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Mechanistic insight into contextual TGF- β signaling

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Abstract

Transforming growth factor- β (TGF- β) controls a wide range of cellular functions by activating both SMADs and non-SMAD pathways. In different tissue or physiological environment, cellular responses to TGF- β can be diverse, even opposite. Complex regulations at the level of ligand mobilization, receptor presentation, and the network of intracellular signal transducers afford the TGF- β pathway with versatile means to induce precise cellular responses in accordance to specific contextual demands. This article summarizes recent development in how cells manage their responses to TGF- β through ligand activation, receptor abundance, as well as SMAD-dependent and SMAD-independent mechanisms.

Introduction

Transforming growth factor- β (TGF- β) is the prototype of a very large family of peptide growth factors with 33 members regulating a wide range of cellular functions during development and in maintaining adult tissue homeostasis. The core conduit of TGF- β signal consists of a complex of type I and type II membrane-associated receptors and downstream SMAD transcriptional effectors [1]. The two types of TGF- β receptors (TGF β Rs) are structurally similar, both containing a glycosylated and disulfide-rich ectodomain, a single transmembrane segment, and a cytoplasmic kinase domain [2]. The kinase domains of TGF β Rs not only possess serine/threonine kinase activity but also are able to induce tyrosine phosphorylation. Among 7 type I receptors, which are also called activin receptor-like kinases (ALKs), ALK5 (also known as TGF β RI) mediates TGF- β signaling in all types of mammalian cells, while ALK1 in endothelial cells and ALK2 in other cells can also function with ALK5. The basic scheme of TGF- β signaling is quite straight forward, with ligand engagement inducing the assembly of receptor complexes that enables type II receptors, which are constitutively active, to phosphorylate the glycine-serine-rich domain (GS domain) of type I receptors. The activated type I receptors in turn phosphorylate SMADs at their carboxyl terminus and set forth their movement into the nucleus for controlling target gene expression. Of 8 SMADs encoded in mammalian genomes, 5

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(Smad1/5/8 for ALK1/2 and Smad2/3 for TGF β RI) are direct substrates of their cognate receptor kinases and are thus called the receptor-specific R-SMADs, and 1 (SMAD4) is common to all receptor pathways, and 2 (SMAD6/7) possess inhibitory functions. TGF- β receptors also activate a number of other signal transducers such as MAPKs, PI3K/AKT, JAK/STAT and small GTPases; these non-SMAD pathways carry out either stand-alone functions or converge back to SMADs to modulate TGF- β signaling output [3]. Because of the share number of pathway components and their crucial functions, it is not surprising that dysregulation of TGF- β signaling has profound impacts on human diseases including cancer, making this pathway a rich ground for therapeutic interventions. Commensurate to the diverse functions of the large repertoire of ligands, components of the TGF- β pathway are heavily regulated to generate exact signaling response in accordance to specific biological and physical cues. However, the complexity of regulatory mechanism is multiplied even further by the fact that cellular responses to a particular TGF- β ligand are often context dependent; it is not uncommon that a same ligand elicits completely opposite sets of responses in different tissue environment or cell types. At present, effort utilizing combinations of traditional and ever-changing advanced genomics/systemic approaches to untangle the web of intricate contextual controls of TGF- β signaling has come into the focus of the field. This review summarizes recent progress in understanding how cell context determines TGF- β signaling output through regulating ligand activation, TGF β R abundance, as well as SMAD and non-SMAD pathways.

