DEVELOPMENTAL DYNAMICS 230:685–699, 2004

RESEARCH ARTICLE

Unique and Redundant Roles of Smad3 in TGF-β–Mediated Regulation of Long Bone Development in Organ Culture

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The most well-characterized intracellular signaling molecules for transforming growth factor-beta (TGF-β) are the Smads. R-Smads interact with and are phosphorylated directly by the TGF-β type I receptor. Phosphorylated R-Smads can then associate with Smad4, translocate to the nucleus and regulate transcription. Specific R-Smads transduce distinct signals for members of the TGF-β superfamily. Smad2 and -3 mediate signaling by TGF-β/activin, whereas Smad1, -5, and -8 mediate bone morphogenetic protein signaling. TGF-β inhibits proliferation and hypertrophic differentiation in metatarsal organ cultures by a perichondrium-dependent mechanism. To determine the mechanism of TGF-β signaling in the perichondrium, we tested the hypothesis that TGF-β–restricted Smad2 and Smad3 regulate chondrocyte proliferation and differentiation in embryonic metatarsal organ cultures. Perichondrium was infected with adenoviruses containing dominant-negative forms of Smad2 (Ad-Smad2-3SA) and Smad3 (Ad-Smad3ΔC). Proliferation and differentiation were measured in response to treatment with TGF-β1. Results were compared with control bones infected with a β-galactosidase reporter virus (Ad-β-gal). Infection with Ad-Smad2-3SA completely blocked the effects of TGF-β1 on metatarsal development while Ad-Smad3ΔC only partially blocked TGF-β1 effects. To further characterize the role of Smad3 in long bone development, TGF-β1 responsiveness in cultures from Smad3$^{+/+}$ and Smad3$^{ex8/ex8}$ mice were compared. Loss of Smad3 only partially blocked the effects of TGF-β1 on differentiation. In contrast, the effects of TGF-β1 on chondrocyte proliferation were blocked completely. We conclude that Smad2 signaling in the perichondrium can compensate for the loss of Smad3 to regulate inhibition of hypertrophic differentiation; however, Smad3 is required for TGF-β1–mediated effects on proliferation. *Developmental Dynamics 230:685–699, 2004.* © 2004 Wiley-Liss, Inc.

Key words: endochondral bone; cartilage; perichondrium; adenovirus; Smads

Received 6 January 2004; Revised 22 February 2004; Accepted 22 March 2004

INTRODUCTION

Long bones develop by a process called endochondral bone formation in which a cartilage model is made and then replaced with bone (Cancedda et al., 1995, 2000; Erlebacher et al., 1995). The first step involves the condensation of mesenchymal cells followed by a complex program of proliferation and differentiation resulting in hypertrophy and apoptosis of the chondrocytes. A sheath of mesenchymal cells called the perichondrium is maintained around the developing skeletal element. These cells can differentiate along both chondrogenic and osteogenic lineages allowing appositional growth of the bone. Hypertrophic chondrocytes can be distinguished by the synthesis and secretion of Type X collagen (Schmid and Conrad, 1982; Schmid and Linsenmayer, 1983). The matrix of the hypertrophic chondrocytes is mineralized and vascularized allowing the hypertrophic cells to be replaced

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Grant sponsor: NIAMS/NIH; Grant numbers: AR45605, AR46982; Grant sponsor: Ministry of Education, Culture, and Sports, Spain; Grant number: PF00 10985154.
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DOI 10.1002/dvdy.20100
Published online 9 June 2004 in Wiley InterScience (www.interscience.wiley.com).

with bone. Proliferation, differentiation, and hypertrophy are recapitulated in the growth plate after birth to allow longitudinal growth of the bones. The rate of chondrocyte proliferation and differentiation must be strictly regulated to achieve the proper length and shape of each skeletal element.

Because endochondral bone development is complex and it involves several steps that must be tightly coordinated, it is likely that signaling through several paracrine factors is involved. Members of the transforming growth factor-beta (TGF-β) superfamily appear to be key regulators of bone development. TGF-β peptides are secreted signaling molecules that regulate many aspects of growth and differentiation in a cell type-specific manner (Roberts and Sporn, 1990; Massague et al., 2000; Chang et al., 2002). This family includes three isoforms of TGF-β, growth and differentiation factors (GDFs), the activin and inhibins, and the bone morphogenetic proteins (BMPs). TGF-β has different effects on proliferation and differentiation, depending on the culture conditions and stage of the cells used (Kato et al., 1988; Ballock et al., 1993; Tschan et al., 1993; Dieudonne et al., 1994; Bohme et al., 1995; Moses and Serra, 1996; Serra et al., 1999). TGF-β proteins signal through heteromeric Type I and Type II serine/threonine kinase receptors (Derynck, 1994; Shi and Massague, 2003). TGF-β binds to TβRII on the cell surface (Wrana et al., 1994). The conformational change allows TβRII to bind TβRI to form a heteromeric complex. The constitutively active TβRII kinase then phosphorylates and activates the TβRI kinase. TβRI directly phosphorylates downstream signaling proteins.

