Transforming Growth Factor-β Stimulates Cyclin D₁ Expression through Activation of β-Catenin Signaling in Chondrocytes

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Abstract

Transforming growth factor-β (TGF-β) plays an essential role in chondrocyte maturation. It stimulates chondrocyte proliferation but inhibits chondrocyte differentiation. In this study, we found that TGF-β rapidly induced β-catenin protein levels and signaling in murine neonatal sternal primary chondrocytes. TGF-β-increased β-catenin induction was reproduced by over-expression of SMAD3 and was absent in Smad3⁻/⁻ chondrocytes treated with TGF-β. SMAD3 inhibited β-transducin repeat-containing protein-mediated degradation of β-catenin and immunoprecipitated with β-catenin following TGF-β treatment. Both SMAD3 and β-catenin co-localized to the nucleus after TGF-β treatment. Although both TGF-β and β-catenin stimulated cyclin D₁ expression in chondrocytes, the effect of TGF-β was inhibited with β-catenin gene deletion or SMAD3 loss of function. These results demonstrate that TGF-β stimulates cyclin D₁ expression at least in part through activation of β-catenin signaling.

Endochondral bone formation involves condensation and differentiation of mesenchymal cells into chondrocytes, followed by chondrocyte proliferation, maturation, hypertrophic differentiation, and apoptosis. Eventually, the calcified cartilage tissue formed in the growth plate is replaced by bone tissue (1). Each step of the endochondral bone formation process is precisely regulated by local growth factors. Among these factors, transforming growth factor-β (TGF-β)³ plays important roles in chondrocyte proliferation and hypertrophy. TGF-β promotes chondrocyte proliferation but inhibits chondrocyte differentiation and hypertrophy (2–6). The mechanism of TGF-β-induced chondrocyte proliferation remains undefined.

In this study, we investigated the interaction between TGF-β and β-catenin signaling in chondrocytes. We found that TGF-β activates β-catenin signaling through SMAD3. SMAD3 interacted with β-catenin and increased β-catenin nuclear translocation and signaling. Although TGF-β stimulated cyclin D₁ expression in chondrocytes, this effect was abolished by inhibition of β-catenin signaling. These results demonstrate for the first time that TGF-β stimulates cyclin D₁ expression at least in part through activation of β-catenin signaling. These findings provide novel insight regarding the mechanism through which TGF-β regulates chondrocyte proliferation.

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MATERIALS AND METHODS

Cell Culture

*Smad3*−/− mice derived from a C57/B6 lineage, in which exon 8 of the *Smad3* gene is deleted (a kind gift from Dr. C. X. Deng, National Institutes of Health, Bethesda, MD) (7), were bred using heterozygote pairs. 3-day-old neonatal mice were killed and genotyped using tail tissues obtained at the time of death. The anterior rib cage and sternum were harvested *en bloc*, washed with sterile phosphate-buffered saline (PBS), and then digested with Pronase (Roche Applied Science) dissolved in PBS (2 mg/ml) in a 37 °C water bath with continuous shaking for 60 min. This was followed by incubation in a solution of collagenase D (3 mg/ml dissolved in serum-free Dulbecco’s modified Eagle’s medium; Roche Applied Science) for 90 min at 37 °C. The soft tissue debris was thoroughly removed. The remaining sterna and costosternal junctions were further digested in fresh collagenase D solution in Petri dishes in a 37 °C incubator for 5 h with intermittent shaking. This step allows remnant fibroblasts to attach to the Petri dish while the chondrocytes remain afloat in the medium. The digestion solution was filtered through Swinex to remove all residual bone fragments. The solution was centrifuged, and the cells were resuspended in complete medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 100 mM L-glutamine, and 50

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3The abbreviations used are:

- TGF-β: transforming growth factor-β
- PBS: phosphate-buffered saline
- FBS: fetal bovine serum
- TCF: T cell factor
- Ad: adenovirus
- CMV: cytomegalovirus
- GFP: green fluorescent protein
- ICAT: inhibitor of β-catenin and TCF
- β-TrCP: β-transducin repeat-containing protein
- BisTris: 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol
- FITC: fluorescein isothiocyanate
- TRITC: tetramethylrhodamine isothiocyanate
- BrdUrd: 5-bromo-2′-deoxyuridine
- Cdk: cyclin-dependent kinases
- Rb: retinoblastoma.
μg/ml ascorbic acid, pH 7.1). The cells were counted and plated at the appropriate density. To remove any remaining fibroblasts, 24-h cultures were treated with 0.05% trypsin for 1 min to lift the fibroblasts from the culture dish while allowing the chondrocytes to remain attached. A similar procedure was used for chondrocyte isolation from β-catenin<sup>flox/flox</sup> mice (8) and β-catenin reporter TOPGAL transgenic mice, in which a β-galactosidase gene is under the control of a lymphoid enhancer factor/T cell factor (TCF)- and β-catenin-inducible promoter. In these transgenic mice, TOPGAL expression is directly stimulated by a stabilized form of β-catenin (9). The chondrocyte cell line RCJ3.1C5.18 (C5.18) was cultured in α-minimal essential medium containing 10% FBS. In cyclin D<sub>1</sub> reporter and Western blot assays, all cells were synchronized for 3 days in serum-free medium prior to different treatments.