Regulation of TGF- β signaling by cell tension and polarity

In their natural tissue locale, cells response not only to biological cues specified by growth factors, but also changes in the physical environment [4]. The stiffness of extracellular matrix (ECM), tension on cell surface, cell density, and other mechanical forces are all integral to morphogenesis during embryonic development and niche factors governing the physiology of tissue stem cells in the adult. Cell tension caused by attachment to ECM is transmitted to intracellular cytoskeleton through integrin receptors [5]. At focal adhesions, interaction between integrin and ECM induces actomyosin contraction, thereby generating cellular tension. The tensile force plays critical roles in releasing active ligands from latent pools embedded in the ECM as well as organizing cell surface distribution of the receptors [6, 7] (Figure 1). Following de novo biosynthesis as propeptides, all three TGF- β isoforms are cleaved by furin protease into dimeric disulfide-linked mature ligands and the noncovalently associated prodomains; the latter of which are tethered to latent TGF- β binding proteins (LTBPs) or glycoprotein-A repetitions predominant proteins (GARPs) to form latent complexes, in which TGF- β is kept inactive by a ring-shaped “straitjacket” of the prodomain, but is otherwise poised to be rapidly activated and released upon biochemical and physical cues from the surrounding milieu [8, 9]. Two integrins, α V β 6 and α V β 8, possess the intrinsic ability to bind RGDLXX(I/L) motifs of the prodomain; however, this interaction, albeit essential, is not sufficient by itself to cause the release of active TGF- β [8, 9]. Several structural and functional studies suggest that cell tension plays a crucial role in active TGF- β ligand releasing. Recently, a proof of this hypothesis has come to light with structural studies comparing the 3-D crystal structures of integrin α V β 6 head fragment with a complex consisting of the head fragment and a pro-TGF- β 1 dimer demonstrated that

binding of integrin induces conformational changes not only at the interdigitated interacting interface but also deep into a hydrophobic region of the prodomain that plays an important role in restraining the active growth factor [10].

Relative to ligand release, effect of tension force on receptor organization at cell surface is less clear. Recent high-resolution imaging and single molecule tracking experiments performed in ATDC5 chondroprogenitor cells showed that integrin discretely arranges TGF β RI in focal adhesions but simultaneously excludes TGF β RII to the surrounding area [11]. This type of spatial organization would confer focal adhesion with mechanosensory control of TGF- β receptor activities. Indeed, disrupting cell tension by a Rho/ROCK inhibitor caused the collapse of the physical separation between TGF β RI and TGF β RII, permitting these two receptors to interact and form an active signaling complex.

Cell-cell contact is another contextual factor that impacts cellular response to TGF- β signaling. The discovery of Hippo pathway has linked the organ size control and cell differentiation to the aged old phenomenon of cell contact inhibition. The activity of the Hippo pathway is closely associated with cell junctions, cellular architecture, and the mechanical properties of the microenvironment [12, 13]. When epithelial cells achieve high density, the assembly of Crumbs apical-basal polarity complex leads to activation of Hippo pathway kinases MST and LATS, which in turn phosphorylate nuclear transducers YAP/TAZ, and redirect their localization back to the cytoplasm [12]. Phosphorylated TAZ/YAP interact with SMAD2/3, causing them to be sequestered in the cytoplasm [14]. This led one group to believe that assembly of cell polarity attenuates TGF- β signaling responses via Hippo pathway [15, 16] (Figure 1). However, this view was challenged by a different group, who reported that the attenuation was caused by polarized distribution of TGF β RI and TGF β RII receptors that deprived the access of apically delivered ligand to its receptors rather than the Hippo pathway activation [17, 18]. TGF- β pathway can also regulate Hippo signaling by interacting with scaffold protein RASSF1A and promoting degradation of RASSF1A through polyubiquitination [18]. This inactivates the MST/LATS kinase cassette, facilitating nuclear localization of the YAP/TAZ and pSMAD2/3 complex. Moreover, ECM stiffness and tension of the actomyosin cytoskeleton also increases YAP/TAZ nuclear localization in many different types of cells independent of the MST/LATS kinases [19] (Figure 1). Nuclear localized YAP/TAZ form complex with TEAD and p-SMAD2/3 to regulate diverse transcriptional programs that maintain pluripotency in embryonic stem cells, regulate differentiation into diverse lineages, and promote tumorigenesis and fibrosis in various models [20–22].

AKT regulates pathway responsiveness by receptor abundance

The abundance of TGF- β receptors at cell surface influences the duration and amplitude of the pathway activity. It is now clear that both TGF β RI and TGF β RII are regulated by complex mechanisms that impose strict control over their rate of turnover [2]. For TGF β RI, the closely related HECT type E3 ligases Smurf1, Smurf2, NEDD4-2, and WWP1 all have been reported to play a role in ubiquitylation and degradation by using SMAD7 as an adaptor. Several deubiquitinating enzymes (DUBs), namely ubiquitin specific protease (USP) 4, 11, 15, and ubiquitin C-terminal hydrolase (UCH) 37 have also been shown to partake in

