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TGF-β SIGNALING IN CHONDROCYTES

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Abstract

Transforming growth factor-beta (TGF-β) regulates a large variety of cellular activities. Binding of TGF-β to its cell surface receptor triggers several signaling cascades, among which the TGF-β-Smad pathway is the most extensively studied. TGF-β also activates protein kinases, including MAPK, PKA and PKC, and modulates gene expression via its delicate interaction with other signaling pathways. During endochondral bone formation, TGF-β acts as a potent inhibitor of the terminal differentiation of epiphyseal growth plate chondrocytes. This effect appears to be primarily mediated by Smad molecules, although MAPK-ATF2 signaling is also involved. The rate of chondrocyte maturation is tightly regulated through the interactions of Smad-mediated signaling, the Wnt signaling pathway, and the transcription factor Runx2. Improving our understanding of the exact mechanisms underlying TGF-β-mediated signaling pathways and their effects may greatly impact the diagnosis and treatment of many common orthopaedic diseases.

Keywords

TGF-β; Receptor; Smad; Chondrocyte; Maturation; Review; Gene

2. INTRODUCTION

TGF-β signaling is involved in a wide array of cellular activities in both physiological and pathological conditions. It regulates gene expression through several signaling pathways. Among them, the TGF-β-Smad pathway is best characterized. The receptors for TGF-β superfamily have intrinsic serine/threonine kinase activity. Binding of the TGF-β type II receptor (TβRII) causes the phosphorylation of the type I receptor (TβRI), which subsequently activates TGF-β-specific Smads. To date, seven type I receptors for TGF-β superfamily members, which are also referred to as activin receptor-like kinase (ALK), have been cloned in mammals. ALK5 predominantly transduces TGF-β signaling, while ALK3 and ALK6 specifically mediate BMP responses (1–3).

Eight known Smad proteins in vertebrates can be classified into three categories: receptor-activated Smads (R-Smads: Smad1, Smad5, Smad8 for BMP signaling, Smad2 and Smad3 for TGF-β and activin signaling); the common mediator Smad (Co-Smad: Smad4); and inhibitory Smads (I-Smads: Smad6 and Smad7). Upon TGF-β activation, Smad2 and Smad3 are phosphorylated by TβRI; and the phosphorylated Smads form protein complexes with Smad4. These complexes translocate into nucleus, where they regulate target gene expression. Smad2 and Smad3 have distinct roles in mediating TGF-β signaling. Smad3 binds DNA directly, whereas Smad2 regulates gene expression in an indirect way: a 30 amino acid insertion in the

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MH1 domain of Smad2 prevents its binding to the DNA. Smad2 is closely involved in the embryonic development, while Smad3 may play a more important role in adult life (4–8). A recent report showed that TRAP1-like protein (TLP), associates with both active and kinase-deficient TβRII, and represses Smad3-mediated transcription. TLP prevents Smad3 from forming a protein complex with Smad4, but it has little effect on Smad2-mediated TGF-β signaling, suggesting that TLP may serve as a molecular switch balancing the effects of Smad3 and Smad2 (9).

3. REGULATION OF TGF-β SIGNALING

TGF-β signaling cascade is tightly controlled by feedback mechanisms at the different levels: extracellular matrix, cell membrane, cytoplasm, and nucleus. Normal physiological functions of Smads depend on a delicately balanced regulation. I-Smads (Smad6 and Smad7) antagonize R-Smads by inhibiting phosphorylation of R-Smads through competitive binding to the activated TβRI. In addition, I-Smads recruit E3 ubiquitin ligases Smurf1 and Smurf2, which target the activated TβRI at the cell membrane causing receptor degradation. I-Smads also repress the function of R-Smads in nucleus by recruiting transcriptional co-repressors. Smad7 preferentially inhibits TGF-β signaling and plays a role in the dephosphorylation of TβRI, whereas Smad6 is a more specific inhibitor of BMP signaling (10,11).

The Ski proto-oncoprotein family members (c-Ski and SnoN) inhibit TGF-β-mediated transcriptional activation by disrupting the formation of the heteromeric Smad complexes. These oncoproteins migrate with R-Smad/Co-Smad complexes to their DNA binding sites, where they recruit histone deacetylase and repress the transcription of target genes. c-Ski also stabilizes inactive Smad complexes on DNA, thereby suppressing gene transcription (12–15).

Ubiquitin-dependent degradation of Smads by Smurf1 or Smurf2 is critical in regulating the turnover of Smads. Smurf2 is a member of the HECT domain family of E3 ubiquitin ligases. It targets Smad1 and Smad2 for a ubiquitin-dependent degradation. Further, Smurf2 associates with Smad7, and the Smurf-Smad complex interacts with TβRI, enhancing receptor degradation (16–19).