The most well-characterized TGF-β signaling molecules are the Smads (Derynck and Zhang, 1996; Massague, 1998; Shi and Massague, 2003). Smads were first identified in genetic screens of *Drosophila* and *Caenorhabditis elegans* (Sekelsky et al., 1995; Savage et al., 1996). Receptor-associated Smads, R-Smads, are phosphorylated directly by TβRI. R-Smads transduce distinct signals for members of the TGF-β superfamily. For example, Smad2 and Smad3 mediate signaling by TGF-β and activin ligands, whereas Smad1, Smad5, and Smad8 mediate BMP signaling. Phosphorylated R-Smads associate with a common Smad, Smad4, translocate to the nucleus, and regulate transcription. Smads can bind DNA directly, but it is more likely that they bind DNA and regulate transcription in combination with various cofactors and corepressors. Dominant-negative forms of the Smads have been used to address the roles of these proteins in signals transmitted by TGF-β. A dominant-negative form of Smad2 containing point mutations at Ser464, Ser465, and Ser467 (Smad2-3SA) in the phosphorylation domain has been described (Abdollah et al., 1997). Phosphorylation of Smad2 by TβRI in this domain is required for association with Smad4 and translocation to the nucleus. Phosphorylation is required for Smad2 to be released from TβRI; therefore, this dominant-negative form of Smad2 blocks signaling by both TGF-β–restricted Smads. It will also block signaling through Type I receptors that mediate activin signaling, some of which are shared with the BMP signaling pathway (Shi and Massague, 2003). Dominant-negative forms of Smad2 and Smad3 containing deletions of the C-terminal domain have also been described (SmadΔC; Zhang et al., 1996; Wu et al., 1997; Ferguson et al., 2000). The C-terminal domain of R-Smads contains the receptor phosphorylation domain and mediates receptor binding, as well as interactions with Smad4. Dominant-negative effects of the deletion mutants appear to be more specific (Ferguson et al., 2000). When the truncated, dominant-negative Smad3 is transfected into chick sternal chondrocytes, activation of a PAI promoter-reporter DNA by TGF-β is blocked. Expression of the truncated Smad2 protein did not block activation of this promoter, although it still had effects on chondrocyte maturation (Ferguson et al., 2000).

It has been shown that the perichondrium synthesizes factors that are essential to coordinate the development of the long bone (Long and Linsenmayer, 1998; Haaijman et al., 1999). The effects of Indian or Sonic Hedgehog (Ihh, Shh) on differentiation are indirect and mediated through the perichondrium while the effects of Ihh on chondrocyte proliferation are direct (Long et al., 2001; Alvarez et al., 2002). TGF-β1, 2, and 3 mRNA are synthesized in the mouse perichondrium and periosteum from 13.5 days post-coitum (p.c.) until after birth (Sandberg et al., 1988; Gatherer et al., 1990; Pelton et al., 1990; Millan et al., 1991). In addition, TGF-β receptor proteins have been detected in the perichondrium as well as in chondrocytes (Serra et al., 1999). Previously, we showed that enzymatic removal of the perichondrium from mouse long bone organ cultures seriously altered responsiveness of the bone to TGF-β (Alvarez et al., 2001). In addition, expression of a dominant-negative form of the TGF-β receptor in the perichondrium of bones in organ culture completely blocked both inhibition of proliferation and hypertrophic differentiation by TGF-β, suggesting the perichondrium mediates both these effects in the developing bone (Alvarez et al., 2002). Dominant-negative interference of TGF-β signaling in the perichondrium of transgenic mice results in increased and inappropriate hypertrophic differentiation in the growth plate, suggesting a role for perichondrial TGF-β signaling in the regulation of chondrocyte differentiation in vivo (Serra et al., 1997a). Likewise, Smad3-null mice demonstrate increased hypertrophic differentiation in the growth plate, but, because every cell is null for Smad3, it is not clear which cell type mediates the phenotype (Yang et al., 2001).

In this study, we use a mouse embryonic metatarsal organ culture model to test the hypothesis that Smads mediate TGF-β signaling in the perichondrium. The advantage of the metatarsal model is that cells are maintained with physiologically relevant cell–cell and cell–matrix interactions. The organ cultures can be easily manipulated to dissect out paracrine interactions required for normal bone development. Previously, we showed that the perichondrium of metatarsal cultures can be infected with replication defective adenoviruses (Alvarez et al., 2002). In this study, we used ad-