deubiquitylating TGF β RI either directly or through recruitment by tumor necrosis factor receptor-associated factor (TRAF) 4. The stability of TGF β RII is controlled by a different ubiquitin E3 ligase complex consisting of valosin-containing protein (VCP) and β TRCP, which is recruited onto the receptor by FAS associated factor 1 (FAF1) [23]. In different cell or tissue context, activities of these E3 ligases and DUBs are regulated in response to various signaling inputs to fine tune TGF- β receptor levels. Multiple studies have demonstrated that by phosphorylating key components in these enzymatic complexes, AKT coordinately stabilizes both TGF β RI and TGF β RII, henceforth the responsiveness to TGF- β [23, 24] (Figure 2). AKT-mediated USP4 phosphorylation was shown to enhance the association of USP4 with TGF β RI at the plasma membrane resulting in TGF β RI deubiquitylation, and directly opposes the TGF β RI ubiquitylation by SMURF2 in association with SMAD7 [24]. AKT also phosphorylates FAF1, thereby disrupting the FAF1-VCP complex that ubiquitylates TGF β RII, leading to increased level of TGF β RII at the cell surface [23]. Moreover, AKT activation was shown in direct control of TGF β R level at the cell surface through phosphorylation of AS160, a RabGAP, thus promoting translocation of TGF β Rs from intracellular pools to the plasma membrane [25]. These underlining mechanisms of AKT likely account for the heightened TGF- β responsiveness of cancer cells with PTEN or RTK mutations that activate AKT.

Contextual switch of SMAD functions in cancer cells

For its name sake, TGF- β was originally identified as a transforming factor of fibroblasts. Now well-known for its ability to inhibit the proliferation of epithelial cells and suppress tumor formation in pre-malignant cells, TGF- β also promotes the growth of fibroblasts and metastasis of advanced tumors [26]. Recent progress in revealing SMAD functions has shed light on how this dichotomous functional switch is achieved (Figure 3). The DNA binding MH1 domains of SMADs are known to have weak affinity and limited sequence specificity, making it necessary for them to partner with other site-specific transcription factors as well as chromatin modifiers to regulate transcription [27]. Many of these SMAD-interacting transcription factors are tissue-specific and are often prime recipients of cross talk from other signaling pathways, thus generating substantial versatility in the transcriptional response in context-dependent manner. It appears that cancer cells have evolved capacity to take advantage of this built-in promiscuity to rewire SMAD interacting network for malignant gains. This is exemplified in breast cancer cells, in which high expression of 14-3-3 ζ was shown to disrupt the tumor-suppressing p53-SMADs partnership, allowing the released SMADs to partner with Gli2 [28]. The 14-3-3 ζ -driven SMAD-Gli2 complex then spurs the metastasis of breast cancer cells to bones by promoting PTHrP expression. A different contextual SMAD4 switch was found at work in advanced gastrointestinal and pancreatic cancers where *SMAD4* is frequently mutated or deleted, a genetic culprit often associated with the ability of TGF- β to promote metastasis and drug resistance [29, 30]. Although critical for the majority of TGF- β -induced transcription, particularly of those genes involved in cell cycle arrest, SMAD4 is not essential for all TGF- β responsive genes [31]. It was known that in SMAD4-deleted ductal pancreatic tumor cells, SMAD2 and SMAD3 are principle mediators of TGF- β -induced transcriptional responses in lieu of SMAD2/3 and SMAD4 complexes that promote apoptosis and tumor suppression [32]. This

“acquired” transcription program drives the expression of endodermal master genes Sox4 and Klf5, which cooperatively promote tumorigenesis.

In addition to acting as transcriptional regulators, R-SMADs have been reported to possess RNA-binding affinity independent of SMAD4. R-SMADs have been reported to bind double strand RNA sequence that closely resembles the DNA binding element (SBE) of SMADs as well as RNA-binding protein DEAD-box RNA helicase p68 (DDX5) to facilitate the processing of a subset pri-miRNAs [33]. The SMAD-regulated miRNA processing plays an important role in gene regulation in SMAD4-deficient tumor cells. Recently, SMAD3 was also shown to form complex with poly(RC) binding protein 1 (PCBP1) in cancer cells and bind RNA directly in the variable exon region of cancer stem cell marker CD44 pre-mRNA, forcing the exclusion of these alternate exons and promoting the production of the shortened mesenchymal CD44s isoform, which is essential for EMT and metastasis [34]. Formation of the SMAD3-PCBP1 complex requires phosphorylation of PCBP1 by an activated RTK signaling cascade and SMAD3 at T179 in the linker region by MAPKs and CDKs. In addition to CD44, the alternative splicing patterns of many EMT genes have been uncovered to be regulated by SMAD3 in the same fashion. Thus, in the context of cancer cells with elevated RAS-MAPK and CDK activities, phosphorylation of SMAD3 at T179 forges a RNA-binding liaison with PCBP1 for controlling RNA splicing and converting TGF- β into a tumor promoter. The same T179 phosphorylation has also been shown to induce monoubiquitination of SMAD3 by HECT-domain containing E3 ligase SMURF2, causing disruption of the SMAD3-SMAD4 complex [35]. Likewise, SMAD4 was also shown to undergo a similar monoubiquitination that disrupts the SMAD3-SMAD4 complex and DNA-binding [36].