**Adenovirus Production and Infection**

The full-length mouse β-catenin cDNA was cloned into the TOPO entry vector using the Gateway system (Invitrogen). By LR reaction, a β-catenin insert was subcloned into the ViraPower™ adenoviral expression vector. The plasmid was linearized using PacI and transiently transfected into 293A cells. After several cycles of amplification, the adenovirus was purified using the CsCl binding method. Ad5-CMV-Cre-GFP and Ad5-CMV-enhanced GFP were purchased from the Baylor College of Medicine. Infection (multiplicity of infection of 10) lasted for 24 h, and cells were allowed to recover for 48 h prior to treatments. β-Catenin<sup>flox/flox</sup> chondrocytes were infected with Ad5-CMV-Cre-GFP for 24 h and recovered in full medium for 48 h. Ad5-CMV-enhanced GFP was used in the same way as a control.

**β-Galactosidase Activity Assay**

TOPGAL chondrocytes were plated for 24 h. TGF-β (1 ng/ml) was added to cultures in serum-free medium for 24 h. β-Galactosidase activity was measured with a luminescent β-galactosidase detection kit (BD Biosciences) on a luminometer (Opticom 1, MGM Instruments, Inc., Hamden, CT). Each cell preparation was tested in triplicate, and the values were standardized by protein concentrations. The results are presented as the means ± S.E.

**Transfections and Luciferase Assay**

Transient transfection was performed using a Targefect F-2 reagent kit (Targeting Systems, Santee, CA). To increase the transfection efficiency, Virofect provided in the kit was added to all reactions. The transfection complex was formed after incubation at 37 °C for 20 min. At the same time, chondrocytes were pretreated with hyaluronidase (200 ng/ml; Sigma) at 37 °C for 30 min. The transfection complex was then added to cell culture dishes containing Dulbecco’s modified Eagle’s medium with 10% FBS. The following plasmids were transfected into chondrocytes for 12 h before treatments: TOPflash and FOPflash reporter plasmids (a gift from Dr. Jennifer Westendorf, Mayo Clinic College of Medicine, Rochester, MN), the cyclin D<sub>1</sub> promoter (-1745CD1-Luc, a gift from Dr. Phyllis LuValle, University of Florida, Gainesville, FL), β-catenin<sup>S33Y</sup> (constitutively active β-catenin, a gift from Dr. Kenneth Kinzler, The Johns Hopkins University Medical Institutions, Baltimore, MD) (10), F-SMAD3 (a gift from Dr. Yin Sun, University of Rochester, Rochester, NY), and inhibitor of β-catenin and TCF (ICAT; a gift from Dr. Tetsu Akiyama, University of Tokyo, Japan) (11,12). An SV40-<i>Renilla</i> luciferase construct was cotransfected with the above firefly reporters to standardize results for transfection efficiency. Luciferase activity in the cell lysate was determined using a luminometer (Opticom 1). Murine β-transducin repeat-containing protein (β-TrCP) cDNA was amplified by real-time PCR using template RNA extracted from 2T3 osteoblast precursor cells and then cloned into the p3XFLAG-CMV vector (Sigma). The nucleotide sequence was verified by sequencing the entire cDNA.
**Immunoprecipitation**

Immunoprecipitation was performed using a Catch and Release Version 2.0 kit (Upstate, Charlottesville, VA). Briefly, cell lysates (100 μg), primary antibody (1 μg), and antibody capture affinity ligand (10 μl) were added to 500 μl of 10× wash buffer (10% NP-40, 2.5% deoxycholic acid, pH 7.4) and incubated in the provided column at 4 °C for 1 h with constant rotation. After thorough washes, the protein samples were eluted using 70 μl of non-denaturing elution buffer and stored at -80 °C until used. Rabbit anti-SMAD3 polyclonal antibody (Zymed Laboratories Inc.) was used in the experiment.

**Western Blotting**

Chondrocytes were lysed in Golden lysis buffer supplemented with protease inhibitor (Roche Applied Science), 1 mM sodium orthovanadate, 1 mM ethylene glycol bis(β-aminoethyl ether), 1 mM sodium fluoride, and 1 μM microcysteine (Sigma). The protein concentration was determined using a Coomassie Plus protein assay kit (Pierce). The protein extracts (10 μg) were separated using NuPAGE™ Bis-Tris gels (Invitrogen). After transfer to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences) and blocking with 5% milk, the blots were probed with the following mouse monoclonal antibodies overnight at 4 °C: anti-β-catenin (Upstate, Lake Placid, NY), anti-active β-catenin (Upstate), and anti-cyclin D1 (Cell Signaling Technology, Beverly, MA). Anti-β-actin monoclonal antibody (Sigma) at a dilution of 1:8000 was used to confirm equal protein loading. Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were then applied to the membrane and incubated for 60 min. The immune complexes were detected using SuperSignal West Femto maximum sensitivity substrate (Pierce). For multiple detection of different antibodies in the same membrane, we used ReBlot Plus strong antibody stripping solution (Chemicon International, Inc., Temecula, CA).

