Review

Molecular Engineering of the TGF-β Signaling Pathway

Zhike Zi

Otto-Warburg Laboratory, *Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany*

Correspondence to Zhike Zi: *zhike.zi@molgen.mpg.de*
https://doi.org/10.1016/j.jmb.2019.05.022

Abstract

Transforming growth factor beta (TGF-β) is an important growth factor that plays essential roles in regulating tissue development and homeostasis. Dysfunction of TGF-β signaling is a hallmark of many human diseases. Therefore, targeting TGF-β signaling presents broad therapeutic potential. Since the discovery of the TGF-β ligand, a collection of engineered signaling proteins have been developed to probe and manipulate TGF-β signaling responses. In this review, we highlight recent progress in the engineering of TGF-β signaling for different applications and discuss how molecular engineering approaches can advance our understanding of this important pathway. In addition, we provide a future outlook on the opportunities and challenges in the engineering of the TGF-β signaling pathway from a quantitative perspective.

Introduction

Cells sense and respond to their environment using a complicated signaling network. External signals are detected, processed and translated into specific cellular responses by different signaling proteins, composed of distinct functional domains with high modularity in their structures. The modularity of signaling proteins makes it possible for researchers to engineer signaling molecules and rewire cellular functions for research and clinical applications [1–3]. By reconstituting signaling proteins with new functional domains and regulatory elements, it is now possible to redirect signaling information flow and control cellular responses to diverse signaling cues [2–7].

Signal transduction in cells is initiated in part by secreted proteins such as cytokines, growth factors and hormones. Transforming growth factor beta (TGF-β) is one of the most important cytokines that plays essential roles in regulating tissue development and homeostasis. Dysfunction of TGF-β signaling has been connected to many human diseases such as cancer, fibrotic diseases and connective tissue disorders [8–11]. Since the discovery of the TGF-β ligand, engineering approaches have been used in studies attempting to probe the molecular mechanisms of this remarkable signaling pathway and develop novel therapeutic drugs for clinical applications. In this paper, we will review recent advances in the engineering of TGF-β signaling for different applications (Table 1). We will also discuss the challenges associated with engineering the TGF-β signaling pathway from a quantitative perspective. Since the TGF-β superfamily is a large group of structurally related cytokines with 33 members, including TGF-β isoforms, bone morphogenetic proteins, growth differentiation factors, activins, inhibins, nodal and other subfamilies [12–14], our discussion in this review will only focus on the TGF-β subfamily.

Modularity of TGF-β Signaling

TGF-β signaling proteins have been identified and well characterized at the molecular level (previously reviewed in Refs. [15–18]). As illustrated in Fig. 1, the TGF-β signaling network is composed of common signaling elements: sensors (TGF-β receptors) that perceive the TGF-β signal input, intracellular effectors (receptor-regulated Smad2/3 and common partner Smad4), feedback regulators (e.g., inhibitory Smads, Smad7) that modulate signaling activities, and

Table 1. Examples of engineered TGF-β signaling proteins and their applications

Target TGF-β signaling proteins	Engineering method	Applications	References
TGF-β ligand	Engineered TGF-β chimeras by changing their binding affinity to TGF-β receptors	Block TGF-β signaling	[31–33]
	Synthetic TGF-β receptor-binding ligands that can tether to monolayer surface	Augment TGF-β signaling response	[35]
	Synthetic latent TGF-β complex conjugated with single-walled carbon nanotubes	Control the release of active TGF-β *in vitro* and *in vivo* using near-infrared light	[36]
	Recombinant TGF-β ligand with chemical immobilization property	Present and deliver TGF-β with spatial control	[37,38]
	Engineered TGF-β CAR T-cells that bind to TGF-β	Block and rewire TGF-β signaling	[83–85,88]
TGF-β receptors	Engineered TGF-β receptor chimeras with extracellular domains that bind to other ligands	Induce TGF-β signaling with Epo or GM-CSF	[39–42]
	Synthetic TGF-β receptors with an optogenetic system	Spatiotemporal control of TGF-β signaling with light	[52–54]
	Engineered TGF-β receptors with fluorescent protein reporters	Visualize and quantify TGF-β receptor signaling activities	[52,65–70,72,73]
	Engineered Smad proteins with fluorescent protein reporters	Visualize and quantify TGF-β signaling responses	[21,23,24,26,52,63,64]
Smad proteins	Synthetic peptide that binds to active Smad3 and block Smad3 nuclear accumulation	Inhibit profibrotic actions of TGF-β signaling	[90]

biological responses (e.g., transcriptional outputs). Once TGF-β binds to the type II receptor (TβRII), TβRII recruits type I receptor (TβRI) and forms a receptor complex in which TβRII phosphorylates and activates TβRI. The TβRI kinase activates Smad proteins, which translocate to the nucleus to activate or repress target genes that regulate different cellular processes. Similar to other signaling pathways, TGF-β signaling is tightly controlled and finely tuned by many feedback regulators [19]. An increasing number of studies have shown that changes in the TGF-β signal input are encoded in the temporal patterns of the Smad transcription factors, which influence diverse transcriptional outputs [20–24]. However, the downstream biological outputs of TGF-β signaling are highly cellular context-dependent: they are determined not only by Smad proteins but also by the activities of non-Smad pathways [9,25–28].

Engineering of TGF-β Ligands to Modulate TGF-β Signaling activity

TGF-β signals by binding to TβRII, which recruits TβRI to form a heteromeric complex on the cell surface. The TGF-β isoforms (TGF-β1, -β2 and -β3) are 25-kDa homodimeric polypeptides. They share common structural elements but show different binding affinities for TβRII. TGF-β1 and -β3 bind TβRII with a high affinity, while TGF-β2 binds TβRII with a low affinity [13,29,30]. Previous studies suggest that the binding epitopes of TGF-β receptors within each monomer of TGF-β superfamily ligands are unique and that their functions are largely independent of one another [13,31]. The high specificity of TGF-β ligands for their receptors provides an opportunity for researchers to develop TGF-β chimeras by mixing, rearranging and mutating their binding epitopes. Modified receptor-binding epitopes can alter the functional properties of natural TGF-β ligands. For example, Hinck's laboratory engineered a TGF-β monomer that lacks the heel helix motif for binding TβRI, but still has the ability to bind TβRII with high affinity [32]. This newly designed ligand can transform TGF-β from a signaling initiator to an inhibitor because it can bind and occupy cell surface TβRII without recruiting TβRI to trigger downstream TGF-β signaling. Therefore, it can potentially be used as an alternative inhibitor to block TGF-β signaling for therapeutic applications in human diseases [31,32]. Similar structure-guided approaches have been applied to the engineering of other TGF-β superfamily ligands (reviewed in Ref. [33]).