Non-SMAD signaling

The wide range of non-SMAD signaling transducers activated by TGF- β afford cells versatile means to strengthen, attenuate or otherwise modulate downstream responses in accordance to physiological and pathological contextual demands [3, 37–39]. The diversity of these non-SMAD pathways reflects the number of proteins with which the TGF- β receptor complex interact, however, much remains to be learned about the identity of those proteins that fall into direct contact with the receptors. To date, the best characterized non-SMAD effector of the activated TGF- β receptor complex is the ubiquitin E3 ligase TRAF6, which was known for the activation of TAK1 and downstream JNK and p38 MAPK [40, 41]. Recent data indicated that polyubiquitination of the PI3K regulatory subunit p85 α by TRAF6 promotes the formation of a TGF β RI and p85 α complex, leading to the activation of PI3K and AKT, although it was argued that the kinase activity of TGF β RI was dispensable [42]. Another significant progress in non-SMAD-mediated TGF- β signaling mechanism is the revelation of the physical interaction between JAK1 and TGF β RI that leads to STAT3 activation [43]. Phosphorylated STAT3 was shown to be essential for the induction of nearly half of TGF- β target genes, at least in hepatic stellate cells, suggesting a close relationship between STAT3 and SMAD3 [43]. In fact, cooperation between activated STAT3 and SMAD3 is critical for the onset of TGF- β -induced liver fibrosis [43, 44].

A general belief in the field is that SMADs are activated by internalized receptor complexes in clathrin-coated pits, whereas activation of some noncanonical signaling transducers, namely MAPKs and PI3K/AKT occurs in the caveoli lipid compartment [45] (Figure 2). The physical separation of canonical and noncanonical pathways enables receptor interacting proteins with propensity to sort TGF β Rs to different endocytic routes to adjust relative strength of these different branches of the TGF- β pathway. In addressing the mechanism of TGF- β -induction of EMT, ShcA was uncovered with an ability to stabilize TGF β RI interaction with caveolin-1, causing further enrichment of the receptor complex in lipid raft/caveolae, thus tilting TGF- β signaling toward ERK MAPK and PI3K-AKT activation at the expense of SMADs [46]. Within the caveolae, ShcA recruits GRB2 and SOS1 to tyrosine phosphorylated TGF β RI to activate RAS and ultimately leads to the ERK1/2 MAPK activation [47]. The SMAD7-SMURF2 complex that directs TGF β RI to ubiquitin-mediated degradation has also been found in the caveolae [48], raising an interesting possibility that non-SMAD signaling and TGF β RI turnover occur in a sequential order. However, recent experiments that track intracellular movement of fluorescently labeled TGF β RI showed that these two endocytic pathways may converge under the control of RAB5, forming endosomal vesicles that are positive for both caveolin-1 and clathrin [49]. Various components of both SMAD and non-SMAD branches including EEA1, caveolin-1, RAB5, TGF β RI, SMAD3/SARA complexes, SMAD7/SMURF2 complexes, and RAB11 were found in this unified fusion organelle. This observation may help to explain why TRAF6, which is a key player in the non-SMAD pathway, also enhances SMAD signaling by promoting TGF β RI and CIN85 interaction and recycling of TGF β Rs [50].