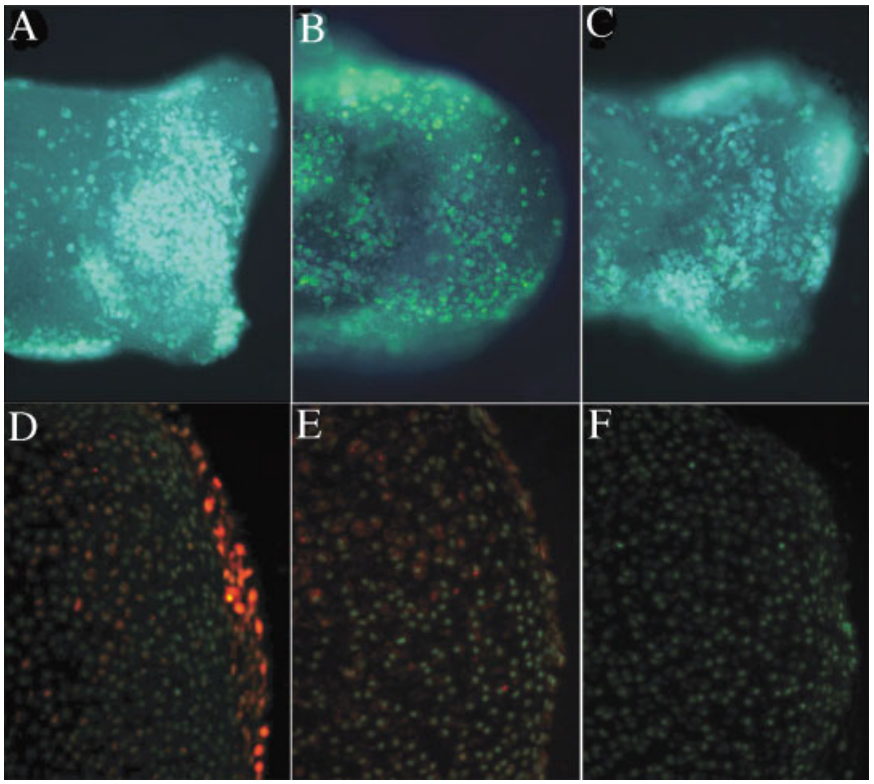

Fig. 1. Adenovirus infection of metatarsal bones. Infection was monitored by whole-mount immunofluorescence with an antibody directed to the adenovirus coat protein. Infected cells appear green. **A–C:** Infection is only detected in the perichondrium and each of the three viruses infected bones to the same level: Ad-β-gal (A), Ad-Smad2-3SA (B), Ad-Smad3ΔC (C). **D:** To confirm localization to the perichondrium, sections from bones that had been infected with Ad-β-gal for 5 days were incubated with an antibody against the β-gal protein. Localization of the β-gal protein was determined by immunofluorescence as red staining. The nuclear counterstain is green. **E:** Sections from uninfected bones were used as a negative control for the specificity of the primary antibody. **F:** Sections from β-gal infected bones that were not incubated with the primary β-gal antibody were used as negative controls to access the level of background staining from the subsequent steps.

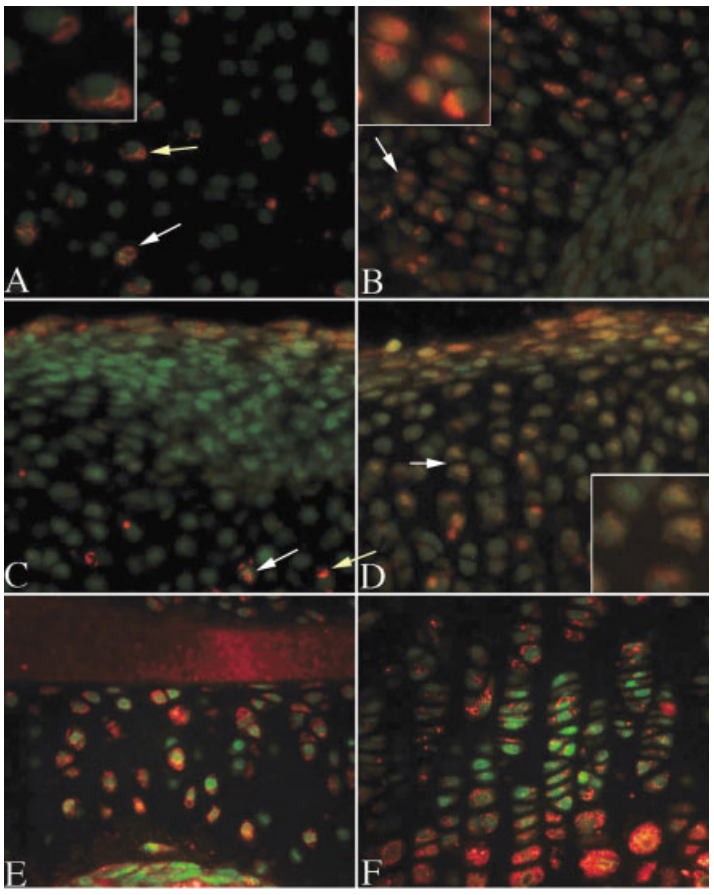

Fig. 8. Smad protein localization. **A–F:** Smad 2 and Smad3 protein were localized in untreated (A,C) or transforming growth factor-beta1 (TGF-β1) treated (B,D) metatarsal cultures using either the I-20 antibody (A,B,E,F) or the PS2 antibody (C,D). Smad staining is red and the nuclear counterstain is green. Yellow arrows point to examples of cells in which staining is restricted to the cytoplasm. White arrows point to examples of cells in which staining is predominantly nuclear. E,F: Sections from articular cartilage (E) and growth plate (F) from mice at 4 weeks of age are also shown stained with the I-20 antibody. High-magnification inserts show the areas indicated by the arrows in A,B,D.

enoviruses containing dominant-negative forms of Smad2 and Smad3 as well as cultures from Smad3-null mice to access the role of Smad signaling with regard to chondrocyte proliferation and hypertrophic differentiation. The data suggest Smad2 can compensate for loss of Smad3 to regulate differentiation but that Smad3 is required to regulate proliferation.

RESULTS

Effects of Dominant-Negative Interference of Smads in the Perichondrium

We previously showed that the perichondrium plays an important role in mediating the effects of TGF-β on embryonic long bone development (Alvarez et al., 2001). Now, to determine the signaling mechanism used, we tested the hypothesis that TGF-β signals through Smad proteins in the perichondrium. To block Smad signaling in the perichondrium, embryonic metatarsal bones in organ culture were infected with adenovirus vectors containing either dominant-negative mutants of Smad2 or Smad3 (Ad-Smad2-3SA, Ad-Smad3ΔC). Cultures infected with a β-galactosidase reporter virus (Ad-β-gal) were used as controls. The Smad2-3SA mutation has been shown to block signaling through several Smads by remaining bound to the Type I receptor (Abdollah et al., 1997). The C-terminal deletion mutant of Smad3 appears to be specific (Ferguson et al., 2000). Freshly dissected metatarsal bones were soaked overnight in conditioned medium from 293 cells containing adenoviruses. In the morning, conditioned medium was removed and replaced with standard culture medium. The adenoviruses used are replication-defective, so only the perichondrium, the outer layer of cells, is infected. Infection was monitored by whole-mount immunofluorescence. Infected bones were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody directed to the adenovirus coat protein. Infected cells in the perichondrium appeared green (Fig. 1A–C). All three adenoviruses used had similar infection efficiency. Perichondrial staining was shown previously by using LacZ staining (Alvarez et al. 2002). To confirm localization of infection in the perichondrium in this study, β-gal protein was localized by immunofluorescent staining in sections from bones that had been infected with Ad-β-gal for 5 days (Fig. 1D). Red staining in the nucleus indicates the presence of the β-gal protein. The nuclear counterstain is green. Red staining was restricted to the perichondrium, indicating that infection is limited to the perichondrium and persists for at least 5 days in culture. Sections from bones that were not infected or not treated with the primary antibody were used as negative controls to determine the specificity of the antibody and the level of background fluorescence (Fig. 1E,F).