TGF-β is secreted into the extracellular matrix as a latent complex referred to as the small latent complex, consisting of TGF-β and the latency-associated peptide [34]. TGF-β can be released from latency-associated peptide by different latent TGF-β activators, including proteases, cell-surface integrins and physicochemical factors. However, it remains difficult to precisely control the activation of latent TGF-β *in vitro* and *in vivo*. To address this challenge, several groups have employed tissue engineering approaches to manipulate the activation of TGF-β signaling with spatial precision. For example, Li and coworkers [35]

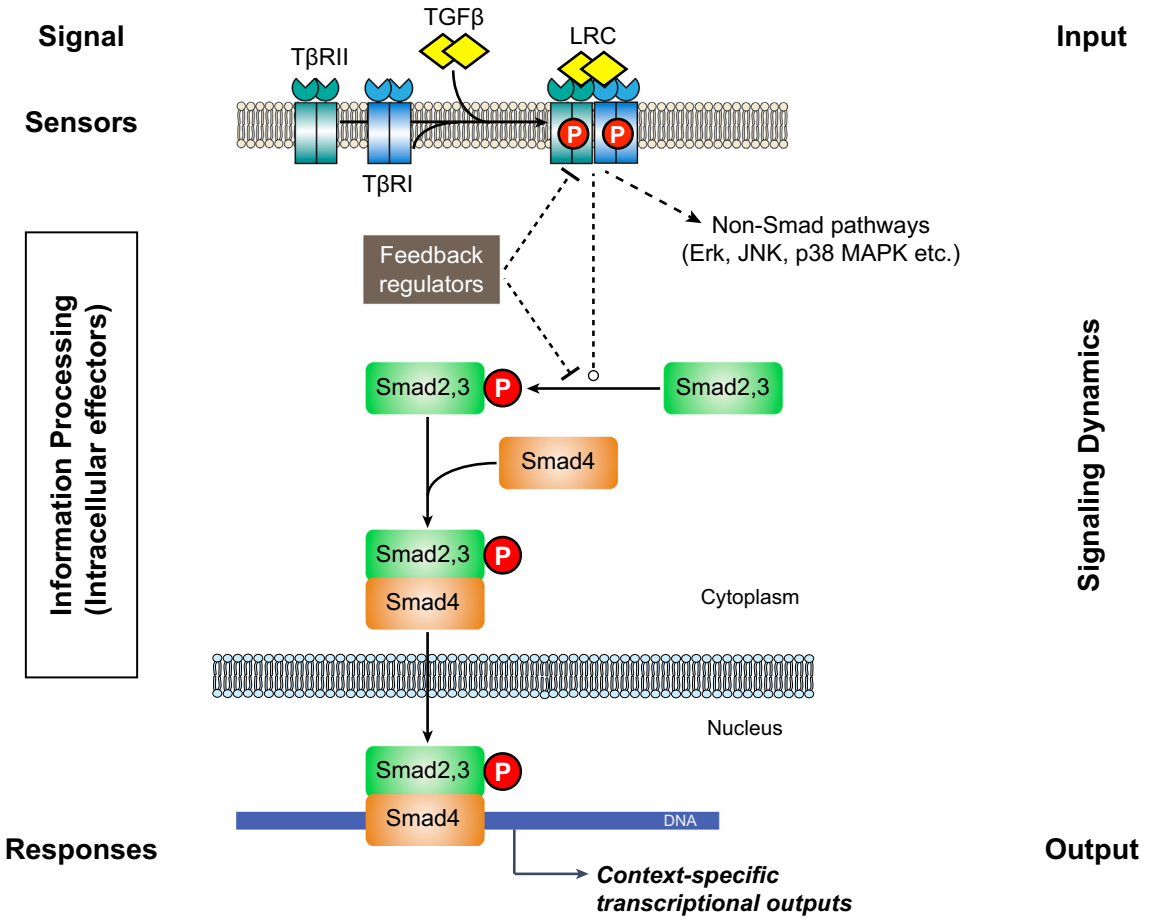

Fig. 1. Schematic illustration of the TGF-β signaling network. Active TGF-β ligand is a signal (input) that is received by the sensors of TGF-β receptors (TβRII and TβRI). Once TGF-β binds to TβRII, TβRII recruits TβRI. In the receptor complex, TβRII phosphorylates and activates TβRI. The TGF-β signaling system is relayed by Smad and non-Smad signaling proteins, which regulate context-specific gene responses and therefore control different cellular processes (Output). The details of non-Smad signaling and feedback regulators are not depicted in this simple scheme.

synthetized TβRI- and TβRII-binding peptide ligands and spatially tethered them to a self-assembled monolayer surface. They showed that these peptide ligands do not compete with TGF-β because they occupy a binding site on TGF-β receptors that is different from that used by TGF-β. TGF-β signaling responses are greatly augmented when cells are grown on surface presenting synthetic TβR-binding ligands. In addition, Chen's laboratory recently used near-infrared irradiation to trigger the release of active TGF-β ligands from a synthetic latent complex conjugated with single-walled carbon nanotubes [36]. With this approach, TGF-β signaling can be activated in living cells and living mice using near-infrared light. Other chemical strategies are used to present and deliver active recombinant TGF-β ligands through spatially controlled immobilization [37,38]. These methods provide new ways to modulate TGF-β signaling activities, but they require the synthesis of specific surface materials, which might limit their broader applications.

Engineering of Synthetic TGF-β Receptors that Provide New Strategies for Studying TGF-β Signaling

Chimeric cytokine receptors are constructed by taking specific functional domains from different proteins and combining them to build new receptors. The engineering of cell surface receptors has been a powerful strategy for generating cells that target a specific protein, redirect cellular responses and reprogram cell–cell interactions. This approach has also been adopted to probe the molecular mechanism of TGF-β signal activation and find new ways to control TGF-β signaling. For instance, in some early studies, chimeric TGF-β receptors were constructed and

expressed in specific cells by combining the cytoplasmic domains of TGF-β receptors with the extracellular domain of the erythropoietin receptor or the granulocyte-macrophage colony-stimulating factor (GM-CSF) [39,40]. By quantifying the growth inhibition effect or luciferase activity in cells expressing different combinations of TGF-β receptor chimeras (TβRI + TβRII chimeras; only TβRI or TβRII chimeras alone), these studies showed that chimeric TβRI and TβRII receptors provide a unique system for inducing TGF-β signaling with Epo or GM-CSF, while homooligomers of TβRI or TβRII alone are TGF-β signaling-incompetent. Various versions of GM-CSF/TβRI and GM-CSF/TβRII receptor chimeras were later established in Leof's laboratory to confirm the basolateral targeting motif of TGF-β receptors [41,42], which demonstrate that the chimeric receptor system is useful for exploring the functions of TGF-β receptors. It is worth noting that point mutation is a very powerful tool for investigating the molecular mechanism of TGF-β signaling. These studies have been previously reviewed [43], and they are not discussed here.

While TGF-β receptor chimeras are able to trigger TGF-β signaling and are useful for studying TGF-β signaling, it remains difficult to regulate TGF-β signaling with high spatial and temporal precision. The invention of optogenetics has provided excellent tools for controlling cell signaling using light stimulation [7,44,45]. Optogenetic tools have been broadly adopted to modulate and dissect intracellular signaling networks including Ras–Erk, FGF, calcium activity, p53 and other types of signaling [46–51], but their application in the field of TGF-β signaling is still in its infancy. In a recent study, Li *et al.* [52] developed an optogenetic system (optoTGFBRs), in which blue light triggers TGFβ receptor complex formation and downstream Smad signaling in single cells. In the optoTGFBRs synthetic tool, spatiotemporal control of TGFβ receptor complex formation is achieved through manipulating the reversible interaction between the N-terminus of CIB1 (CIBN) and the PHR domain of cryptochrome 2 (CRY2) with blue-light illumination (Fig. 2A). As TβRII is constitutively active, the light-activated interaction of CIBN–PHR brings TβRII and TβRI into close proximity, inducing the formation of TβRI/TβRII complex and the activation of Smad2 in the absence of TGF-β ligand. Using the optoTGFBRs system, the authors demonstrated that TGF-β signaling can be selectively and sequentially activated in individual cells by illuminating cells with blue light in a temporally and spatially dependent manner. In addition, guided by model simulations of TGF-β signaling dynamics, it is possible to generate transient, sustained and oscillated Smad2 dynamics by manipulating the frequency of blue light pulses (Fig. 2B).

