**TGFβ STIMULATES CYCLIN D1 EXPRESSION IN CHONDROCYTES THROUGH ACTIVATION OF β-CATENIN SIGNALING**

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**Introduction:**
TGF-β is an important regulator of the endochondral ossification. TGF-β inhibits colX, alkaline phosphatase, MMP13 and other genes associated with chondrocyte maturation in various cell culture models. Mice deficient in TGF-β signaling are normal until 4 weeks of age. At that point, Smad3−/− mice and mice over-expressing dominant-negative TβRII develop disorganized growth plates with premature chondrocyte maturation and reduce long bone growth.

In addition to its inhibitory effects on maturation, TGF-β also stimulates chondrocyte proliferation. While prior studies have established p38 kinase/ATF-2 as a downstream mediator of TGF-β effects on proliferation, mice deficient in Smad3 signaling also have reduced proliferation. Thus, Smad3 is a potential regulator of proliferation in chondrocytes. The current study examines mechanisms involved in a Smad3 mediated proliferation and establishes that effects are mediated through an induction of β-catenin with subsequent induction of the cyclin D1 gene.

**Materials and Methods**
Methods used in this study include in vivo and in vitro BrdU labeling, transient transfection and luciferase assay with TopFlash and cyclin D reporters, real time PCR using RotorGene DNA amplification system, western blot with antibodies against axin, cyclin D1, total β-catenin, and active form β-catenin, immunoprecipitation of Smad3 and β-catenin using Catch and Release System from Upstate, double immunofluorescence labeling and confocal microscopy for the colocalization of Smad3 and β-catenin.

Sternal chondrocytes were isolated from Smad3−/− and Smad3+/+ neonatal mice of 3-days old. Western blotting and transient transfection experiments were performed in these cells. Chondrocytes from β-catenin reporter (Topgal) mice were treated with TGF-β and galactosidase activity was examined. Adenoviral construct expressing β-catenin was generated using Gateway adenovirus system, amplified in 293A cells, and purified using traditional CsCl binding method.

**Results:**
**TGF-β stimulates β-catenin expression and signaling**
Mouse sternal chondrocytes isolated from Topgal transgenic mice were treated with various concentrations of TGF-β for 24 hours. Treatment with TGF-β resulted in a dose-dependent stimulation of β-galactosidase activity with maximal effects observed at 5ng/ml where a 2-fold induction occurred. Consistent with these findings, TGF-β (2ng/ml) increased β-catenin protein levels in a time-dependent manner (0-8h) in primary mouse sternal chondrocytes and in C5.18 chondrocytes. Protein levels of β-catenin were increased within 15 minutes and reached maximal level after 2 hours. In contrast, TGF-β had no effect on β-catenin mRNA expression in either primary cells or C5.18 chondrocytes.

**Smad3 associates with β-catenin and regulates its expression.**
To determine whether Smad3 is involved in the regulation of β-catenin protein levels and signaling, β-catenin expression was examined in Smad3−/− chondrocytes. In Smad3−/− chondrocytes, β-catenin protein levels were significantly reduced compared with wild type chondrocytes. To examine if direct Smad3/β-catenin interactions are involved the induction of β-catenin by TGF-β, C5.18 chondrocytes were cultured in the presence or absence of TGF-β for 30 minutes and cell lysates were immunoprecipitated with anti-Smad3 antibody followed by Western blot using an anti-β-catenin antibody. Minimal association between Smad3 and β-catenin was observed in the absence of TGF-β. In contrast, TGF-β stimulated a strong interaction between Smad3 and β-catenin. Similar results were also obtained when anti-Smad3 antibody was utilized in the immunoprecipitation assay. Finally, co-localization experiments using double immunofluorescence labeling and confocal microscopy demonstrate that under basal conditions, both Smad3 and β-catenin localize to the cytoplasm. However, within 30-minutes following TGF-β stimulation, Smad3 and β-catenin co-localize within the nucleus.

**β-TrCP mediated catabolism of β-catenin is inhibited by Smad3**
β-TrCP is an E3 ligase in the SCFβ-TrCP complex that promotes β-catenin degradation in many cell types. Similarly, we found that transfection of C5.18 cells with β-TrCP reduced β-catenin protein levels. However, co-transfection of Smad3 with β-TrCP significantly reduced β-TrCP-mediated β-catenin degradation. The results suggest that inhibition of β-catenin degradation by β-TrCP is a mechanism through which TGF-β/Smad3 stimulate β-catenin levels in chondrocytes.

**Proliferative effects of TGF-β/Smad3 are mediated by β-catenin and targeted to the cyclin D1 promoter**
To determine the role of Smad3 in chondrocyte proliferation, we examined proliferation, cyclin D1 reporter activity and cyclin D1 protein expression in chondrocytes derived from Smad3−/− mice. Compared with wild type littermates, Smad3−/− chondrocytes had reduced BrdU incorporations and rates of proliferation both in vivo and in vitro. The cyclin D1 promoter contains five putative TCF/LEF binding sites that are potentially responsive to β-catenin. Expression of constitutively active β-catenin (β-cateninS33Y) in C5.18 chondrocytes stimulated cyclin D1 reporter activity and protein expression confirming the importance to these binding sites. In contrast, cyclin D1 reporter activity and protein expression were reduced in Smad3−/− chondrocytes. Since β-catenin is reduced in Smad3−/− chondrocytes, the findings suggest that β-catenin may mediate effects of TGF-β/Smad3 on chondrocyte proliferation.

To directly determine if β-catenin is a downstream mediator of TGF-β/Smad3, the effect of TGF-β on cyclin D1 expression was examined in β-catenin-deficient chondrocytes. Primary mouse sternal chondrocytes were isolated from β-catenin-loxp mice and β-catenin expression eliminated by gene recombination through infection with adenovirus expressing Cre recombinase (Ad-Cre). Deletion of the β-catenin gene reduced basal cyclin D1 protein levels and completely blocked the ability of TGF-β to induce cyclin D1 expression in chondrocytes. The results suggest that β-catenin signaling is a critical regulator of cyclin D1 expression and acts downstream of TGF-β/Smad3.

**Discussion:**
While mechanisms involved in the suppression of maturation by TGF-β have been extensively investigated, less is known concerning the effect of TGF-β on chondrocyte proliferation. The current findings provide new evidence concerning cross-talk between TGF-β and β-catenin signaling pathways and demonstrate an important physiological role in the regulation of chondrocyte proliferation.

Previous studies have established that β-catenin activation requires signals that prevent constitutive degradation of the β-catenin protein. Although treatment with TGF-β had no effect on gene expression, treatment with TGF-β resulted in rapid induction in β-catenin protein levels and stimulated β-catenin signaling. Our experiments establish that the activated, phosphorylated Smad3 associates with β-catenin and prevents its degradation by β-TrCP. Following TGF-β stimulation, both Smad3 and β-catenin translocate to the nucleus and activate expression of target genes. The cyclin D1 gene contains multiple TCF/LEF binding sites and is an important target of β-catenin. Loss of Smad3 signaling resulted in decreased β-catenin levels, and reduced cyclin D1 expression and cellular proliferation. Finally, the ability of TGF-β to stimulate proliferation was absent in chondrocytes lacking β-catenin expression.

Altogether the results establish important interactions between β-catenin and TGF-β/Smad3 that mediate critical signals controlling chondrocyte proliferation. These findings suggest that TGF-β stimulates chondrocyte cell growth at least in part through activation of β-catenin signaling while it inhibits chondrocyte differentiation.