Next, cultures that had been infected with Ad-Smad2-3SA, Ad-Smad3ΔC, or Ad-β-gal were treated with varying concentrations of TGF-β1 for 5 days. As expected, in control Ad-β-gal–infected cultures, treatment with TGF-β1 resulted in a dose-dependent decrease in total length of the metatarsal bones as well as a decrease in the clear area of the culture representing the hypertrophic zone (Fig. 2A). In contrast, bones infected with Ad-Smad2-3SA did not respond to TGF-β at any dose (Fig. 2B). Cultures infected with Ad-Smad3ΔC appeared to have a partial response (Fig. 2C). The total length of bones after treatment with 10 ng of TGF-β1/ml was significantly shorter than untreated Ad-Smad3ΔC–infected controls; however, the reduction in length was significantly less than that in Ad-β-gal–infected cultures treated with the same dose of TGF-β1 (Fig. 2C). Furthermore, there was a shift in the dose response to TGF-β1 in Ad-Smad3ΔC infected cultures so that treatment with 1 ng of TGF-β1/ml did not significantly alter the total length of the bone. There were no significant differences in the total length of untreated metatarsal bones infected with any of the three viruses used (Fig. 2C).

The total length of the bone is a combined effect of proliferation and hypertrophic differentiation. To further characterize the effects of TGF-β1 on hypertrophic differentiation in the adenovirus infected cultures, bones were embedded, sectioned, and stained with hematoxylin and eosin (Fig. 3A). The zones of round and flat proliferating cells, as well as the hypertrophic zone were clearly demarcated in untreated bones that had been infected with any of the three viruses. The cells appeared healthy and the perichondrium was intact. To clarify the effects of TGF-β1 on hypertrophic differentiation in the adenovirus infected cultures, Col10a1 mRNA was localized using in situ hybridization in sections from metatarsals (Fig. 3B). The percentage of hypertrophic cartilage in each culture was calculated as the length of the area containing Col10a1 over the total length as previously described (Fig. 3D; Alvarez et al., 2001). Significant differences in the area of hypertrophic cartilage were not detected in untreated metatarsals infected with any of the three viruses used in this study (Fig. 3D). The expression domain of Col10a1 was reduced in a dose-dependent manner in cultures infected with Ad-β-gal and treated with TGF-β1 (Fig. 3B,D). TGF-β1 did not inhibit hypertrophic differentiation as measured by the percentage area of Col10a1-expressing cartilage in Ad-Smad2-3SA–infected cultures (Fig. 3B,D). TGF-β1 had a partial effect on hypertrophic differentiation in cultures infected with Ad-Smad3ΔC compared with control Ad-β-gal–infected cultures (Fig. 3B,D). Infection with Ad-Smad3ΔC altered the dose response of inhibition so that there was no effect on differentiation with 1 ng of TGF-β1/ml; however, the Col10a1 expression domain was reduced in Ad-Smad3ΔC–infected cultures treated with 10 ng of TGF-β1/ml to the same extent as Ad-β-gal–infected cultures treated with the same dose.

Previously, we showed that TGF-β1 inhibits DNA synthesis in chondrocytes and that this effect is dependent on the perichondrium (Alvarez et al., 2001). It is likely that reduced cell proliferation is at least partially responsible for the decrease in total length observed in metatarsal cultures after treatment with TGF-β1. To determine whether Smads transduce signals that lead to decreased chondrocyte proliferation in the or-