Recently, Sako and coworkers [53] applied a light-oxygen-voltage optogenetic tool to construct a photoactivatable Nodal receptor Opto-acvr1b/2b system in which Nodal signaling can be induced with blue light. While this Opto-acvr1b/2b system is a very useful tool for studying the importance of the Nodal signaling duration in zebrafish embryos, it does not include a reporter for visualizing the activation of upstream receptors. Similarly, Hill's group built the Opto-TGFBR1* and Opto-ACVR1 systems by fusing the light-oxygen-voltage domain with the intracellular domains of constitutively activated TGFBR1 or that of a wild-type ACVR1, respectively [54]. By checking whether cotransfected FLAG-SMAD1 is phosphorylated by light in the system involving Opto-ACVR1 alone or both Opto-ACVR1 and Opto-TGFBR1*, the authors confirmed that TGFBR1 activates ACVR1, which phosphorylates SMAD1/5. The design of the optoTGFBRs system is different from that of the Opto-acvr1b/2b or Opto-TGFBR1* system. The optoTGFBRs system provides a platform for simultaneously monitoring the dynamics of upstream TGF-β receptors and downstream Smad signaling in single cells. In the optoTGFBRs system, optoTβRI is expressed at the plasma membrane, while optoTβRII is expressed in the cytoplasm and thus is spatially separated from optoTβRI. Upon light stimulation, the spatial localization of the optoTβRII receptor is changed, which indicates the formation and dissociation of TGF-β receptor complexes. In addition, iRFP-Smad2 is coexpressed as a reporter of downstream Smad signaling. This design is useful for establishing a quantitative relationship between the upstream TGF-β receptor activation and downstream Smad signaling at the single-cell level.

Engineering of Reporters for the Visualization and Quantification of TGF-β Signaling

The engineering of TGF-β signaling reporters combined with imaging methods, such as live-cell imaging and single molecule imaging, has provided quantitative information on TGF-β signaling in single cells. By transfecting Smad proteins with fluorescent or epitope tags into cells, an extensive literature demonstrates that Smad proteins, similar to β-catenin, ERK and p53, undergo nucleocytoplasmic shuttling and regulate gene expression [55–62]. In later work, cell lines that stably express fluorescent Smad2 or Smad4 protein were constructed. These cell lines allow researchers to use live cell imaging and mathematical modeling approaches to investigate the quantitative relationship between downstream signaling outputs and upstream TGF-β signaling [21,23,63]. Schmierer *et al.* [63] showed that Smad nucleocytoplasmic dynamics serve as a robust interpretation system for TGF-β signaling and that this system is robust to fluctuations in upstream receptor activity. Warmflash and coworkers [21] further demonstrated that, for certain genes, their transcriptional dynamics correlate with the nuclear localization of Smad4, but not Smad2, at the

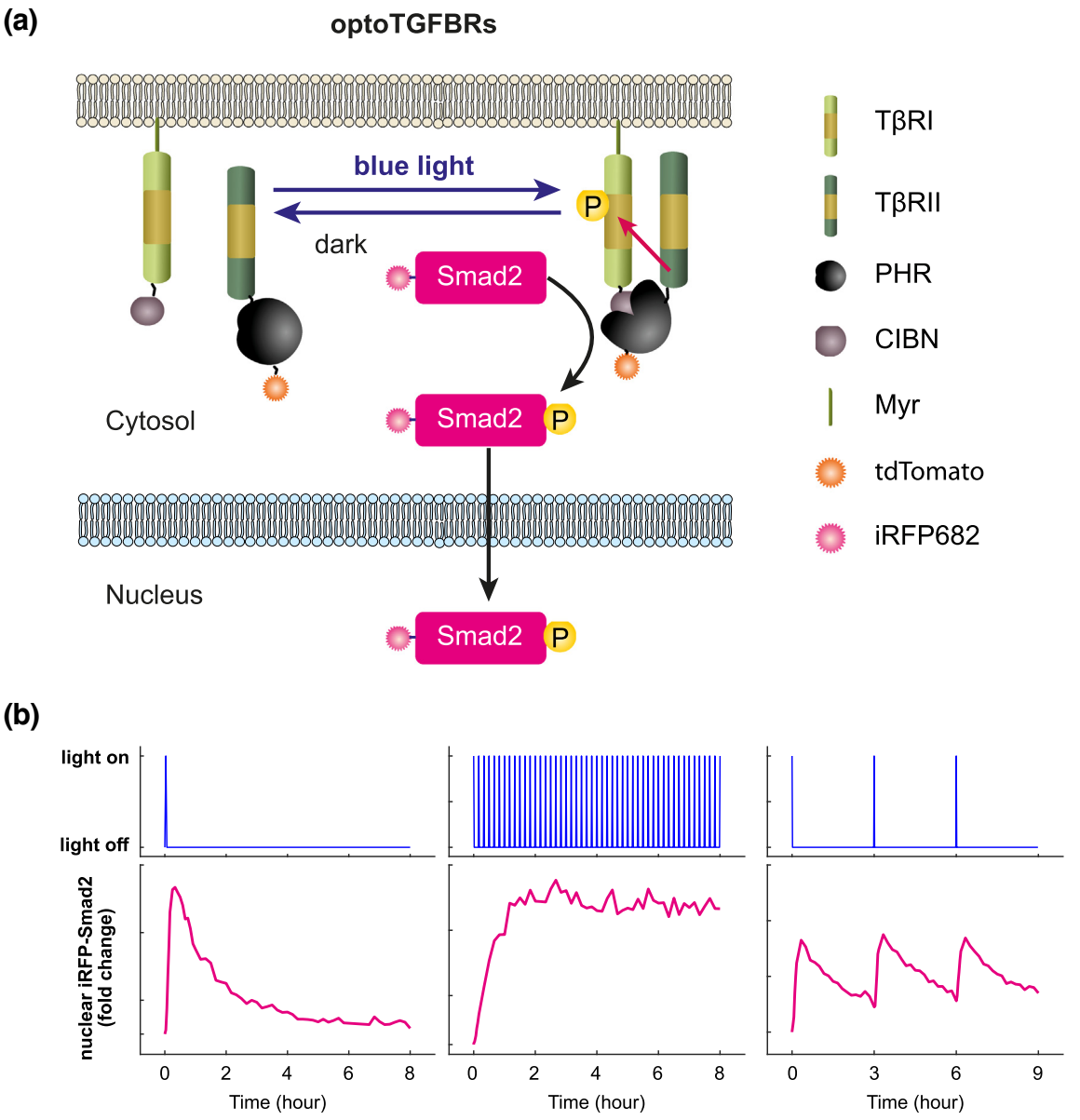

Fig. 2. The optogenetic control of TGF-β signaling (the optoTGFBRs system). (A) Schematic representation of the optoTGFBRs system. (B) Different patterns of Smad2 signaling can be generated in optoTGFBRs-expressing cells by modulating the frequency of blue light pulses. Detailed information is available in Ref. [52]. Adapted with permission from Ref. [52]. Copyright (2018) American Chemical Society.

population level. In addition, they found that TGF-β signaling dynamics depend on the speed of the increase in TGF-β concentrations [23]. It seems that the dependence of transcriptional dynamics on Smad proteins is gene-specific. Tidin *et al.* [64] recently measured the dynamics of Smad2/4 nuclear translocation and the transcriptional response of the endogenous connective tissue growth factor (*ctgf*) gene in individual cells. They found that Smad2/4 nuclear translocation efficiency had little impact on *ctgf* transcriptional activation.