**Double Immunofluorescence Labeling and Confocal Microscopy**

C5.18 chondrogenic cells (13) were plated in 2-well chamber slides (2000 cells/well; Nalgene Nunc International, Rochester, NY) for 24 h and then treated with TGF-β (1 ng/ml) for 4 h. After washes with PBS, the cells were fixed with acetone/methanol (1:1) at 4 °C for 30 min. Nonspecific binding was blocked by incubation with PBS containing 10% normal goat serum at room temperature for 1 h. After excess serum was removed, mouse anti-β-catenin and rabbit anti-SMAD3 antibodies diluted to 1:50 in PBS containing 0.1% saponin (Research Organics, Inc., Cleveland, OH) were applied to slides and incubated overnight at 4 °C. After thorough washes with PBS, the slides were incubated in the dark for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:100 in PBS containing 0.1% saponin and 10% goat serum. The slides were then rinsed with tap water for 30 min and mounted with VECTASHIELD medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was detected using a Zeiss microscope with different filters. Similarly, after double labeling, specimens were viewed either through a ×40 objective on a Nikon Diaphot inverted fluorescence microscope or through a ×100 oil immersion objective on a Leica TCS SP confocal laser scanning microscope equipped with two lasers working simultaneously with excitation wavelengths of 543 and 488 nm to detect TRITC and FITC, respectively. The confocal three-dimensional data were processed using the Leica confocal LCS software program.

**Real-time PCR**

Total RNA was extracted from cultures using an RNeasy kit (Qiagen Inc., Valencia, CA). 1 μg of RNA was reverse-transcribed using an Advantage RT-for-PCR kit (BD Biosciences).
Real-time PCR was performed using the Rotor-Gene real-time DNA amplification system (Corbett Research, New South Wales, Australia) and the fluorescent dye SYBR Green I to monitor DNA synthesis (SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA). The primers used in this study were as follow: β-actin, 5′-TGT TAC CAA CTG GGA CGA CA and 3′-CTG GGT CAT CTT TTC ACG GT; and cyclin D1, 5′-GTC ACC TGG ATT GTT CTG TT and 3′-CAG CTT GCT AGG GAA CTT GG. The PCR protocol included a denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing for 20 s, and extension at 72 °C for 30 s. Detection of the fluorescent product was carried out at the end of the 72 °C extension period. PCR products were subjected to a melting curve analysis, and the data were analyzed and quantified with the Rotor-Gene analysis software. Dynamic tube normalization and noise slope correction were used to remove background fluorescence. Each sample was tested at least in triplicate and repeated for three independent cell preparations.

**In Vivo 5-Bromo-2′-deoxyuridine (BrdUrd) Labeling and Histology**

Concentrated BrdUrd solution (Zymed Laboratories Inc.) was injected into the peritoneal cavities of 4-day-old neonatal mice (1 ml/100 g of body weight). Mice were killed 4 h later, and lower limb samples centered at knee joints were harvested and processed. Decalcified tissue sections were labeled using a BrdUrd staining kit (Zymed Laboratories Inc.), counterstained with hematoxylin, and mounted with Permount. All BrdUrd-positive chondrocytes in the growth plate were counted, and at least three samples from each group were used.

**In Vitro BrdUrd Labeling and Flow Cytometry**

BrdUrd (10 μM) was added to cultures containing primary chondrocytes and incubated for 2 h. The cells (~2 × 10^6) were trypsinized, washed with PBS, and fixed with 75% ethanol at 4 °C for 24 h. On the day of flow cytometry, the cells were washed with PBS and incubated with pepsin solution for 30 min. After spinning and decanting, the cells were resuspended with 1 ml of PBS containing 0.5% FBS and 0.5% Tween 20. Then, the cells were incubated with 1 ml of PBS containing 2% FBS at room temperature for 30 min. FITC-conjugated anti-BrdUrd antibody (Roche Applied Science) was added to the above solution and incubated for 60 min in the dark. After washes, cells were treated with RNase A (1 mg/ml) at room temperature for 30 min. Finally, cells were labeled with propidium iodide (20 μg/ml in PBS) for 10 min at room temperature, filtered through 37-μm mesh, and loaded onto a FACSCanto flow cytometer (BD Biosciences).

**Statistics**

Statistical comparisons were made between groups using either analysis of variance or Student’s t test as appropriate. p values <0.05 were considered significant and are denoted in the figures.

**RESULTS**

**TGF-β Stimulates β-Catenin Signaling**

Initial experiments examined β-catenin signaling in C5.18 cells and in primary chondrocytes isolated from the sterna of TOPGAL transgenic mice. Cotransfection of the TOPflash reporter with the constitutively active β-catenin expression plasmid β-catenin^{S33Y} in C5.18 cells resulted in stimulation of reporter activity. Similarly, infection of TOPGAL chondrocytes with Ad-β-catenin resulted in increased β-galactosidase activity (Fig. 1, a and b).
To determine whether TGF-β activates β-catenin signaling in chondrocytes, we examined the effect of TGF-β (1 ng/ml) on β-galactosidase activity in primary chondrocytes isolated from TOPGAL transgenic mice and found that TGF-β stimulated β-galactosidase activity in a dose-dependent manner (Fig. 1c). To investigate whether TGF-β increases β-catenin protein levels in chondrocytes, we treated C5.18 cells with TGF-β (1 ng/ml) for different periods of time and examined changes in β-catenin protein levels by Western blot analysis. TGF-β significantly increased the protein levels of the total and active non-phosphorylated forms of β-catenin in a time-dependent manner. TGF-β increased β-catenin protein levels within 15 min, and its maximum effect was achieved at the 2-h time point after TGF-β treatment (Fig. 1d). In contrast, TGF-β had no effect on β-catenin mRNA expression in these cells (data not shown). These results suggest that TGF-β activates β-catenin signaling by increasing β-catenin protein levels in chondrocytes.