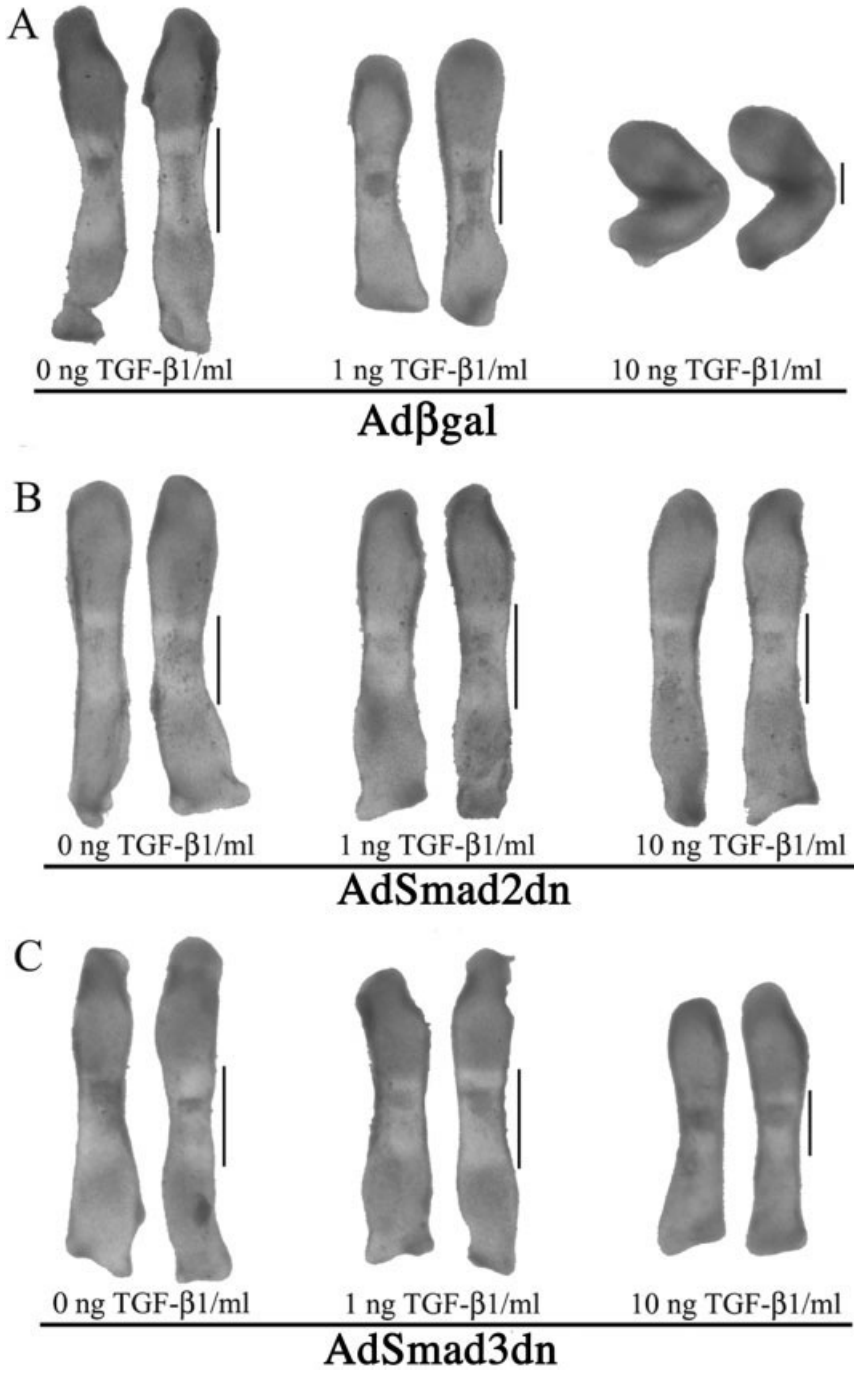

Fig. 2. Role of Smads in the perichondrium. **B,C:** Metatarsals were infected with dominant-negative forms of Smad2 (B; Ad-Smad2-3SA) or Smad3 (C; Ad-Smad3ΔC). **A:** Bones infected with a reporter virus containing the β-galactosidase gene (Ad-β-gal) were used as controls. Cultures were treated with 0, 1, or 10 ng of transforming growth factor-beta1 (TGF-β1) /ml for 5 days.

gan cultures, adenovirus–infected cultures were treated with 10 ng of TGF-β1/ml for 24 hr followed by incubation with bromodeoxyuridine (BrdU) for 2.5 hr. Incorporation of BrdU was detected by using immunofluorescent staining. The level of proliferation was determined by counting the percentage labeled nuclei (Fig. 4). There were no significant differences in the percentage of labeled nuclei in untreated cultures infected with Ad-β-gal, Ad-Smad2-3SA, and Ad-Smad3ΔC (Fig. 4). TGF-β1 inhibited the percentage of nuclei labeled with BrdU in Ad-β-gal–infected cultures. Proliferation was also inhibited in Ad-Smad3ΔC–infected cultures, whereas inhibition of proliferation was blocked in Ad-Smad2-3SA–infected cultures (Fig. 4). Treatment with TGF-β1 resulted in a close to 60% inhibition of proliferation relative to the untreated control in Ad-β-gal–infected cultures. Proliferation was inhibited only 40% in Ad-Smad3ΔC–infected cultures, suggesting a partial response. The data suggest that Smad2/3 signaling in the perichondrium is required for the effects of TGF-β on proliferation and differentiation. Dominant-negative interference of Smad3 only partially affects TGF-β signaling, suggesting that Smad2 can compensate for interference with Smad3 signaling.

Effects of the Loss of Smad3 on Metatarsal Development

To clarify the role of Smad3 in TGF-β signaling in long bone development, we compared the effects of TGF-β1 on metatarsal cultures from mice in which exon 8 of the Smad3 gene was disrupted by homologous recombination. The exon deletion results in a null allele. Mice homozygous for the disrupted allele ($Smad3^{ex8/ex8}$) survive to birth and demonstrate alterations in postnatal differentiation of both growth plate and articular chondrocytes (Yang et al., 2001) similar to that observed in transgenic mice expressing a dominant-negative mutation of TβRII (Serra et al., 1997a). Previously, it was shown that metatarsal cultures from embryonic day 17.5 $Smad3^{ex8/ex8}$ embryos only partially respond to TGF-β1 (Yang et al., 2001). In the present study, we used metatarsal bones from mice at 15.5 days of gestation. Bones were either untreated or treated with 1 ng or 10 ng of TGF-β1/ml for 5 days at which time the total length and percentage of hypertrophic cartilage in the cultures was calculated (Fig. 5). There were no significant differences in the length or percentage of hypertrophic cartilage in the untreated cultures from $Smad3^{+/+}$, $Smad^{+/ex8}$, or $Smad^{ex8/ex8}$ mice. Cultures from $Smad3^{+/+}$ mice demonstrated the