Fluorescently tagged Smad proteins are expressed heterogeneously in individual cells. Accordingly, the nuclear translocation of these proteins varies substantially across cells. The large amount of data generated from live-cell imaging experiments poses a challenge in the analysis of heterogeneous Smad signaling in individual cells. Fortunately, recent advances in imaging analysis have facilitated the quantification of signaling dynamics in single cells. For example, by tracking the dynamics of nuclear Smad3 signals with single-cell resolution, Frick and his

colleagues [24] found that the fold change in nuclear Smad3 relative to its basal level is a robust response in single cells after TGF-β stimulation, although the level of nuclear Smad3 is heterogeneous among individual cells. They further observed that the expression of Smad3 regulated genes correlates strongly with the fold change in nuclear Smad3. Using a fluorescent reporter system, Strasen *et al.* [26] performed long-term quantitative measurements of Smad2 signaling in the breast epithelial cell line MCF10A. Cells present various dynamic patterns of Smad2 nuclear signaling, which can be clustered into six signaling classes using dynamic time warping. Additional kinetic modeling analyses indicate that the variation of protein abundance could resemble the observed heterogeneous Smad2 signaling. Further studies are needed to clarify which protein's abundance plays a dominant role in determining the heterogeneity of TGF-β signaling.

The visualization of TGF-β receptors has been attempted by different groups. By transfecting cells with various combinations of epitope-tagged TβRI and TβRII receptors, Lodish's laboratory found that homodimers of TβRI and TβRII receptors form in resting cells and that hetero-oligomers exist after TGF-β stimulation [65,66]. Recently, researchers engineered fluorescently tagged TβRII/TβRI proteins and applied cutting-edge microscopy to investigate the stoichiometry of TGF-β receptors in cells. The groups of Chen and Fang expressed GFP-tagged TβRI and EGFP-tagged TβRII at a low expression level and monitored individual TβRI-GFP and TβRII-EGFP on the living cell surface with total internal reflection fluorescence microscopy [67,68]. These single-molecule imaging studies revealed that both the TβRI and TβRII receptors mainly form monomers in resting cells and that they undergo dimerization after TGF-β treatment, providing new information on the oligomeric status of the TGF-β receptors [69,70]. Similar approaches have been extended to study TGF-β receptor endocytosis and have revealed some new insights. Previous work indicated that TGF-β receptors could be internalized through two distinct routes: clathrin-mediated endocytosis and caveolae-mediated endocytosis [71]. Surprisingly, He *et al.* [72] found that these two types of endocytic vesicles could be fused into one compartment, in which the TβRI receptor is internalized into caveolin-1 and EEA1 double-positive early endosomes. It has been estimated that only 5%–10% of TGF-β receptors are located at the cell surface due to their rapid internalization and relatively slower recycling [71]. However, a high-resolution image of the spatial organization of TGF-β receptors is lacking. Rys *et al.* [73] simultaneously expressed fluorescently tagged mCherry-TβRI and mEmerald-TβRII in cells and evaluated the localization of these receptors with the combination of high-resolution imaging and single particle tracking methods. They found that TGF-β receptors are spatially separated and located in segregated domains at the cell surface.

Rewiring of TGF-β Signaling Toward Clinical Applications

Given the importance of TGF-β signaling in development and diseases, targeting TGF-β signaling has potential clinical applications. Almost all the components of the canonical TGF-β signaling pathway have been targeted by various types of drugs including small-molecule inhibitors, antisense oligonucleotides and antisense RNA, ligand traps, and antibodies. The progress in this area has been well summarized in recent reviews [11,74–76]. Here, we highlight recent progress in the engineering of signaling proteins that rewire TGF-β signaling for therapeutic applications. The rewiring of TGF-β signaling information flow has been performed in two different ways: (1) by constructing a new signaling system that specifically binds the TGF-β ligand and generates new customized signaling outputs, and (2) by engineering synthetic proteins that target TGF-β signaling proteins and inhibit endogenous TGF-β signaling activities.

Immunotherapy based on checkpoint inhibitors and chimeric antigen receptor (CAR) T cells represents a breakthrough in cancer therapy [77]. Mounting evidence indicates that TGF-β is a potent immunosuppressive cytokine involved in suppressive immune responses [9,78–80]. In addition, it has been reported that high levels of TGF-β are frequently found in colorectal carcinomas, which are clinically associated with poor survival in patients [81,82]. Therefore, it is not surprising that tremendous effort has been made to target TGF-β signaling for cancer treatment [11]. Researchers have started to engineer CAR-expressing T cells that specifically bind the TGF-β ligand and overcome its immunosuppressive effect [83,84]. Chang *et al.* [83] designed TGF-β CAR T cells with a rewired input–output system, in which TGF-β was transformed from an immunosuppressive ligand to a strong cytokine for primary human T cells. In this design, the TGF-β-binding CAR consists of an extracellular TGF-β-binding scFv fused to the CD28 and CD3ζ endodomains (Fig. 3). The scFv is able to block TGF-β-induced Smad2 phosphorylation, while the CD28 costimulatory domain is helpful for converting the T-cell response from endogenous TGF-β signaling to the immunostimulatory pathway.

A second strategy is to engineer proteins and peptides that interfere with TGF-β signaling. In a recent study [85], the dnTGFβRII-T2A-Pbbz CAR was constructed to specifically target prostate cancer cells with an anti-prostate specific membrane antigen, which is derived from mouse J591 monoclonal

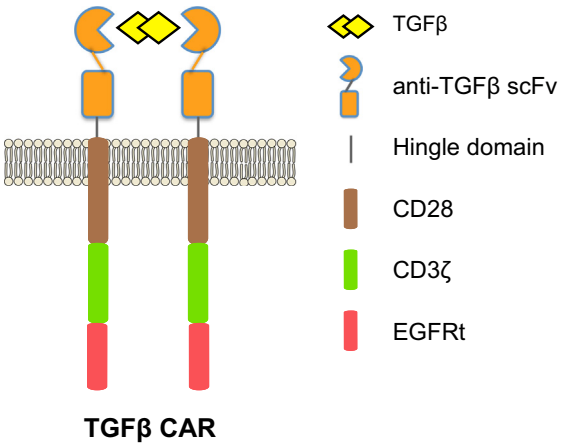

Fig. 3. The design of the TGF-β CAR. The TGF-β CAR contains an extracellular TGF-β-binding scFv, the CD28 and CD3ζ endodomains and a truncated epidermal growth factor receptor (EGFRt). For details, see Ref. [83].

antibody [86]. The dnTGFβRII-T2A-Pbbz CAR also carries a dominant negative TGFBRII (dnTGFβRII) that can block TGF-β signaling [87]. The authors have initiated a phase I trial (NCT03089203) to evaluate the safety and feasibility of using this CAR in patients with metastatic castrate-resistant prostate cancer. Similar dual prostate-specific membrane antigen-specific/TGFβ-resistant CAR T cells were reported by Zhang *et al*. [88]. In addition, another study attempted to design proteins that target activated TGF-β signaling proteins. Wilkes and coworkers [89] previously discovered that the sorting nexin 9 (SNX9) protein binds phosphorylated Smad3 (pSmad3) and specifically promotes pSMAD3 nuclear import after TGFβ treatment. They later found that the SNX9 SH3 domain binds pSMAD3 similarly to the full-length SNX9 protein and acts as a dominant inhibitor to block pSMAD3 nuclear accumulation. Finally, a TAT-SNX9/SH3 peptide was constructed by fusing the SNX9 SH3 domain to the cell-penetrating TAT peptide from HIV [90]. This TAT-SNX9/SH3 peptide can prevent the nuclear import of pSMAD3 and inhibit the profibrotic actions of TGF-β in cells and in living mice. As TGF-β1 is considered a key driver of tissue fibrosis [8], this finding provides proof of concept that it is possible to decrease profibrotic activity by specifically targeting pSmad3 with a synthetic peptide.

