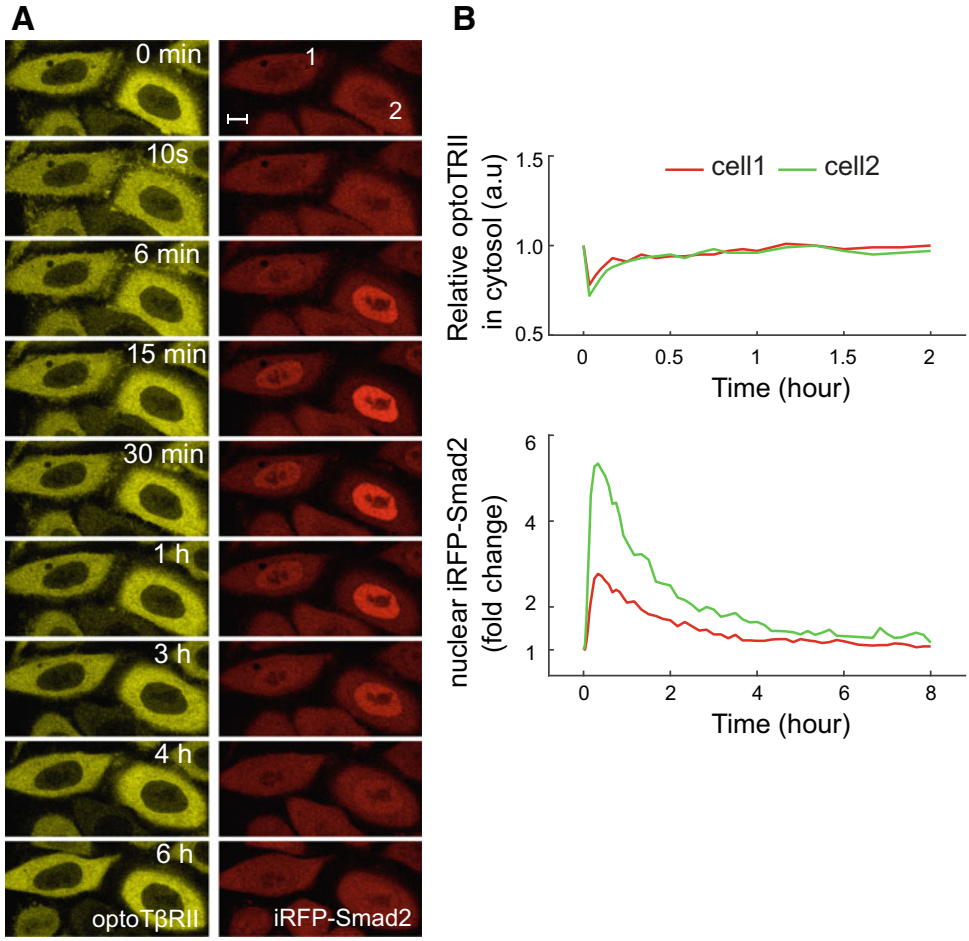

Fig. 1 TGF-β signaling dynamics in optoHeLa-TGFBRs cells with one short pulse of blue light stimulation. (**a**) Representative images for the optoTβRII and iRFP-Smad2 proteins. (**b**) Quantification of nuclear Smad2 and cytoplasmic optoTβRII signaling dynamics. (Reproduced with permission from ref. 21. Copyright (2018) American Chemical Society)

4. Use a 633 nm laser and a 543 nm laser with 5% intensity for tdTomato channel and iRFP682 channel, respectively. Use the detection wavelength (band-pass filter set) of 548–647 nm for collecting emission light of tdTomato, and 638–759 nm for iRFP682 signal.
5. Find focus with the tdTomato channel. Take images for both the tdTomato channel and the iRFP682 channel.
6. Use the Bleaching function to stimulate the optogenetic system. Set the Bleaching tab parameters. Specifically, use the 488 nm laser with intensity of 5% and scan speed of 6, which results in an illumination power of 12.4 μW in our microscope setup (*see* **Note 13–16**). Draw a rectangle covering the entire field of view for bleaching.

7. Perform a bleaching and take an image of tdTomato signal immediately.
8. To measure TGF-β signaling dynamics 8 h after blue light stimulation (Fig. 1a), we take images per second for 1 min for only the tdTomato channel, followed by taking images per minute for 9 min for both tdTomato and/or iRFP682 channel. After that, take images every 5 min for another 50 min. Then, take images every 30 min for another 7 h (*see* **Note 17**).

3.5 Spatiotemporal Control of the TGF-β Signaling in the optoTGFBRs System

1. Follow the **steps 1–5** described in Subheading 3.3 to prepare the cells and set up the microscope for the light stimulation of optoHeLa-TGFBRs cells.
2. Use the bleaching function to stimulate the optogenetic system. Set the "Bleaching" tab parameters. Specifically, use the 488 nm laser with intensity of 5% and scan speed of 6. Draw a small circle within the cytoplasmic region of one cell for bleaching (*see* **Note 18**).
3. Perform a bleaching and take an image in tdTomato channel immediately. Following that, take images per minute for 12 min for both tdTomato and/or iRFP682 channel (*see* **Note 19**).
4. Repeat **steps 2** and **3** for imaging more cells.