**TGF-β Activates β-Catenin Signaling through SMAD3**

To determine whether TGF-β-regulated β-catenin protein levels are mediated by SMAD3, we examined the steady-state protein levels of β-catenin in Smad3 knock-out chondrocytes. Cell lysates were extracted from sternal chondrocytes derived from wild-type and Smad3 knock-out mice, and changes in β-catenin protein levels were detected by Western blotting using anti-β-catenin monoclonal antibody. The results showed that both total and active non-phosphorylated β-catenin levels were significantly reduced in Smad3-deficient chondrocytes compared with wild-type chondrocytes (Fig. 2a). In addition, the cyclin D1 protein level was also reduced in Smad3−/− chondrocytes (Fig. 2a). To further determine whether TGF-β activates β-catenin signaling through SMAD3, we transfected the TOPflash reporter into chondrocytes isolated from wild-type or Smad3 knock-out mice and treated these cells with TGF-β (1 ng/ml). TGF-β stimulated TOPflash reporter activity in wild-type but not Smad3 knock-out chondrocytes (Fig. 2b). To further determine the role of SMAD3 in β-catenin signaling, the SMAD3 expression plasmid was cotransfected with the β-catenin reporter into C5.18 cells. Although transfection of SMAD3 alone significantly increased β-catenin reporter activity, cotransfection of SMAD3 with β-cateninS33Y only slightly enhanced β-catenin-induced TOPflash reporter activity (Fig. 2c), suggesting that overexpression of β-catenin may have already achieved a maximum level of reporter induction. These results demonstrate that TGF-β activates β-catenin signaling through SMAD3 in chondrocytes.

**SMAD3 Interacts with β-Catenin in Chondrocytes**

To determine whether SMAD3 directly interacts with β-catenin in chondrocytes, we performed immunoprecipitation assays. C5.18 chondrocytes were cultured in the presence or absence of TGF-β for 30 min, and cell lysates were extracted and subjected to immunoprecipitation using anti-SMAD3 antibody or anti-His control antibody, followed by Western blot analysis using anti-β-catenin antibody. A weak interaction between SMAD3 and β-catenin was detected in the absence of TGF-β. In contrast, a strong interaction between SMAD3 and β-catenin was detected in the presence of TGF-β (Fig. 2d, upper panels). In contrast, SMAD2 did not interact with β-catenin in C5.18 cells (data not shown). These results suggest that β-catenin may interact mainly with phospho-SMAD3 upon TGF-β treatment in chondrocytes.

**TGF-β Induces β-Catenin Nuclear Translocation**

To determine whether TGF-β induces β-catenin nuclear translocation, we performed immunostaining experiments using anti-β-catenin monoclonal antibody. C5.18 chondrocytes were serum-starved overnight and then treated with TGF-β (1 ng/ml) for 4 h. Immunostaining and confocal microscopic analysis showed that treatment with TGF-β induced the nuclear localization of both SMAD3 and β-catenin in C5.18 cells (Fig. 3a). The merged image shows
that β-catenin colocalized with SMAD3 in the nucleus of C5.18 cells (Fig. 3b), suggesting that these two proteins may coactivate target genes in chondrocytes.

**SMAD3 Prevents β-TrCP-induced β-Catenin Degradation**

β-TrCP is a ubiquitin-protein isopeptide ligase in the SCFβ-TrCP (Skp1/Cul1/F-box) protein complex that induces β-catenin degradation in many cell types (14-18). To determine whether β-TrCP induces β-catenin degradation in chondrocytes, we transfected the β-TrCP expression plasmid into C5.18 cells and examined the effect of β-TrCP on the steady-state protein levels of β-catenin. Transfection of β-TrCP significantly reduced the β-catenin protein level in C5.18 cells. Transfection of SMAD3 enhanced the basal level of β-catenin and attenuated β-TrCP-induced β-catenin degradation in C5.18 cells (Fig. 4). These results suggest that SMAD3 may prevent β-TrCP-induced β-catenin degradation.