The examples discussed above imply that studies on the TGF-β-responsive/irresponsive CAR-T cell provide a new opportunity to develop cell therapies for cancer [91]. Despite the potential application of these cells, great caution should be taken due to the unpredictable toxicities of engineered T cells [1]. The activation of TGF-β *in vivo* is a complicated process, which makes it difficult to distinguish the activity of TGF-β in normal tissue from that in solid tumors. It is not clear whether the current generation of TGF-β CAR-T cells can avoid unacceptable toxicity in normal tissues when they are applied *in vivo*. The next generation of TGF-β CAR-T cells will require precise targeting and control mechanisms to avoid toxicity while maintaining their on-target effects.

Challenges and Future Outlook

Many efforts have been made in the engineering of TGF-β signaling. These studies have provided excellent tools for exploring the molecular mechanism of TGF-β signaling and targeting TGF-β signaling for potential clinical applications. However, there are still great challenges to overcome. Because TGF-β signaling is highly cellular context-dependent and different types of ligands sharing common TGF-β receptors give rise to heterogeneous signaling outputs even in the same type of cells, it is not easy to engineer a “magic bullet” that will optimally perturb TGF-β signaling and cure a human disease. In addition, we still lack a clear quantitative understanding of TGF-β signaling and its crosstalk with other signaling pathways. Therefore, the off-target effects in the modulation of TGF-β signaling are difficult to predict. Here, we describe some unmet needs in the engineering of the TGF-β pathway that might improve the understanding of TGF-β signaling dynamics.

The study of TGF-β signaling is hampered by the lack of a suitable visualization tool for reporting TGF-β ligand activity *in vitro* and *in vivo*. It remains an outstanding challenge to quantitatively determine the distribution, production, activation and degradation of TGF-β ligands. The current methods for the measurement of mature TGF-β ligands mainly involve radiolabeled TGF-β, a specific TGF-β antibody or indirect responses based on Smad phosphorylation and TGF-β-responsive gene expression [92]. These experiments can provide semiquantitative information about TGF-β activity, but they are time consuming and do not involve direct visualization of the TGF-β ligand in live cells. An alternative approach to qualitatively study the function of the TGF-β ligand is to establish a reporter system that can directly monitor active TGF-β ligand. Such a solution has been successfully established for other subsets of TGF-β superfamily ligands. Fluorescent-tagged Decapentaplegic (GFP-Dpp) has been extensively used to study the role of Dpp in patterning and growth [93–96]. In addition, bone morphogenetic protein 2 (BMP2) has been fluorescently labeled, and this construct has been used to quantitatively analyze its dynamics in cells [97]. In the future, the field of TGF-β signaling will benefit from the engineering of sensors and reporters that allow direct quantification and tracking of active TGF-β ligand in cells and tissues.

TGF-β signaling can regulate the expression of hundreds of genes. Smad complexes mediate the transcription of these genes. However, the correlation

between Smad signaling dynamics and downstream gene expression has mainly been studied at the population level. In addition, TGF-β-regulated gene expression is often indirectly measured at the protein level. The use of fluorescent proteins to report the dynamics of corresponding target gene expression is hindered by effects on protein maturation and stability. In addition, previous studies have clearly shown that the correlation between protein levels and corresponding mRNA levels in the cell is not always strong [98]. Therefore, it will be necessary to revisit the regulation of TGF-β responsive genes at the single-cell level through engineering a direct reporter for quantifying nascent RNA dynamics. For example, the transcriptional output of a TGF-β-responsive gene in single cells can be measured using approaches involving fluorescent protein-labeled MS2/PP7 [99–102]. As MS2/PP7 fluorescent protein accumulates at the transcription site, nascent RNAs can be detected as fluorescent spots in engineered cells, allowing real-time quantification of transcriptional activities regulated by TGF-β.

The TGF-β signaling pathway involves complicated feedback and crosstalk regulations [19,27,103]. Such a complexity makes it difficult to associate the specific contributions of canonical Smad signaling and non-Smad signaling with the biological functions of TGF-β signaling. Recent studies have provided a synthetic biology approach for bypassing this problem by expressing mammalian signaling circuits in yeast [104–106], which creates an insulated environment to avoid the complicated factors in the original mammalian cellular context. This approach provides new possibilities for mimicking orthogonal signaling cascades and understanding how perturbations change cell signaling in an insulated environment. Such an engineering method would be useful for reevaluating the roles of feedback regulators in controlling TGF-β signaling as well. To date, dozens of proteins have been reported to regulate TGF-β signaling in different contexts [107]. Perturbation of these proteins might have a direct or indirect effect on TGF-β signaling. It will be interesting to test the function of these key regulators by engineering a human TGF-β signaling circuit in yeast. It would also be greatly appreciated if the Smad signaling and non-Smad signaling cascades could be simultaneously included in a constructed yeast model. However, many difficulties must still be solved to reconstruct a functional TGF-β signaling system in yeast.

It becomes clear that optogenetic tools are very powerful for controlling cell signaling. The reported applications of optogenetic tools in TGF-β signaling are mainly focused on the induction of TGF-β receptors complexes with light. Activating TGF-β signaling might induce many branches of TGF-β signaling network, which are difficult to separate when the downstream signaling responses are analyzed as a whole. For example, a recent study showed that TGF-β also induces phosphorylation of Smad1 and Smad5, in addition to the well-known activation of Smad2 and Smad3 [54]. One option for addressing such a complexity is to use optogenetic tools to selectively activate specific downstream Smad nodes with light, thereby isolating a single Smad activation event from a series of Smad signaling activities. This method has been demonstrated through the successful application of opotogenetics to control the activity of the mitogen-activated protein kinase (MAPK) cascade [46]. Other challenges remain in the engineering of optogenetic tools for controlling TGF-β-induced physiological responses, such as the regulation of cell proliferation and epithelial-to-mesenchymal transition.

It has been a long road to the elucidation and disentangling of the puzzle of TGF-β signaling. Despite the great progress that has been made in understanding many components of TGF-β signaling, we are still far away from revealing how heterogeneous TGF-β signaling is quantitatively determined by the context in individual cells and different types of cells. Successful drugs and cell therapies targeting the TGF-β pathway have yet to be applied in the clinical setting. To address these questions, interdisciplinary studies that integrate engineering, experimental and modeling approaches have moved the field closer to a better quantitative and predictive understanding of TGF-β signaling at the systems level. In this context, we envisage that molecular engineering at the intersection of systems and synthetic biology will continue to play a vital role in the study of TGF-β biology.

Acknowledgments

We would like to thank Yuchao Li for her critical reading of this manuscript. This work was supported by a grant to Z.Z. from the Federal Ministry of Education and Research (BMBF)-funded e:Bio SyBioT project (031A309).

Conflict of Interest Statement: The authors have no competing interests to declare.

Received 30 January 2019;
Received in revised form 5 May 2019;
Available online 21 May 2019

Keywords:
TGF-beta;
Smad;
protein engineering;
synthetic biology

Abbreviations used:
TGF-β, transforming growth factor beta; GM-CSF, granulocyte-macrophage colony-stimulating factor; CAR, chimeric antigen receptor.