Sumolyation is a ubiquitination-like protein conjugation process. However, it does not target specific substrates for proteasomal degradation. SUMO-1 is actively involved in the regulation of transcription factors. It modulates Smad4 activity through a p38 MAP kinase pathway. The protein inhibitor of activated STAT, PIASy, prevents TGF-β-mediated Smad3 activation by inducing sumolyation of Smad3 (20,21).

4. SMAD-DEPENDENT AND -INDEPENDENT PATHWAYS

In addition to TβRI, increasing numbers of cellular kinases have been shown to participate in the functional modulation of Smad proteins. The interdependent relationship of Smads and these kinases is essential for maintaining the balance between different signaling pathways in response to TGF-β. The phosphorylation of Smads by these kinases not only activates Smads but also changes their capability for nuclear translocation and DNA binding.

TGF-β elicits cellular response through TGF-β activating kinase 1 (TAK1), a member of the MAPKKK family. TAK1 is involved in the activation of several MAP kinases (MAPK), including ERK, p38, and JNK, which ultimately results in activation of ATF2. This MAPK can be activated through both Smad-dependent and Smad-independent mechanisms. TGF-β induces a biphasic JNK response: a rapid Smad-independent activation followed by a Smad-dependent activation. JNK, in turn, phosphorylates Smad3 and facilitates its nuclear translocation. Functional and physical interactions between Smad3/Smad4 and c-Jun/c-Fos have been observed upon TGF-β stimulation, indicating that Smad and MAPK signalings

converge at AP-1 binding sites of target gene (22–27). A recent report demonstrates that TGF-β activates PKA in the absence of increased cAMP. This may occur through complex formation between Smad proteins and the regulatory subunit of PKA with subsequent release of the catalytic subunit. TGF-β may also stimulate the translocation of α-catalytic subunit of PKA to the nucleus (28,29).

Calmodulin-dependent protein kinase II (PKII) and PKC provide negative feedback for Smad2 and Smad3, respectively. In the presence of Ca^{++}, calmodulin interacts with Smad2, 3 and 4 through the conserved N-terminal domain of Smad proteins leading to the phosphorylation of these Smads by calmodulin-dependent PKII. PKC phosphorylates the MH1 domain of Smad3 and interferes its DNA binding (30–33). In osteoblasts, PTH induces Smad3 expression through PKA as well as PKC pathways (34).

5. TGF-β AND OTHER SIGNALING CASCADES

There is evidence for TGF-β-dependent activation of PKB/Akt through PI3 kinase and Rho-dependent signaling pathways. PKB/Akt sequentially regulates TGF-β signaling through a direct interaction with Smad3. PKB/Akt represses TGF-β-Smad3 signaling through a kinase-activity-independent mechanism. It prevents Smad3 phosphorylation, Smad3/4 complex formation and nuclear translocation. In contrast, Smad3 does not inhibit PKB (35–38).

The STAT pathway also modulates Smad activity by the induction of Smad7. PIAS3, a member of the protein inhibitor of activated STAT (PIAS) family, activates TGF-β-Smad transcriptional responses. PIAS3 interacts with the MH2 domain of Smad3 and with the general co-activator p300/CBP. The formation of a PIAS3/Smad3/p300 complex enhances TGF-β signaling. PIASy, another member of the PIAS family, interacts strongly with both Smad3 and Smad4, and inhibits TGFβ/Smad transcriptional responses through a negative feedback loop. PIASy also stimulates the sumolyation of Smad3 *in vivo* (39–42). Several other signals affect TGF-Smad pathway including Notch/and NF-κB (43–45).

6. ENDOCHONDRAL BONE FORMATION

Chondrogenesis begins with mesenchymal condensation, which is driven by transcription factor Sox9 and is characterized by expression of type II collagen (Col-II). Other chondrocyte-specific genes (Col IX, Col-XI, N-cadherin, and aggrecan) are subsequently expressed. Once committed, these chondrocytes are destined to a well-coordinated process referred to as endochondral ossification consisting of proliferation, prehypertrophy, hypertrophy, and apoptosis. Blood vessels together with osteoblasts invade and replace the hypertrophic areas of chondrocytes, which eventually leads to the formation of major axial bones. Several molecules, e.g. Indian hedgehog (Ihh), BMP6, type X collagen (Col-X) and alkaline phosphatase, are detected in hypertrophic chondrocytes. They have been well accepted as chondrocyte maturation marker genes. Conversely, articular chondrocytes do not express these genes: their differentiation is arrested before the chondrocytes become hypertrophic (46–49).