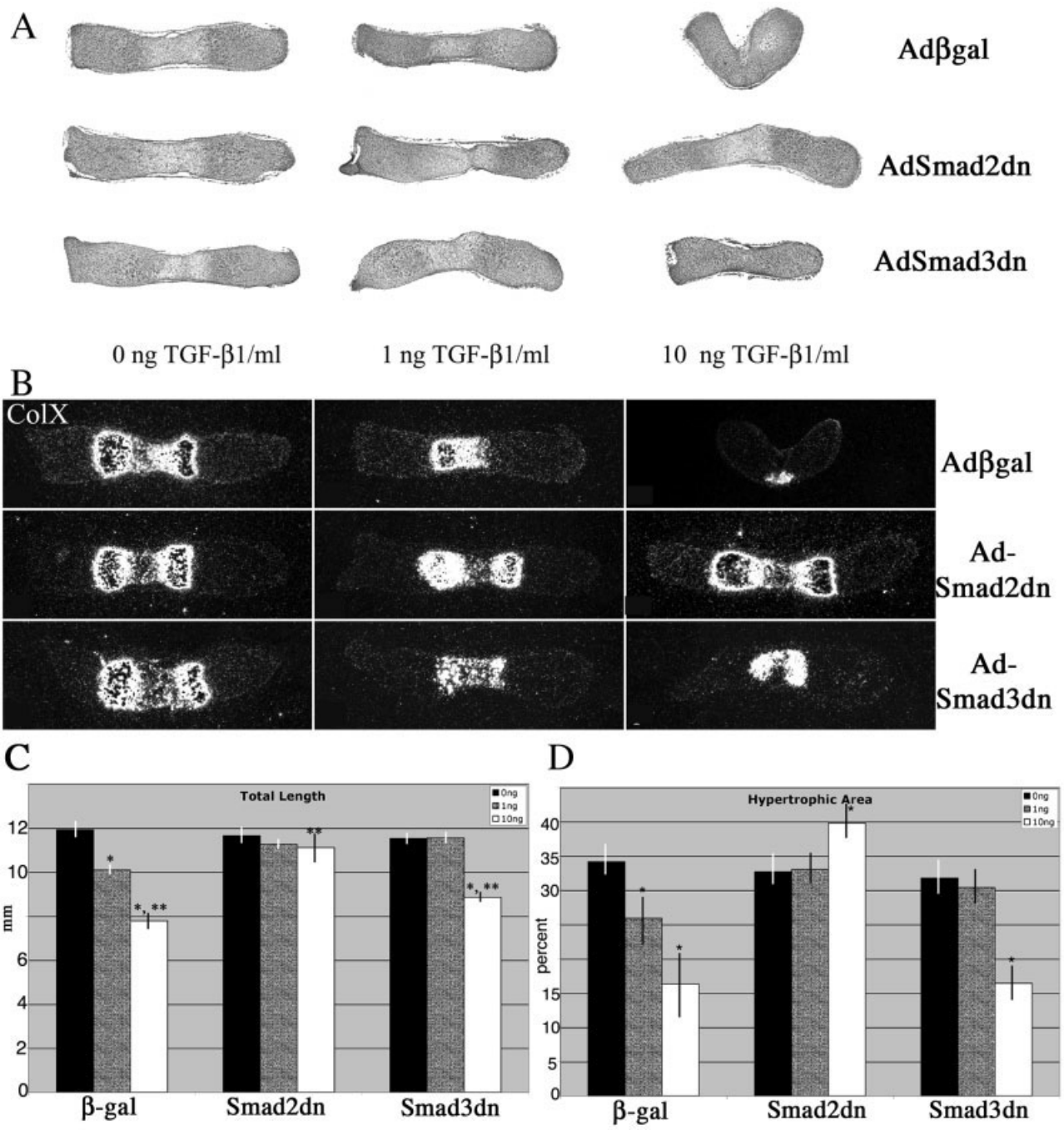

Fig. 3. Effects of dominant-negative forms of Smad2 and Smad3 on the development of metatarsal cultures. **A:** Histology. Metatarsals in organ culture were infected with the indicated adenoviruses, then were either untreated or treated with 1 or 10 ng of transforming growth factor-beta1 (TGF-β1) for 5 days. Metatarsals were fixed, embedded, sectioned, and stained with hematoxylin and eosin. **B:** Col10a1 (ColX) expression. The level of hypertrophic differentiation was measured by the expression domain of Col10a1 synthesis determined by in situ hybridization. Darkfield images are shown. Hybridization is seen as bright white grains. **C:** Lengths. Total bone length was measured from pictures of bones (×25). Between 9 and 20 bones were measured for each condition. The averages are shown. Small bars represent the standard deviation. Single asterisks indicate $P < 0.05$ compared with control untreated. Double asterisks indicate $P < 0.05$ compared with β-gal +10 ng TGF-β. **D:** Differentiation. The percentage hypertrophic area was calculated as the length of the area containing Col10a1 mRNA over the total length. Between 3 and 12 samples under each condition were measured. The averages are shown. Small bars represent the standard deviation. Asterisks indicate $P < 0.05$ compared with control untreated.

expected response, that is, a dose-dependent reduction in total length and inhibition of hypertrophic differentiation. Loss of Smad3 demonstrated a dose-dependent effect on reduced length in response to TGF-β1. That is, even though the total length of bones from $Smad3^{ex8/+}$ and $Smad3^{ex8/ex8}$ mice were significantly reduced after treatment with TGF-β1, the reductions were significantly less than in $Smad3^{+/+}$ cultures

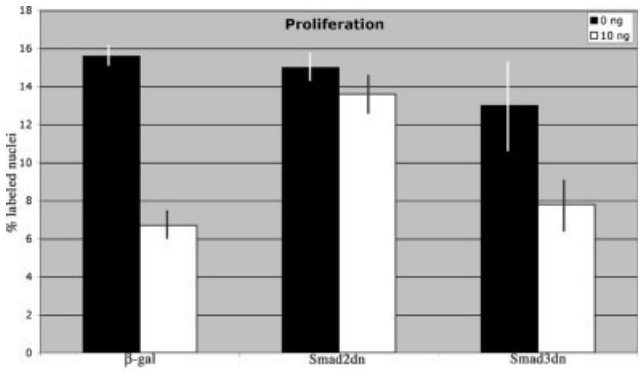

Fig. 4. Chondrocyte proliferation in adenovirus infected bones. Bones were untreated or treated with 10 ng/ml transforming growth factor-beta1 (TGF-β1) for 24 hr followed by treatment with bromodeoxyuridine for 2.5 hr. Labeled cells were detected using immunofluorescence, and the percentage labeled nuclei were counted. The average from four to six sections is shown. The vertical lines represent the standard deviation. WT, wild-type.