References

[1] W.A. Lim, C.H. June, The principles of engineering immune cells to treat cancer, Cell. 168 (2017) 724–740.
[2] C. Kiel, E. Yus, L. Serrano, Engineering signal transduction pathways, Cell. 140 (2010) 33–47.
[3] R.M. Gordley, L.J. Bugaj, W.A. Lim, Modular engineering of cellular signaling proteins and networks, Curr. Opin. Struct. Biol. 39 (2016) 106–114.
[4] C.J. Bashor, J.J. Collins, Understanding biological regulation through synthetic biology, Annu. Rev. Biophys. 47 (2018) 399–423.
[5] Y.E. Antebi, N. Nandagopal, M.B. Elowitz, An operational view of intercellular signaling pathways, Curr. Opin. Syst. Biol. 1 (2017) 16–24.
[6] A. PT, M. Fussenegger, Engineering mammalian cells for disease diagnosis and treatment, Curr. Opin. Biotechnol. 55 (2018) 87–94.
[7] D. Tischer, O.D. Weiner, Illuminating cell signalling with optogenetic tools, Nat Rev Mol Cell Biol. 15 (2014) 551–558.
[8] K.K. Kim, D. Sheppard, H.A. Chapman, TGF-beta1 signaling and tissue fibrosis, Cold Spring Harb. Perspect. Biol. 10 (2018).
[9] C.J. David, J. Massague, Contextual determinants of TGFbeta action in development, immunity and cancer, Nat Rev Mol Cell Biol. 19 (2018) 419–435.
[10] P.M. van der Kraan, The changing role of TGFbeta in healthy, ageing and osteoarthritic joints, Nat. Rev. Rheumatol. 13 (2017) 155–163.
[11] S. Colak, P. ten Dijke, Targeting TGF-β signaling in cancer, Trends in Cancer 3 (2017) 56–71.
[12] M. Morikawa, R. Derynck, K. Miyazono, TGF-beta and the TGF-beta family: context-dependent roles in cell and tissue physiology, Cold Spring Harb. Perspect. Biol. 8 (2016).
[13] A.P. Hinck, T.D. Mueller, T.A. Springer, Structural biology and evolution of the TGF-beta family, Cold Spring Harb. Perspect. Biol. 8 (2016).
[14] C.H. Heldin, A. Moustakas, Signaling receptors for TGF-beta family members, Cold Spring Harb. Perspect. Biol. 8 (2016).
[15] E.H. Budi, D. Duan, R. Derynck, Transforming growth factor-beta receptors and Smads: regulatory complexity and functional versatility, Trends Cell Biol. 27 (2017) 658–672.
[16] Y. Shi, J. Massague, Mechanisms of TGF-beta signaling from cell membrane to the nucleus, Cell. 113 (2003) 685–700.
[17] C.S. Hill, Transcriptional control by the SMADs, Cold Spring Harb. Perspect. Biol. 8 (2016).
[18] A. Hata, Y.G. Chen, TGF-beta signaling from receptors to Smads, Cold Spring Harb. Perspect. Biol. 8 (2016).
[19] X. Yan, X. Xiong, Y.G. Chen, Feedback regulation of TGF-beta signaling, Acta Biochim. Biophys. Sin. Shanghai 50 (2018) 37–50.
[20] Z. Zi, Z. Feng, D.A. Chapnick, M. Dahl, D. Deng, E. Klipp, et al., Quantitative analysis of transient and sustained transforming growth factor-beta signaling dynamics, Mol. Syst. Biol. 7 (2011) 492.
[21] A. Warmflash, Q. Zhang, B. Sorre, A. Vonica, E.D. Siggia, A.H. Brivanlou, Dynamics of TGF-beta signaling reveal adaptive and pulsatile behaviors reflected in the nuclear localization of transcription factor Smad4, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E1947–E1956.
[22] Z. Zi, D.A. Chapnick, X. Liu, Dynamics of TGF-beta/Smad signaling, FEBS Lett. 586 (2012) 1921–1928.
[23] B. Sorre, A. Warmflash, A.H. Brivanlou, E.D. Siggia, Encoding of temporal signals by the TGF-beta pathway and implications for embryonic patterning, Dev. Cell 30 (2014) 334–342.
[24] C.L. Frick, C. Yarka, H. Nunns, L. Goentoro, Sensing relative signal in the Tgf-beta/Smad pathway, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E2975–E2982.
[25] Y.E. Zhang, Mechanistic insight into contextual TGF-beta signaling, Curr. Opin. Cell Biol. 51 (2018) 1–7.
[26] J. Strasen, U. Sarma, M. Jentsch, S. Bohn, C. Sheng, D. Horbelt, et al., Cell-specific responses to the cytokine TGFbeta are determined by variability in protein levels, Mol. Syst. Biol. 14 (2018) e7733.
[27] Y.E. Zhang, Non-Smad signaling pathways of the TGF-beta family, Cold Spring Harb. Perspect. Biol. 9 (2017).
[28] J. Massague, TGFbeta signalling in context, Nat Rev Mol Cell Biol. 13 (2012) 616–630.
[29] G. De Crescenzo, C.S. Hinck, Z. Shu, J. Zuniga, J. Yang, Y. Tang, et al., Three key residues underlie the differential affinity of the TGFbeta isoforms for the TGFbeta type II receptor, J. Mol. Biol. 355 (2006) 47–62.
[30] J. Baardsnes, C.S. Hinck, A.P. Hinck, M.D. O'Connor-McCourt, TbetaR-II discriminates the high- and low-affinity TGF-beta isoforms via two hydrogen-bonded ion pairs, Biochemistry. 48 (2009) 2146–2155.
[31] A.P. Hinck, Structure-guided engineering of TGF-betas for the development of novel inhibitors and probing mechanism, Bioorg. Med. Chem. 26 (2018) 5239–5246.
[32] S.K. Kim, L. Barron, C.S. Hinck, E.M. Petrunak, K.E. Cano, A. Thangirala, et al., An engineered transforming growth factor beta (TGF-beta) monomer that functions as a dominant negative to block TGF-beta signaling, J. Biol. Chem. 292 (2017) 7173–7188.
[33] W. Kwiatkowski, P.C. Gray, S. Choe, Engineering TGF-beta superfamily ligands for clinical applications, Trends Pharmacol. Sci. 35 (2014) 648–657.
[34] I.B. Robertson, D.B. Rifkin, Regulation of the bioavailability of TGF-beta and TGF-beta-related proteins, Cold Spring Harb. Perspect. Biol. 8 (2016).
[35] L. Li, J.R. Klim, R. Derda, A.H. Courtney, L.L. Kiessling, Spatial control of cell fate using synthetic surfaces to potentiate TGF-beta signaling, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 11745–11750.
[36] L. Lin, L. Liu, B. Zhao, R. Xie, W. Lin, H. Li, et al., Carbon nanotube-assisted optical activation of TGF-beta signalling by near-infrared light, Nat. Nanotechnol. 10 (2015) 465–471.
[37] T. Satav, J. Huskens, P. Jonkheijm, Effects of variations in ligand density on cell signaling, Small. 11 (2015) 5184–5199.
[38] J. Cabanas-Danes, J. Huskens, P. Jonkheijm, Chemical strategies for the presentation and delivery of growth factors, J. Mater. Chem. B 2 (2014) 2381–2394.
[39] R.A. Anders, E.B. Leof, Chimeric granulocyte/macrophage colony-stimulating factor/transforming growth factor-beta (TGF-beta) receptors define a model system for investigating the role of homomeric and heteromeric receptors in TGF-beta signaling, J. Biol. Chem. 271 (1996) 21758–21766.