7. EXPRESSION OF TGF-β AND RELATED MOLECULES IN CHONDROCYTES

TGF-β is produced by chondrocytes as a latent, high molecular weight molecule in association with latent TGF-β binding protein (LTBP). In growth plate chondrocytes, the storage of TGF-β by LTBP is cell maturation dependent. Plasmin, transglutaminase and MMPs help release and activate TGF-β (50–54). In the epiphyseal growth plate, TGF-β1 and TGF-β3 are expressed in the resting, proliferating, and hypertrophic zones in 6 to 24-week-old rats. TGF-β2 is expressed at similar areas at 6-weeks of age, but decreased during growth. The expression of TGF-βs in hypertrophic chondrocytes is weak. TβRI is co-expressed with TGF-β ligands in resting, proliferating, and hypertrophic zones throughout the process of development. TβRII

is detected in 6-week-old rats but decreased after that. In the growing human bone, TGF-β2 exits in all zones of endochondral ossification, with the highest expression seen in hypertrophic and mineralizing zones. TGF-β3 is expressed in the chondrocytes of proliferative and hypertrophic zones. TGF-β1 is only found in the proliferative and upper hypertrophic zones. TβRI and TβRII are expressed intensely in the hypertrophic and mineralizing zones. The expression of TGF-β specific Smads is correlated with that of TGF-β1 and its receptors. Whereas Smad2 is strongly expressed in proliferating chondrocytes, Smad3 is found predominantly in mature chondrocytes. Smad3 is also found in perichondrium in developing cartilage undergoing endochondral bone formation. Smad4 is seen in all zones of the growth plate. Smad6 and Smad7 are mainly detected in mature chondrocytes (55–58). These findings suggest that TGF-β and its associated Smad signaling cascade may play an important role in chondrocyte differentiation and maturation in the epiphyseal growth plate.

8. FUNCTION OF TGF-β IN CHONDROCYTES

Although TGF-β is a well-known inhibitor of the cell cycle, its overall *in vitro* effect on chondrocyte proliferation is stimulatory. The response to TGF-β may be affected by cell culture conditions, the origin of the cell line, the extent of differentiation, and presence of other growth factors such as IGF and FGF (59–63). In cultured rat chondrocytes, TGF-β1 stimulates cell proliferation and extracellular matrix formation. Transient expression of c-fos through PKC activation is required for the mitogenic effect of TGF-β. TGF-β transduces its signal through a MEK-ERK-Elk1 pathway to regulate chondrocyte proliferation. The MEK-ERK pathway activated by TGF-β is negatively regulated by PKA but transactivated by PKC (64–66). The pro-proliferative effect of TGF-β is at least partially mediated through induction of cyclin D1 (67).

Treatment with TGF-β increases cartilage matrix synthesis, especially aggrecan. Gene transfer with adenoviral or retroviral vectors expressing TGF-β increases the production of Col-II and proteoglycans. TGF-β increases total glycosaminoglycan synthesis in immature cartilage, but not in mature cartilage. TGF-β helps maintain the matrix components of cartilage in an immature state (68,69). Through a Smad-dependent pathway, TGF-β induces aggrecan expression in chondrogenic cell lines. In response to TGF-β, Smad2 is rapidly phosphorylated, leading to the initial activation of the aggrecan gene. This rapid phosphorylation of Smad2 is, however, not necessary for maintenance of this increased aggrecan expression. In contrast, TGF-β-induced phosphorylation of MAP kinases (ERK and p38) is critical in keeping aggrecan at the constantly elevated level. TGF-β simultaneously upregulates the expression of aggrecanase, and thus accelerates the turnover of cartilage matrix. Overexpression of Smad7 in chondrocytes completely blunts TGF-β1-mediated effects on cell proliferation and proteoglycan synthesis (70,71).

While TGF-β enhances chondrocyte proliferation, it inhibits the terminal differentiation of chondrocytes and helps chondrocytes remain in the prehypertrophic stage. TGF-β acts downstream of Ihh and upstream of PTHrP. However, it has both PTHrP-dependent and -independent effects on chondrocyte maturation. TGF-β1, 2, and 3 all stimulate PTHrP expression, at least partially through Smad-mediated signaling events. In response to TGF-β, ATF2 is rapidly phosphorylated via the activation of p38 MAP kinase. The phosphorylated ATF2 cooperates with Smad3 to inhibit the rate of chondrocyte maturation. Smad3 has stronger inhibitory effect on the terminal differentiation of chondrocytes than Smad2 (26,72–77). As chondrogenesis proceeds, ERK activity is reduced, whereas p38 activity is continuously increased. Inhibition of ERK induces chondrogenesis, whereas inhibition of p38 activity represses chondrogenesis (78).

TGF-β1 stimulates the release of PLA-2 and prostaglandin via the induction of COX-1 in growth plate chondrocytes. PGE2 activates the EP2 receptor, leading to G-protein-dependent activation of PKA. PKA signaling in turn upregulates PKC activity, ultimately resulting in suppression of chondrocyte proliferation and maturation. Over-expression of ALK5, a TGF-β specific type I receptor, hampers chondrocytes maturation and hypertrophy (79,80).