and the reduction in $Smad3^{ex8/ex8}$ bones was significantly less that that seen in $Smad^{+/ex8}$ bones (Fig. 5B). The dose response for inhibition of hypertrophic differentiation in response to TGF-β1 was altered in both $Smad3^{ex8/+}$ and$Smad3^{ex8/ex8}$ cultures so that that the percentage of hypertrophic cartilage was not altered after treatment with 1 ng of TGF-β1 /ml, but it was inhibited with 10 ng TGF-β1/ml to the same degree as observed in $Smad3^{+/+}$ mice (Fig. 5B). This response is similar to what was observed in cultures infected with Ad-Smad3ΔC and suggests that factors other than Smad3 can mediate inhibition of hypertrophic differentiation by TGF-β1 at the higher dose. It also suggests that responsiveness to TGF-β1 is sensitive to the amount of Smad3 available, especially at low doses of TGF-β1.

Next, to determine the role of Smad3 signaling in TGF-β–mediated effects on proliferation, metatarsal cultures from $Smad3^{+/+}$, $Smad3^{ex8/+}$, and $Smad3^{ex8/ex8}$ mice were treated with 10 ng of TGF-β1/ml for 24 hr followed by incubation with BrdU for 2.5 hr. BrdU was detected in sections from the cultures by using immunofloures-cence. The percentage of nuclei that incorporated BrdU was determined (Fig. 6). Treatment with TGF-β1 resulted in a decrease in the percentage labeled nuclei when compared with untreated controls in $Smad3^{+/+}$ cultures. The level of inhibition was approximately 50%. Loss of Smad3 demonstrated a dose-dependent effect on the inhibition mediated by TGF-β1. Proliferation was not inhibited by TGF-β1 in cultures fromSmad3$^{ex8/ex8}$ mice, whereas cultures from $Smad3^{ex8/+}$ mice demonstrated an intermediate level of inhibition. The data suggest Smad3 is required for inhibition of proliferation meditated by TGF-β1.

Smad2 and Smad3 Expression

To determine whether there were any significant differences in the expression domain for Smad2 or Smad3 that might account for their differential roles in chondrocyte proliferation and differentiation, the localization of Smad2 and Smad3 mRNA was determined by using in situ hybridization (Fig. 7). Smad2 mRNA was detected at high levels in the perichondrium and in maturing prehypertrophic cells close to the hypertrophic zone (Fig. 7A). Smad3 mRNA was detected in a similar domain (Fig. 7B). After treatment with TGF-β1 for 5 days, Smad2 and Smad3 mRNA was detected in all the round chondrocytes as well as the thick perichondrium (Fig. 7D and data not shown). Only background hybridization by the Smad3 probe was seen on sections from Smad3-null bones (Fig. 7E). Furthermore, Smad2 expression was not significantly altered in Smad3-null cultures (Fig. 7C). The results suggest that differences in expression patterns of Smad2 and Smad3 alone cannot predict unique or redundant roles for Smad3 and Smad2.

To localize Smad protein expression and to assay activation of Smads in the metatarsal cultures, antibodies directed to Smad proteins were used in immunohistochemical studies (Fig. 8, page 687). Two antibodies were used. The first, Smad3 (I-20), is commercially available (Santa Cruz Biotechnology) and reacts with both Smad2 and Smad3 proteins from several species. The antibody is directed to the N-terminal domain of the protein, and it does not distinguish between phosphorylated (activated) and unphosphorylated Smads. The second antibody, PS2, is specific for phosphorylated Smad2 and does not cross-react with Smad3 (kind gift from Dr. Carl-Henrik Heldin, Ludwig Institute; Persson et al., 1998; Piek et al., 1999). To localize Smad2 and Smad3, sections from metatarsal bones that were either untreated or treated with 10 ng of TGF-β1/ml for 24 hr or 5 days wereincubated with each antibody. Proteins were visualized by fluorescence so that Smad proteins were seen as red staining with a green nuclear counterstain. Sections from $Smad3^{ex8/ex8}$ bones were used to try to distinguish between Smad2 and Smad3 staining with the I-20 antibody (data not shown). We could not detect any clear differences in Smad staining in sections from $Smad3^{+/+}$ or $Smad3^{ex8/ex8}$ bones, suggesting significant overlap in expression of Smad2 and Smad3 in the cultures. The expression pattern of Smad2/3 seen with the I-20 antibody was similar in untreated metatarsal bones at 24 hr and at 5 days of culture. Very little staining was observed in the perichondrium with this antibody. Smad2/3 protein in the perichondrium was detected primarily in the cytoplasm, although staining in the nucleus was also observed (data not shown). Staining in round chondrocytes near the ends of the bones was sparse and was detected predominantly in the cytoplasm, although a few cells demonstrating nuclear staining were found (Fig. 8A). In flat cells, closer to the hypertrophic zone, sparse Smad2/3 staining was observed in both the cytoplasm and nucleus of cells (data not