[40] K. Luo, H.F. Lodish, Signaling by chimeric erythropoietin–TGF-beta receptors: homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction, EMBO J. 15 (1996) 4485–4496.
[41] X. Yin, J.H. Kang, M. Andrianifahanana, Y. Wang, M.Y. Jung, D.M. Hernandez, et al., Basolateral delivery of the type I transforming growth factor beta receptor is mediated by a dominant-acting cytoplasmic motif, Mol. Biol. Cell 28 (2017) 2701–2711.
[42] S.J. Murphy, K.E. Shapira, Y.I. Henis, E.B. Leof, A unique element in the cytoplasmic tail of the type II transforming growth factor-beta receptor controls basolateral delivery, Mol. Biol. Cell 18 (2007) 3788–3799.
[43] J. Massague, TGF-beta signal transduction, Annu. Rev. Biochem. 67 (1998) 753–791.
[44] L.J. Bugaj, G.P. O'Donoghue, W.A. Lim, Interrogating cellular perception and decision making with optogenetic tools, J. Cell Biol. 216 (2017) 25–28.
[45] K. Kolar, W. Weber, Synthetic biological approaches to optogenetically control cell signaling, Curr. Opin. Biotechnol. 47 (2017) 112–119.
[46] J.E. Toettcher, O.D. Weiner, W.A. Lim, Using optogenetics to interrogate the dynamic control of signal transmission by the Ras/Erk module, Cell. 155 (2013) 1422–1434.
[47] M. Grusch, K. Schelch, R. Riedler, E. Reichhart, C. Differ, W. Berger, et al., Spatio-temporally precise activation of engineered receptor tyrosine kinases by light, EMBO J. 33 (2014) 1713–1726.
[48] T. Kyung, S. Lee, J.E. Kim, T. Cho, H. Park, Y.M. Jeong, et al., Optogenetic control of endogenous Ca(2+) channels in vivo, Nat. Biotechnol. 33 (2015) 1092–1096.
[49] P. Hannanta-Anan, B.Y. Chow, Optogenetic control of calcium oscillation waveform defines NFAT as an integrator of calcium load, Cell Syst. 2 (2016) 283–288.
[50] D. Niopek, P. Wehler, J. Roensch, R. Eils, B. Di Ventura, Optogenetic control of nuclear protein export, Nat. Commun. 7 (2016), 10624.
[51] H.E. Johnson, Y. Goyal, N.L. Pannucci, T. Schupbach, S.Y. Shvartsman, J.E. Toettcher, The spatiotemporal limits of developmental Erk signaling, Dev. Cell 40 (2017) 185–192.
[52] Y. Li, M. Lee, N. Kim, G. Wu, D. Deng, J.M. Kim, et al., Spatiotemporal control of TGF-beta signaling with light, ACS Synth. Biol. 7 (2018) 443–451.
[53] K. Sako, S.J. Pradhan, V. Barone, A. Ingles-Prieto, P. Muller, V. Ruprecht, et al., Optogenetic control of nodal signaling reveals a temporal pattern of nodal signaling regulating cell fate specification during gastrulation, Cell Rep. 16 (2016) 866–877.
[54] A. Ramachandran, P. Vizan, D. Das, P. Chakravarty, J. Vogt, K.W. Rogers, et al., TGF-beta uses a novel mode of receptor activation to phosphorylate SMAD1/5 and induce epithelial-to-mesenchymal transition, Elife. 7 (2018).
[55] G.J. Inman, F.J. Nicolas, C.S. Hill, Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity, Mol. Cell 10 (2002) 283–294.
[56] B. Schmierer, C.S. Hill, Kinetic analysis of Smad nucleocytoplasmic shuttling reveals a mechanism for transforming growth factor beta-dependent nuclear accumulation of Smads, Mol. Cell. Biol. 25 (2005) 9845–9858.
[57] T. Reguly, J.L. Wrana, In or out? The dynamics of Smad nucleocytoplasmic shuttling, Trends Cell Biol. 13 (2003) 216–220.
[58] L. Xu, Y. Kang, S. Col, J. Massague, Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus, Mol. Cell 10 (2002) 271–282.
[59] L. Xu, Y.G. Chen, J. Massague, The nuclear import function of Smad2 is masked by SARA and unmasked by TGFbeta-dependent phosphorylation, Nat. Cell Biol. 2 (2000) 559–562.
[60] C.E. Pierreux, F.J. Nicolas, C.S. Hill, Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus, Mol. Cell. Biol. 20 (2000) 9041–9054.
[61] L. Xu, J. Massague, Nucleocytoplasmic shuttling of signal transducers, Nat. Rev. Mol. Cell Biol. 5 (2004) 209–219.
[62] M. Watanabe, N. Masuyama, M. Fukuda, E. Nishida, Regulation of intracellular dynamics of Smad4 by its leucine-rich nuclear export signal, EMBO Rep. 1 (2000) 176–182.
[63] B. Schmierer, A.L. Tournier, P.A. Bates, C.S. Hill, Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 6608–6613.
[64] O. Tidin, E.T. Friman, F. Naef, D.M. Suter, Quantitative relationships between SMAD dynamics and target gene activation kinetics in single live cells, Sci. Rep. 9 (2019) 5372.
[65] Y.I. Henis, A. Moustakas, H.Y. Lin, H.F. Lodish, The types II and III transforming growth factor-beta receptors form homo-oligomers, J. Cell Biol. 126 (1994) 139–154.
[66] L. Gilboa, R.G. Wells, H.F. Lodish, Y.I. Henis, Oligomeric structure of type I and type II transforming growth factor beta receptors: homodimers form in the ER and persist at the plasma membrane, J. Cell Biol. 140 (1998) 767–777.
[67] W. Zhang, J. Yuan, Y. Yang, L. Xu, Q. Wang, W. Zuo, et al., Monomeric type I and type III transforming growth factor-beta receptors and their dimerization revealed by single-molecule imaging, Cell Res. 20 (2010) 1216–1223.
[68] W. Zhang, Y. Jiang, Q. Wang, X. Ma, Z. Xiao, W. Zuo, et al., Single-molecule imaging reveals transforming growth factor-beta-induced type II receptor dimerization, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 15679–15683.
[69] R. Zhao, N. Li, J. Xu, W. Li, X. Fang, Quantitative single-molecule study of TGF-beta/Smad signaling, Acta Biochim. Biophys. Sin. Shanghai 50 (2018) 51–59.
[70] M. Zhang, Z. Zhang, K. He, J. Wu, N. Li, R. Zhao, et al., Quantitative characterization of the membrane dynamics of newly delivered TGF-beta receptors by single-molecule imaging, Anal. Chem. 90 (2018) 4282–4287.
[71] G.M. Di Guglielmo, C. Le Roy, A.F. Goodfellow, J.L. Wrana, Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover, Nat. Cell Biol. 5 (2003) 410–421.
[72] K. He, X. Yan, N. Li, S. Dang, L. Xu, B. Zhao, et al., Internalization of the TGF-beta type I receptor into caveolin-1 and EEA1 double-positive early endosomes, Cell Res. 25 (2015) 738–752.
[73] J.P. Rys, C.C. DuFort, D.A. Monteiro, M.A. Baird, J.A. Oses-Prieto, S. Chand, et al., Discrete spatial organization of TGFbeta receptors couples receptor multimerization and signaling to cellular tension, Elife. 4 (2015), e09300.
[74] A. de Gramont, S. Faivre, E. Raymond, Novel TGF-beta inhibitors ready for prime time in onco-immunology, Oncoimmunology. 6 (2017), e1257453.
[75] R.J. Akhurst, Targeting TGF-beta signaling for therapeutic gain, Cold Spring Harb. Perspect. Biol. 9 (2017).
[76] R.J. Akhurst, A. Hata, Targeting the TGFbeta signalling pathway in disease, Nat. Rev. Drug Discov. 11 (2012) 790–811.