Concluding remarks and perspectives

Recent progresses in the field of TGF- β research have centered on better understanding the contextual nature of TGF- β signaling. In a nut shell, the lesson learned is that biological outcomes of TGF- β signaling in specific cellular environment is governed by dynamic changes in signaling complex and interactions between cells and tissues. A major challenge going forward is to integrate various regulatory elements to predict which and how cells read TGF- β signals in different cellular context. Furthering our knowledge on details of molecular mechanisms of TGF- β signaling will pave ways for therapeutic advances, especially in clinically relevant processes such as cancer, fibrosis, inflammation and immunity. Currently several drugs targeting TGF- β or TGF β Rs are in development, however, it is possible they may have considerable variations in therapeutic response among patients with different genetic and disease background. Therefore, there is a need to develop new drugs against TGF- β signaling components that are active only in specific cell types or context, or determine the most efficacious drug combinations for each disease conditions, whether it is immunotherapy, chemotherapy or pathway targeted therapies.

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Figure 1. Regulation of TGF- β signaling by cell tension and polarity

Newly synthesized TGF- β is secreted and stored in the ECM as a latent complex consisting of its dimeric propeptide (referred to as latency-associated peptide [LAP]) and latent TGF- β -binding protein (LTBP). Integrin-mediated cell tension force causes a steady release of the active TGF- β for receptor binding and formation of ligand-bound TGF β RII (II) and TGF β RI (I) complexes that phosphorylate SMAD2 or SMAD3 at their respective C-terminus. ECM stiffness and cell tension also promote the nuclear localization of YAP/TAZ. So in unpolarized cells (i), activated YAP/TAZ and SMAD2/3 along with DNA-binding TEAD to drive diverse transcriptional programs. In emerging polarized cells (ii), the assembly of polarity complex at epithelial cell junctions leads to activation of MST1/2 and LATS1/2, which in turn phosphorylate YAP/TAZ, causing sequestration of YAP/TAZ and dragging along phosphorylated SMAD2/3 to the cytoplasm, thereby blocking transcriptional responses. In the highly polarized epithelial cells (iii), TGF β Rs are restrained to the basolateral side, thus denying the access of apically released paracrine ligands. Moreover, in the absence of ligands, integrins arrange TGF β RI and TGF β RII in discrete membrane compartments, precluding the formation of TGF β R complexes and the activation of SMAD2/3 for TGF- β signaling.

Figure 2. Cell surface receptor abundance and internalization as determinants of TGF- β signaling responses

The SMAD-dependent TGF- β signaling can be initiated in clathrin-coated pits or alternatively in EEA1-positive early endosomes due to the recruitment of the receptors and SMADs by endosomal membrane-bound SARA. Internalized TGF β Rs follow one of the two fates: either being recycled back to cell surface or ending up in lysosomes for degradation. Signaling of some non-SMAD pathways occur in the caveolin-positive lipid compartment, wherein polyubiquitination of TRAF6 is required for the activation of TAK1 and AKT, and ShcA promotes the recruitment of TGF β RI as well as induces tyrosine phosphorylation-mediated MAPK activation. In this model, SMAD7 acts as an adaptor to bring several ubiquitin ligases and DUBs to TGF β RI to control TGF β RI ubiquitylation and degradation, whereas VCF controls TGF β RII ubiquitylation and degradation. AKT plays an important role in inhibiting VCF-mediated TGF β RII ubiquitylation and enhances USP4 activity, thereby increasing the stability of TGF β Rs. AKT also exhibits a propensity to increase cell surface TGF β R through phosphorylation of AS160. Similarly, TRAF6 also possess the ability to promote the recycling of TGF β Rs to cell surface via CIN85.

Figure 3. SMAD function switches in cancer cells

In normal cells, activated SMAD2/3 are set forth to move into the nucleus in partner with SMAD4, where the SMAD complexes cooperate with p53 to activate transcription of cell growth inhibitor p21, leading to tumor suppression. The SMAD complexes also exerting their anti-tumor roles by promoting apoptosis through activating SOX4 and suppressing KLF5. In cancer cells that sustained loss of PTEN, p53, or acquired activating RAS or BRAF mutations, the activated MAPK, AKT, and CDK kinases render SMAD3 to be heavily phosphorylated in its linker region, causing SMAD3 to partner with a different set of transcriptional factors or engaging RNA-binding proteins to partake in pre-miRNA processing and alternative splicing, thus switching its roles from tumor suppression to promoting oncogenic growth and metastasis. Loss of SMAD4 has similar effect in forging a different set of interacting partnership with SMAD3 that converts it into a tumor promoter.