**β-Catenin Induces Cyclin D1 Expression in Chondrocytes**

We then determined the effect of β-catenin on cyclin D1 gene transcription and expression in chondrocytes. C5.18 chondrocytes were cotransfected with the β-cateninS33Y plasmid and the 1.7-kb human cyclin D1 promoter. Overexpression of β-cateninS33Y stimulated cyclin D1 promoter activity in C5.18 chondrocytes (Fig. 5a). Cyclin D1 protein expression was also increased when C5.18 chondrocytes were infected with Ad-β-catenin (Fig. 5b). In contrast, C5.18 chondrocytes cotransfected with the cyclin D1 promoter and ICAT had reduced cyclin D1 promoter activity (Fig. 5c). To further determine the role of β-catenin in cyclin D1 expression in chondrocytes, we isolated primary chondrocytes from β-catenin-loxp mice (8) and infected these cells with Ad-Cre. Cells infected with Ad-GFP were used as a control. The results showed that, in Ad-Cre-infected chondrocytes, cyclin D1 expression was significantly reduced compared with Ad-GFP-infected cells (Fig. 5d). Taken together, these results demonstrate that β-catenin plays an important role in cyclin D1 expression in chondrocytes.

**TGF-β Stimulates Cyclin D1 Expression and Cell Growth through SMAD3**

To determine the role of TGF-β in chondrocyte cell growth, we examined changes in cell proliferation in Smad3 knock-out mice and found that BrdUrd-positive chondrocytes in the proliferating zone of the growth plate were decreased by ~30% (Fig. 6, a-e). This finding was confirmed by an in vitro BrdUrd labeling experiment using primary chondrocytes isolated from Smad3 knock-out mice and their wild-type littermates. The results showed that BrdUrd-positive cells were significantly reduced in Smad3 knock-out chondrocytes, independent of serum starvation (Fig. 6f). In Smad3 knock-out chondrocytes, expression of cyclin D1 mRNA and protein was significantly decreased (Fig. 2a and Fig. 6g). Taken together, these results indicate that TGF-β stimulates cyclin D1 expression and cell growth through SMAD3 in chondrocytes.

**TGF-β-induced Cyclin D1 Expression Is Mediated by β-Catenin**

Because both TGF-β and β-catenin stimulate cyclin D1 expression and because TGF-β activates β-catenin signaling in chondrocytes, we hypothesized that TGF-β induces cyclin D1 expression through activation of β-catenin signaling. To test this hypothesis, primary chondrocytes were isolated from the β-catenin-loxp mice, infected with Ad-Cre or Ad-GFP, and treated with TGF-β. TGF-β stimulated cyclin D1 expression in Ad-GFP-infected but not Ad-Cre-infected chondrocytes (Fig. 7a). Consistent with these findings, transfection of the constitutively active type I TGF-β receptor (19) increased cyclin D1 expression, which was completely inhibited by cotransfection of ICAT (Fig. 7b). ICAT is an 82-amino acid protein that interferes with the binding of β-catenin to TCF and inhibits β-catenin/TCF-induced transcription of downstream target genes (11,12). These results strongly suggest that TGF-β regulates cyclin D1 expression in a β-catenin-dependent manner.

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DISCUSSION

TGF-\(\beta\) has been shown to stimulate cyclin D\(_1\) expression and chondrocyte proliferation (2,7, 20). A recent study demonstrated that \(\beta\)-catenin controls chondrocyte proliferation and terminal differentiation (21). The interaction of TGF-\(\beta\) and \(\beta\)-catenin signaling in chondrocytes has not been previously described. In this study, we have demonstrated that TGF-\(\beta\) activated \(\beta\)-catenin signaling through SMAD3. SMAD3 directly interacted with \(\beta\)-catenin. TGF-\(\beta\) may increase \(\beta\)-catenin protein levels by preventing \(\beta\)-catenin degradation because TGF-\(\beta\) increased \(\beta\)-catenin protein levels but had no effect on \(\beta\)-catenin mRNA expression. TGF-\(\beta\) lost its ability to induce cyclin D\(_1\) expression when the \(\beta\)-catenin gene was deleted in chondrocytes, suggesting that TGF-\(\beta\)-induced cyclin D\(_1\) expression is mediated by \(\beta\)-catenin in chondrocytes. These findings provide novel insights into the interaction between TGF-\(\beta\) and \(\beta\)-catenin signaling pathways and the regulatory mechanism of chondrocyte proliferation.

TGF-\(\beta\) stimulates cell proliferation through induction of cyclin D\(_1\) (20,2), which is critical for progression through G\(_1\) to S phase of the cell cycle. Regulation of the cell cycle is controlled by a combination of cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors, which, together with the retinoblastoma tumor suppressor (Rb), are involved in the tight control of cell cycle machinery. Cdks, in association with their regulatory partners, the cyclins, are key regulators of cell cycle progression. Cdk2-cyclin A/E and Cdk4/6-cyclin D are involved in the G\(_2\)/M transition of the cell cycle (22,23). Cdk4/6-cyclin D promotes the phosphorylation of Rb. Hypophosphorylated Rb proteins are known to inhibit the function of E2F proteins, which promote transcription of factors essential for DNA synthesis (24). Thus, phosphorylation of Rb by Cdk-cyclin D complexes relieves inhibition of Rb on the E2F function, promoting the entry of cells into S phase. In cyclin D\(_1\) knock-out mice, chondrocyte proliferation is inhibited, and the proliferating zone is reduced by >50% in the growth plate, indicating that cyclin D\(_1\) plays a critical role in chondrocyte proliferation (2).