3.6 Image Analysis

1. Export the images as tiff files from ZEN 2 software.
2. Load the tiff images into Fiji. Put images of the same channel and the same series to a stack.
3. To measure the signal of the cytoplasmic or nuclear region, draw an elliptical region within the cytoplasmic or nuclear region of the cell using the "Oval" tool. Measure the intensity by the shortcut "ctrl+m". Data of the "Mean" column correspond to the signal intensity (*see* **Note 20**).
4. Quantify the Smad2 signal as the ratio of nuclear-to-cytoplasmic iRFP-Smad2 signal. Due to the difficulty of quantifying the fluorescence signal at the plasma membrane, the dynamics of optoTβRII can be represented by the depletion of the cytoplasmic optoTβRII level.
5. For comparison among different images, the mean tdTomato intensity (level of optoTβRII) in cytoplasmic area can be normalized to that in the entire imaging field.
6. Quantify the signal intensities from multiple corresponding areas and use the average values.
7. For live cell imaging analysis, plot the dynamics of signal intensity against time (*see* Fig. 1b).

4 Notes

1. The pLNCX2-optoTβRs plasmid was a gift from Won_Do Heo (Addgene plasmid # 118965).
2. The detailed construction procedure for the iRFP682-Smad2 plasmid. To generate the iRFP682-C1 plasmid, the mCitrine sequence in the pmCitrine-C1 vector was first replaced with the FP-encoding sequence from the piRFP682-N1 plasmid. Then, the cDNA encoding Smad2 was amplified and inserted to the piRFP682-C1 plasmid. Following that, the iRFP682-Smad2 was amplified and replaced the EYFP-Smad2 of pREX-EYFP-Smad2-IRES-BSD (a gift from Xuedong Liu) to generate the pREX-iRFP682-Smad2 for retroviral transduction.
3. Due to the lack of nuclear marker, it is difficult to perform automatic image quantification. The live cell imaging data was manually quantified with ImageJ.
4. Centrifuge (1200 × *g* for 60 min at 32 °C) the T75 cell culture flask to improve infection efficiency if possible.
5. Blue light should be avoided when handling cells that express optoTβRI and optoTβRII. Especially, ambient light should be avoided. Generally, wrap the plates/flasks with aluminum foil to protect the cells from light before placing them in the incubator or transferring to other places. Keep clean bench and ambient lights off when the cells are not wrapped in aluminum foil. Only use the red LED flashlights or strip lights when necessary. Perform all the liquid handling operation steps under red LED light.
6. The concentration of antibiotic for each cell line should be individually determined by drawing an IC50 curve before applying the antibiotic selection procedure.
7. The growth time depends on the titer of the virus, as well as how fast the cell grows. In general, cells should reach at least 50% confluency before being trypsinized and transferred.
8. DMEM with phenol-red could be used too. Here we routinely use phenol-red free medium for better signal detection.
9. It is better not to stimulate cells with blue light in this step. Check the growth of the colonies under an epifluorescence microscope using a 4× objective with an autofocus module. However, if the condition is not allowed, bright field can be used to find colonies. In this case, only use a short pulse of white light for each well.
10. For widefield microscopy, a weak light intensity is enough to activate the optogenetic system. Excitation light with strong

intensity might scatter to the neighboring wells and cause the activation of those cells.

11. Temperature changes might cause focus drift, especially for microscopy without the autofocus function, stable temperature should be kept during the live cell imaging experimentation.
12. The microscopy should be covered in a light-proof box to avoid the exposure of optoHeLa-TGFBRs cells to the other sources of light (e.g., accidently turning on the room light by other people).
13. The light intensity is measured and determined by the optical power sensor on the focus plane.
14. The illumination power could be adjusted by changing the scan speed to fine-tune the pixel dwell time.
15. For 2-photon activation, we use a chameleon laser with 5% power, and a wavelength 860 nm.
16. Light intensity is only related to the power of laser and the area of bleach region. Scan speed doesn't matter for measurement by the optical power sensor, as the total amount of light that reach the sensor (during 1 s) stays the same as long as the light power does not change. In general, light intensity increases linearly with the laser power (*see* Fig. 2).
17. Membrane translocation of tdTomato tagged optoTβRII upon blue light stimulation happens at a time scale of seconds and its recovery happens at a time scale of minutes. Nuclear translocation of iRFP682-labeled Smad2 takes place at a time scale of minutes, and it recovers at the scale of hours. Therefore, in order to capture all of these dynamics, we apply different image taking frequency at different stages after light stimulation.