[77] S. Guedan, M. Ruella, C.H. June, Emerging cellular therapies for cancer, Annu. Rev. Immunol. 37 (2019) 145–171.
[78] S. Sanjabi, S.A. Oh, M.O. Li, Regulation of the immune response by TGF-beta: from conception to autoimmunity and infection, Cold Spring Harb. Perspect. Biol. 9 (2017).
[79] M.A. Travis, D. Sheppard, TGF-beta activation and function in immunity, Annu. Rev. Immunol. 32 (2014) 51–82.
[80] S. Budhu, D.A. Schaer, Y. Li, R. Toledo-Crow, K. Panageas, X. Yang, et al., Blockade of surface-bound TGF-beta on regulatory T cells abrogates suppression of effector T cell function in the tumor microenvironment, Sci. Signal. 10 (2017).
[81] A. Calon, E. Lonardo, A. Berenguer-Llergo, E. Espinet, X. Hernando-Momblona, M. Iglesias, et al., Stromal gene expression defines poor-prognosis subtypes in colorectal cancer, Nat. Genet. 47 (2015) 320–329.
[82] H. Friess, Y. Yamanaka, M. Buchler, M. Ebert, H.G. Beger, L.I. Gold, et al., Enhanced expression of transforming growth factor beta isoforms in pancreatic cancer correlates with decreased survival, Gastroenterology. 105 (1993) 1846–1856.
[83] Z.L. Chang, M.H. Lorenzini, X. Chen, U. Tran, N.J. Bangayan, Y.Y. Chen, Rewiring T-cell responses to soluble factors with chimeric antigen receptors, Nat. Chem. Biol. 14 (2018) 317–324.
[84] A.J. Hou, Z.L. Chang, M.H. Lorenzini, E. Zah, Y.Y. Chen, TGF-beta-responsive CAR-T cells promote anti-tumor immune function, Bio. Eng. Transl. Med. 3 (2018) 75–86.
[85] C.C. Kloss, J. Lee, A. Zhang, F. Chen, J.J. Melenhorst, S.F. Lacey, et al., Dominant-negative TGF-beta receptor enhances PSMA-targeted human CAR T cell proliferation and augments prostate cancer eradication, Mol. Ther. 26 (2018) 1855–1866.
[86] N.H. Bander, E.J. Trabulsi, L. Kostakoglu, D. Yao, S. Vallabhajosula, P. Smith-Jones, et al., Targeting metastatic prostate cancer with radiolabeled monoclonal antibody J591 to the extracellular domain of prostate specific membrane antigen, J. Urol. 170 (2003) 1717–1721.
[87] R. Wieser, L. Attisano, J.L. Wrana, J. Massague, Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region, Mol. Cell. Biol. 13 (1993) 7239–7247.
[88] Q. Zhang, B.T. Helfand, B.A. Carneiro, W. Qin, X.J. Yang, C. Lee, et al., Efficacy against human prostate Cancer by prostate-specific membrane antigen-specific, transforming growth factor-beta insensitive genetically targeted CD8(+) T-cells derived from patients with metastatic castrate-resistant disease, Eur. Urol. 73 (2018) 648–652.
[89] M.C. Wilkes, C.E. Repellin, J.H. Kang, M. Andrianifahanana, X. Yin, E.B. Leof, Sorting nexin 9 differentiates ligand-activated Smad3 from Smad2 for nuclear import and transforming growth factor beta signaling, Mol. Biol. Cell 26 (2015) 3879–3891.
[90] J.H. Kang, M.Y. Jung, X. Yin, M. Andrianifahanana, D.M. Hernandez, E.B. Leof, Cell-penetrating peptides selectively targeting SMAD3 inhibit profibrotic TGF-beta signaling, J. Clin. Invest. 127 (2017) 2541–2554.
[91] T.B. Dolberg, P.S. Donahue, Leonard J.N. Synthetic biology, Reframing cell therapy for cancer, Nat. Chem. Biol. 14 (2018) 204–205.
[92] V. Jurukovski, B. Dabovic, V. Todorovic, Y. Chen, D.B. Rifkin, Methods for measuring TGF-b using antibodies, cells, and mice, Methods Mol. Med. 117 (2005) 161–175.
[93] S. Matsuda, M. Affolter, Dpp from the anterior stripe of cells is crucial for the growth of the *Drosophila* wing disc, Elife. 6 (2017).
[94] O. Wartlick, P. Mumcu, A. Kicheva, T. Bittig, C. Seum, F. Julicher, et al., Dynamics of Dpp signaling and proliferation control, Science. 331 (2011) 1154–1159.
[95] E.V. Entchev, A. Schwabedissen, M. Gonzalez-Gaitan, Gradient formation of the TGF-beta homolog Dpp, Cell. 103 (2000) 981–991.
[96] S. Harmansa, F. Hamaratoglu, M. Affolter, E. Caussinus, Dpp spreading is required for medial but not for lateral wing disc growth, Nature. 527 (2015) 317–322.
[97] H. Alborzinia, H. Schmidt-Glenewinkel, I. Ilkavets, K. Breitkopf-Heinlein, X. Cheng, P. Hortschansky, et al., Quantitative kinetics analysis of BMP2 uptake into cells and its modulation by BMP antagonists, J. Cell Sci. 126 (2013) 117–127.
[98] Y. Liu, A. Beyer, R. Aebersold, On the dependency of cellular protein levels on mRNA abundance, Cell. 165 (2016) 535–550.
[99] X. Pichon, M. Lagha, F. Mueller, E. Bertrand, A growing toolbox to image gene expression in single cells: sensitive approaches for demanding challenges, Mol. Cell 71 (2018) 468–480.
[100] D.R. Larson, D. Zenklusen, B. Wu, J.A. Chao, R.H. Singer, Real-time observation of transcription initiation and elongation on an endogenous yeast gene, Science. 332 (2011) 475–478.
[101] E. Tutucci, M. Vera, J. Biswas, J. Garcia, R. Parker, R.H. Singer, An improved MS2 system for accurate reporting of the mRNA life cycle, Nat. Methods 15 (2018) 81–89.
[102] S. Yunger, L. Rosenfeld, Y. Garini, Y. Shav-Tal, Single-allele analysis of transcription kinetics in living mammalian cells, Nat. Methods 7 (2010) 631–633.
[103] K. Luo, Signaling cross talk between TGF-beta/Smad and other signaling pathways, Cold Spring Harb. Perspect. Biol. 9 (2017).
[104] E.C. O'Shaughnessy, S. Palani, J.J. Collins, C.A. Sarkar, Tunable signal processing in synthetic MAP kinase cascades, Cell. 144 (2011) 119–131.
[105] M. Mathur, J.S. Xiang, C.D. Smolke, Mammalian synthetic biology for studying the cell, J. Cell Biol. 216 (2017) 73–82.
[106] Z.B. Zhang, Q.Y. Wang, Y.X. Ke, S.Y. Liu, J.Q. Ju, W.A. Lim, et al., Design of tunable oscillatory dynamics in a synthetic NF-kappaB signaling circuit, Cell Syst. 5 (2017) 460–70 e5.
[107] E.H. Budi, J. Xu, R. Derynck, Regulation of TGF-beta receptors, Methods Mol. Biol. 1344 (2016) 1–33.