\(\beta\)-TrCP-Skp1 is a ubiquitin-protein isopeptide ligase complex that is involved in \(\beta\)-catenin degradation (14-18). In this study, we found that \(\beta\)-TrCP-induced \(\beta\)-catenin degradation was reduced in the presence of SMAD3, suggesting that the binding of SMAD3 to \(\beta\)-catenin may protect \(\beta\)-catenin degradation. Skp2 (\(\beta\) phase kinase-interacting protein-2) is a homolog of Skp1 and has been reported to be involved in the ubiquitination of the Cdk inhibitor p27\(^{Kip1}\); in epithelial cells, TGF-\(\beta\) causes cell cycle arrest in part through inhibition of Skp2 and stabilizing p27\(^{Kip1}\) (25). In contrast to epithelial cells, TGF-\(\beta\) promotes chondrocyte proliferation through activation of cyclin D\(_1\) (Refs. 2 and 19 and this study). TGF-\(\beta\) had no effect on p21\(^{Kip1}\) expression but slightly increased p27\(^{Kip1}\) levels in chondrocytes (data not shown), and the effect of TGF-\(\beta\) on Skp2 function in chondrocytes is currently unknown.

It has been reported that TGF-\(\beta\) regulates cyclin D\(_1\) expression through activation of activating transcription factor-2, which directly binds the cyclin D\(_1\) promoter and activates cyclin D\(_1\) gene transcription in chondrocytes (20). Our results indicate that TGF-\(\beta\) also regulates cyclin D\(_1\) gene expression through activation of \(\beta\)-catenin signaling. Multiple TCF-binding sites have been identified on the cyclin D\(_1\) promoter (26). These findings suggest that TGF-\(\beta\) may control cyclin D\(_1\) expression through at least two separate pathways and that induction of \(\beta\)-catenin is necessary for TGF-\(\beta\)-induced cyclin D\(_1\) induction. Further studies are required to determine whether \(\beta\)-catenin/TCF interacts with activating transcription factor-2 at the cyclin D\(_1\) promoter.

Members of the TGF-\(\beta\) superfamily are expressed in embryonic and adult skeletal tissue. mRNA and protein expression of TGF-\(\beta\) (including TGF-\(\beta\)1, \(\beta\)2, and \(\beta\)3) and TGF-\(\beta\) receptors (including types I and II) is detected in the mouse perichondrium and in proliferating and hypertrophic chondrocytes in the growth plate from 13.5 days postcoitus until after birth.
In proliferating chondrocytes, TGF-β promotes chondrocyte proliferation (2,20), and in hypertrophic chondrocytes, TGF-β inhibits chondrocyte differentiation (3-6).

β-Catenin plays a critical role in chondrocyte development. Although β-catenin suppresses the differentiation of mesenchymal cells into Sox9-expressing chondrocyte precursors (31,32), it promotes chondrocyte maturation in growth plate chondrocytes by stimulating chondrocyte proliferation and increasing chondrocyte marker gene expression. When the β-catenin gene is specifically deleted in Col2a1-expressing chondrocytes, chondrocyte proliferation is decreased, and hypertrophic chondrocyte differentiation is delayed (21). The effect of β-catenin on chondrocyte maturation is regulated by transcription factor SOX9. SOX9 binds the armadillo repeats of β-catenin and inhibits the interaction of β-catenin with TCF (21). Our recent findings demonstrate that Wnt3a stimulates type X collagen expression through activation of bone morphogenetic protein signaling in chondrocytes (33). These observations demonstrate that β-catenin interacts with other transcription factors or signaling pathways during chondrocyte maturation. Although the detailed mechanism of how TGF-β prevents β-catenin degradation and induces β-catenin nuclear translocation requires further investigation, our current findings clearly demonstrate that TGF-β regulates cyclin D1 expression through activation of β-catenin signaling.

We (6) and others (34) have shown that β-catenin induces prehypertrophic chondrocytes to complete terminal maturation. Overexpression of β-catenin or addition of Wnt3a, which induces the canonical β-catenin signaling pathway, stimulates expression of colX and other maturational markers (6,34). Furthermore, we demonstrated that TGF-β treatment inhibits activation of the TOPflash promoter by β-catenin in chick upper sternal chondrocytes (6). Although these findings seemingly contradict the current observation that TGF-β induces β-catenin signaling through a SMAD3-mediated mechanism, important differences between the culture models likely account for the discrepancy in the findings. The previous work utilized embryonic chick upper sternal chondrocytes (6). These chondrocytes express colX and spontaneously complete maturation and thus are in a relatively differentiated state at the time of harvest (6). Although the murine sternal chondrocytes used in the current experiments respond to BMP-2 and other differentiation signals and are capable of undergoing maturation, under basal conditions, colX expression is absent, and the cells are in a much less mature state and do not undergo spontaneous maturation (35).