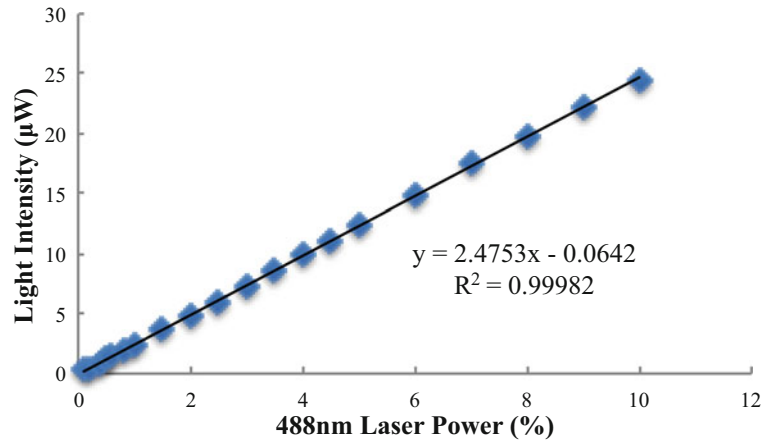

Fig. 2 Light intensity increases linearly with the laser power

18. Due to the light reflection, refraction, and scattering, illumination region should be restricted to a small area inside the target cell, to avoid accidental activation of its neighboring cells. A region covering approximately 10% of the whole cell area is enough to activate the cell.
19. To capture the dynamics of tdTomato-labeled optoTβRII, you can perform the same image acquisition scheme as in Subheading 3.4 (i.e., take images every second for the first minutes after bleaching).
20. If no "Mean" column is shown, or there are too many columns, modify the options in the "Set Measurements" window under the "Analyze" menu.

References

1. Massague J (2012) TGF beta signalling in context. Nat Rev Mol Cell Biol 13:616–630
2. Blobe GC, Schiemann WP, Lodish HF (2000) Mechanisms of disease: role of transforming growth factor beta in human disease. N Engl J Med 342:1350–1358
3. Gordon KJ, Blobe GC (2008) Role of transforming growth factor-beta superfamily signaling pathways in human disease. BBA-Mol Basis Dis 1782:197–228
4. Sporn MB, Roberts AB (1990) The transforming growth factor-betas - past, present, and future. Ann N Y Acad Sci 593:1–6
5. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J (1992) Tgf-Beta signals through a heteromeric protein-kinase receptor complex. Cell 71: 1003–1014
6. Feng XH, Derynck R (2005) Specificity and versatility in TGF-beta signaling through Smads. Annu Rev Cell Dev Biol 21:659–693
7. Hata A, Chen YG (2016) TGF-beta signaling from receptors to Smads. Cold Spring Harb Perspect Biol 8:a022061
8. Massague J, Seoane J, Wotton D (2005) Smad transcription factors. Genes Dev 19: 2783–2810
9. Hill CS (2016) Transcriptional control by the SMADs. Cold Spring Harb Perspect Biol 8: a022079
10. Chang CB (2016) Agonists and antagonists of TGF-beta family ligands. Cold Spring Harb Perspect Biol 8:a021923
11. Schlingensiepen KH, Fischer-Blass B, Schmaus S, Ludwig S (2008) Antisense therapeutics for tumor treatment: the TGF-beta2 inhibitor AP 12009 in clinical development against malignant tumors. Recent Results Cancer Res 177:137–150
12. Kwiatkowski W, Gray PC, Choe S (2014) Engineering TGF-beta superfamily ligands for clinical applications. Trends Pharmacol Sci 35: 648–657
13. Lonning S, Mannick J, McPherson JM (2011) Antibody targeting of TGF-beta in cancer patients. Curr Pharmacol Biotechnol 12: 2176–2189
14. Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci U S A 100:13940–13945
15. Guru A, Post RJ, Ho YY, Warden MR (2015) Making sense of Optogenetics. Int J Neuropsychopharmacol 18:pyv079
16. Motta-Mena LB, Reade A, Mallory MJ, Glantz S, Weiner OD, Lynch KW, Gardner KH (2014) An optogenetic gene expression system with rapid activation and deactivation kinetics. Nat Chem Biol 10:196–202
17. Bugaj LJ, Choksi AT, Mesuda CK, Kane RS, Schaffer DV (2013) Optogenetic protein clustering and signaling activation in mammalian cells. Nat Methods 10:249–252
18. Wu YI, Frey D, Lungu OI, Jaehrig A, Schlichting I, Kuhlman B, Hahn KM (2009) A genetically encoded photoactivatable Rac controls the motility of living cells. Nature 461:104–U111
19. Levskaya A, Weiner OD, Lim WA, Voigt CA (2009) Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature 461:997–1001

20. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL (2010) Rapid blue-light-mediated induction of protein interactions in living cells. Nat Methods 7:973–U948

21. Li Y, Lee M, Kim N, Wu G, Deng D, Kim JM, Liu X, Heo WD, Zi Z (2018) Spatiotemporal control of TGF-beta signaling with light. ACS Synth Biol 7:443–451