9. ANIMAL MODELS TO INVESTIGATE THE ROLES OF TGF-β IN CHONDROCYTES

Transgenic mice over-expressing a cytoplasmically truncated, dominant-negative form of the TβRII in cartilage develop joint diseases similar to human osteoarthritis. In the epiphyseal growth plate, the expression of some maturation markers such as Col-X and Ihh is greatly enhanced. Hyperplasia and cartilaginous metaplasia of synovium is observed in multiple joints. The mice also display several other skeletal deformities including bifurcation of the xiphoid process and sternum, and kyphoscolisosis (81).

Smad3 deficient mice have been created on the basis of a targeted disruption of exon 8 of the Smad3 gene. At birth, Smad3$^{-/-}$ mice have no developmental or skeletal abnormalities and are indistinguishable from their wild-type littermates both grossly and histologically, except for an angular distortion of the forelimb seen in approximately 30% of the knockout mice. Skeletal deformities start to appear after three weeks. The abnormalities are characterized by premature chondrocyte maturation with increased length of the hypertrophic region, disorganization of the chondrocyte columns, and early expression of Col-X (82).

Sternal chondrocytes from Smad3 knock-out mice have higher expression levels of Col-X and other chondrocyte maturation markers compared to wild-type mice. While no compensatory changes of the TGF-β signaling pathway (upregulation of TβRI, TβRII, Smad2, or Smad4) are observed in Smad3 null mutant mice, both BMP2 and BMP6 are upregulated in Smad3 deficient chondrocytes, suggesting a possible reason for early chondrocyte maturation and degenerative arthritis in these mice. TGF-β inhibits Col-II expression in wild-type cells, but increases Col-II expression in Smad3$^{-/-}$ chondrocytes. Smad3 deficient chondrocytes are more sensitive to BMP treatment than wild-type cells (unpublished data).

10. TGF-β AND RUNX2

Runx2 is a key transcription factor in osteoblast differentiation and chondrocyte maturation (83). Treatment with TGF-β results in phosphorylation of Runx2 at threonine residues, possibly through the ERK signaling pathway. The effect of TGF-β on Runx2 expression varies in different cells. In mesenchymal cells, TGF-β induces Runx2 expression via the MAPK pathway. JunB is the upstream molecule of Runx2 in response to TGF-β. Runx2 in turn regulates TGFβ-mediated responses. The promoter of the TβRI gene contains several Runx2 binding sites and forced expression of Runx2 enhances TβRI promoter activity (84–87). In osteoblasts, TGF-β inhibits the expression of Runx2 through Smad3. Similarly, over-expressing Smad2 represses Runx2 expression in osteoblatic cells. Physical interaction of Smad2 and Smad3 with Runx2 is essential for the collagenase 3 expression in osteoblastic and breast cancer cells (88–90). Recent findings demonstrate that E3 ubiquitin ligase Smurf1 mediates Runx2 degradation in osteoblasts (91,92). Since both Smad2 and 3 directly interacts with Runx2, it would be interesting to determine if Smurf1-mediated Runx2 degradation is TGF-β signaling-dependent in chondrocytes.

11. TGF-β AND WNT SIGNALING

TGF-β and Wnt signaling pathways independently or cooperatively regulate LEF1/TCF target genes. The cooperative enhancement of TGF-β and Wnt signaling relies not only on the physical association of transcription factors. This occurs both at the protein level and also at the DNA level at the Smad binding element (SBE) adjacent to the LEF1 binding site. After TGF-β stimulation, Smad3 interacts with LEF1 to activate target gene transcription. Deletion of SBEs near the LEF1/TCF abrogates Smad-dependent transcription. TGF-β-dependent activation of LEF1/TCF target genes occurs independently of β-catenin. Axin, a negative regulator of Wnt signaling, modulates the effects of Smad3. Axin functions as an adapter for Smad3 and facilitates its activation by TβRI for efficient TGF-β signaling. In human marrow stromal cells, TGF-β upregulates the expression of Wnt2, 4, 5a, 7a, 10a, and Wnt co-receptor LRP5. TGF-β also increases nuclear accumulation and stability of β-catenin. Working synergistically with Wnt signal pathways, TGF-β stimulates chondrocyte differentiation from mesenchymal cells. Wnt 7 may be a critical mediator of this process (93–95). In chicken upper sternal chondrocytes, TGF-β inhibits the expression of Wnt 4, 5, 8, and 14. It also suppresses the β-catenin and TCF-induced Col-X expression (unpublished data). A possible explanation for the discrepancy is that the interdependent relationship between TGF-β and Wnt signaling pathways may vary at the different stages of cell differentiation.

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