It has been established that β-catenin has a complex role during chondrogenesis and subsequent endochondral ossification, and in vivo findings in β-catenin-deficient mice support a role for β-catenin in both proliferation and differentiation (21). In mesenchymal stem cells, β-catenin inhibits chondrogenesis and causes cells to differentiate toward an osteoblast phenotype (32). Interestingly, TGF-β and SMAD3 have been shown to enhance β-catenin signaling in embryonic maxillary mesenchymal cells (36). In prehypertrophic and hypertrophic chondrocytes committed to maturation, β-catenin induces terminal differentiation (6,34). The current findings similarly suggest that, in proliferating and immature chondrocytes, β-catenin acts downstream of TGF-β and functions to stimulate chondrocyte proliferation, consistent with an effect on proliferation in other cell types (37-39). Thus, a possible explanation of the role of TGF-β signaling in modulating Wnt canonical signaling during cell growth versus differentiation is that SMAD3 enhances β-catenin signaling during cell proliferation but inhibits signaling in more differentiated chondrocytes progressing toward terminal maturation. Although, at a later stage, β-catenin clearly functions to stimulate terminal differentiation, the mechanisms involved in the transition from proliferation to differentiation and the dual role of β-catenin in these processes remain elusive. However, given the current findings, it is likely that interactions with the TGF-β and bone morphogenetic protein signaling pathways are involved.
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**FIGURE 1. β-catenin signaling is active in chondrocytes**

*a*, the constitutively active β-catenin expression plasmid β-catenin$^{S33Y}$ ($β$-$cat^{S33Y}$) was cotransfected with the TOPflash reporter into C5.18 chondrocytes. The luciferase activity was measured 24 h after transfection. Compared with the empty vector control (Vect), transfection of β-catenin$^{S33Y}$ caused an ~2-fold increase in reporter activity. *b*, primary chondrocytes isolated from TOPGAL transgenic mice were infected with Ad-β-catenin (Ad-β-Cat). Ad-GFP was used as a control. Ad-β-catenin infection resulted in a 2-fold increase in β-galactosidase (β-gal) activity in TOPGAL chondrocytes. TGF-β stimulated β-catenin signaling. *c*, TOPGAL chondrocytes were treated with different concentrations of TGF-β (0-10 ng/ml), and β-galactosidase activity was measured 24 h later. TGF-β increased β-galactosidase activity in a dose-dependent manner in TOPGAL chondrocytes. *d*, C5.18 chondrocytes were treated with TGF-(1 ng/ml) for different periods of time (0-480 min), and changes in β-catenin protein were examined by Western blotting. TGF-β up-regulated the protein levels of both total and active non-phosphorylated β-catenin. TGF-β significantly increased the β-catenin protein level within 15 min, and the maximum effect was reached at the 2-h time point.
FIGURE 2. TGF-β activates β-catenin signaling through SMAD3

a, primary chondrocytes were isolated from Smad3−/− knock-out (KO) mice and their wild-type littermates. Expression of β-catenin protein was analyzed by Western blotting. Cell lysates containing 20 μg of protein were used for Western blotting. Compared with wild-type cells, Smad3-deficient chondrocytes showed lower protein levels of both total and active β-catenin. In addition, cyclin D1 expression was also reduced in Smad3-deficient chondrocytes. b, wild-type and Smad3-deficient chondrocytes were transfected with the TOPflash reporter construct for 24 h. FOPflash (FOP) was used as control. TGF-β was added to cell cultures for 24 h prior to luciferase assay. The basal and TGF-β-induced TOPflash reporter activities were decreased in Smad3−/− chondrocytes. The FOPflash reporter showed no response to TGF-β, confirming the specific effect of TGF-β on β-catenin signaling. c, the β-cateninS33Y (β-catS33Y) plasmid was cotransfected with the TOPflash reporter into C5.18 chondrocytes in the presence or absence of SMAD3, and cell lysates were collected at 24 h for luciferase assay. β-CateninS33Y stimulated TOP-flash reporter activity, and SMAD3 slightly enhanced β-cateninS33Y-induced TOPflash reporter activity. d, total cell lysates were harvested from C5.18 chondrocytes treated with or without TGF-β (1 ng/ml) for 1 h. Cell lysates were immunoprecipitated (IP) with anti-SMAD3 antibody, and Western blotting (WB) was
performed using anti-β-catenin antibody (upper panels). Anti-His antibody was used as a control. The results demonstrate an interaction between SMAD3 and β-catenin in C5.18 chondrocytes. Endogenous β-catenin and SMAD3 protein expression was detected without immunoprecipitation in C5.18 chondrocytes (lower panels).
FIGURE 3. TGF-β induces β-catenin nuclear translocation

a, C5.18 chondrocytes were cultured with or without TGF-β (1 ng/ml). After 4 h of TGF-β treatment, cells were fixed and incubated with either anti-β-catenin or anti-SMAD3 antibody, followed by incubation with either FITC- or TRITC-conjugated secondary antibody. The results showed that treatment with TGF-β induced the nuclear translocation of SMAD3 as well as β-catenin.  
b, similarly, treated cells were examined under a confocal microscope. In the absence of TGF-β, both β-catenin and SMAD3 stayed in the cytoplasm. Treatment with TGF-β promoted both SMAD3 and β-catenin nuclear translocation and co-localization.
C5.18 cells

FIGURE 4. SMAD3 prevents β-TrCP-induced β-catenin degradation
C5.18 chondrocytes were transfected with β-TrCP with or without SMAD3 for 24 h. Cells were lysed, and Western blotting was performed using anti-β-catenin antibody. Transfection of β-TrCP significantly reduced the level of β-catenin, whereas cotransfection of SMAD3 and β-TrCP partially rescued β-TrCP-mediated β-catenin degradation.
FIGURE 5. β-Catenin induces cyclin D1 expression in chondrocytes

a. C5.18 chondrocytes were synchronized in serum-free medium for 72 h. The cyclin D1-luciferase (Luc) reporter construct was cotransfected with either the empty vector control (Vect) or β-catenin<sup>S33Y</sup> (β-cat<sup>S33Y</sup>) for 24 h, and luciferase assay was performed. Transfection of β-catenin<sup>S33Y</sup> increased cyclin D1 reporter activity by ~5-fold compared with the empty vector control.

b. C5.18 chondrocytes were infected with Ad-GFP or Ad-β-catenin (Ad-β-cat) for 24 h. After the medium was changed, cells were cultured in serum-free medium for 72 h. Western blotting showed that overexpression of β-catenin (Ad-β-catenin) increased the protein level of cyclin D1 in C5.18 chondrocytes. c. C5.18 chondrocytes were synchronized in

\[ \text{Cyclin D1-Luc reporter} \]

| Vect | β-cat<sup>S33Y</sup> |
|------|----------------------|
| 0.4  | 1.2                  |

\[ \text{C5.18 cells} \]

\[ \text{β-catenin} \]

\[ \text{Cyclin D1} \]

\[ \text{β-Actin} \]

\[ \text{Ad-GFP} \]

\[ \text{Ad-β-cat} \]

\[ \text{β-catenin}^{\text{flax/flax}} \text{ mice} \]

| Vect | ICAT |
|------|------|
| 0.4  | 1.2  |

\[ \text{C5.18 cells} \]

\[ \text{β-Actin} \]

\[ \text{Ad-GFP} \]

\[ \text{Ad-Cre} \]

\[ \text{β-catenin}^{\text{flax/flax}} \text{ mice} \]

\[ \text{Cyclin D1} \]

\[ \text{β-catenin} \]

\[ \text{β-Actin} \]
serum-free medium for 72 h. The cyclin D$_1$ reporter construct was cotransfected with either the empty vector control or ICAT for 24 h, and luciferase assay was then performed. Transfection of ICAT significantly reduced cyclin D$_1$ reporter activity. 

$d.$, primary chondrocytes were isolated from β-catenin$^{floxflox}$ mice and their wild-type littermates and infected with Ad-Cre or Ad-GFP. Cell lysates were collected 48 h after infection. Infection with Ad-Cre almost completely abolished the protein expression of β-catenin in β-catenin$^{floxflox}$ chondrocytes. The protein level of cyclin D$_1$ was reduced when the β-catenin gene was deleted. In contrast, Ad-GFP had no effect on the protein levels of β-catenin and cyclin D$_1$. 

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FIGURE 6. Chondrocyte proliferation is reduced in Smad3 knock-out mice

- a-d, BrdUrd labeling reagent was injected into the peritoneal cavities of 4-day-old neonatal mice. Mice were killed 4 h later, and hind limbs were harvested. The decalcified tissue sections were stained using a BrdUrd staining kit and counterstained with hematoxylin. a and c, wild-type mice; b and d, Smad3−/− knock-out (KO) mice. e, morphometric analysis revealed that the number of BrdUrd-positive chondrocytes was significantly lower in Smad3−/− mice than in wild-type (Wt) mice.

- f, primary chondrocytes isolated from either wild-type or Smad3-deficient mice were cultured with BrdUrd reagent. After fixation, cells were incubated with FITC-conjugated anti-BrdUrd antibody. The results from flow cytometry showed that the numbers of BrdUrd-positive chondrocytes were reduced in Smad3−/− mice with or without serum starvation.

* indicates a significant difference.
primary chondrocytes isolated from either wild-type or Smad3-deficient mice were lysed, and total RNA was extracted. After reverse transcription, real-time PCR was performed using cyclin D1 primers. The results showed that cyclin D1 mRNA expression was decreased in Smad3−/− chondrocytes.
FIGURE 7. TGF-β stimulates cyclin D1 expression through β-catenin

a, primary chondrocytes isolated from β-catenin<sup>flox/flox</sup> mice were infected with Ad-GFP or Ad-β-catenin for 24 h. After the medium was changed, cells were cultured in serum-free medium for 72 h. Deletion of the β-catenin gene in the β-catenin<sup>flox/flox</sup> chondrocytes by Ad-Cre resulted in loss of responsiveness to TGF-β-induced cyclin D1 protein levels. b, C5.18 chondrocytes were transfected with the constitutively active type I TGF-β receptor (caTβR1) with or without ICAT. Cells were lysed, and Western blotting was performed using anti-cyclin D1 antibody. Transfection of the constitutively active type I TGF-β receptor alone greatly increased the protein level of cyclin D1, whereas transfection of ICAT significantly reduced the constitutively active type I TGF-β receptor-induced up-regulation of cyclin D1 protein. The empty vector transfection control is shown in the first lane.