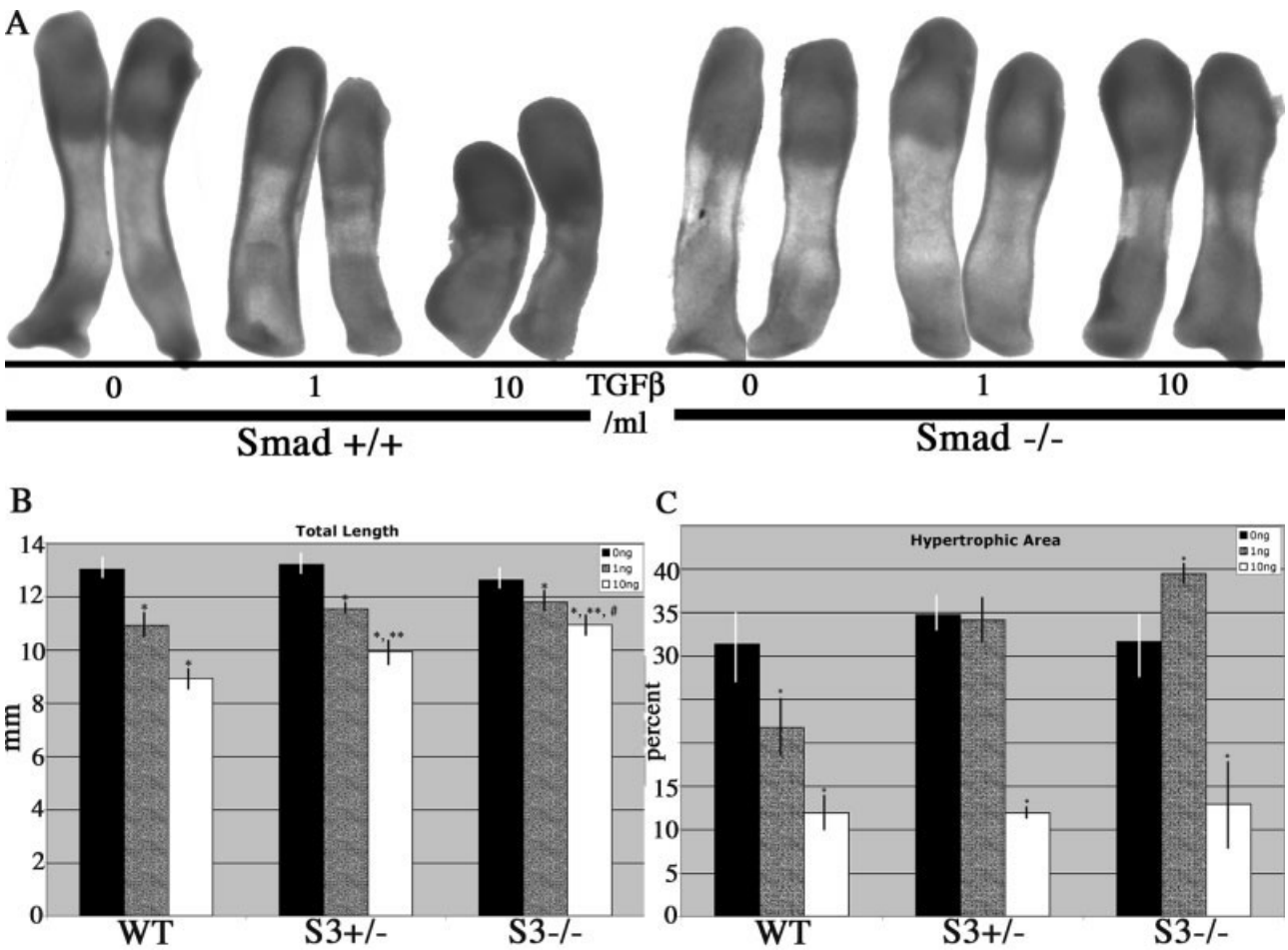

Fig. 5. Role of Smad3 in transforming growth factor-beta (TGF-β) signaling in metatarsal organ cultures. **A:** Bones were isolated from wild-type mice ($Smad^{+/+}$) and mice containing disruption in either one ($Smad3^{+/ex8}$) or both alleles of Smad3 ($Smad3^{ex8/ex8}$). Cultures were untreated or treated with 1 or 10 ng of TGF-β1/ml for 5 days. **B:** Length. The total length of each bone was measured from pictures of each metatarsal (×25). **C:** Differentiation. Hypertrophic area was measured as the clear area in the center of each bone over the total length. Between 9 and 24 bones were measured under each condition. The averages are shown, and the bars represent the standard deviation. Single asterisks indicate $P < 0.05$ relative to untreated control. Double asterisks indicate $P < 0.05$ relative to wild-type (WT) +10 ng of TGF-β. Pound signs indicate $P < 0.05$ relative to $S3^{+/ex8}$ 10 ng of TGF-β.

shown). After 24 hr of treatment with TGF-β1, staining in the round chondrocytes shifted to a predominantly nuclear pattern (Fig. 8B). Staining observed with the PS2 antibody was also sparse in the perichondrium of untreated cultures (Fig. 8C). Staining in the perichondrium of untreated cultures appeared to be localized to both the cytoplasm and the nucleus of cells in the most outer layers of the perichondrium. Phospho-Smad2 staining was seen in only a few chondrocytes (Fig. 8C). Staining could be seen both in the nucleus and in the cytoplasm. After treatment with TGF-β1 for 24 hr, (1) there was an increase in the number of cells staining for Smad2 in the perichondrium and cartilage and (2) most of the staining in the perichondrium and chondrocytes was in the nucleus (Fig. 8D). The data suggest that treatment with TGF-β activates Smad signaling in both the perichondrium and chondrocytes.

Differences in Smad staining at varying stages of chondrocyte differentiation were also observed in the growth plates of 4-week-old mice (Fig. 8F). Smad2/3 staining with the I-20 antibody was primarily cytoplasmic in resting cells at the top of the growth plate. Cells located closer to the hypertrophic zone demonstrated increasingly predominant nuclear Smad2/3 staining. In contrast, most of the cells in the articular cartilage had staining in both the nucleus and cytoplasm (Fig. 8E). Because nuclear Smad staining indicates an active TGF-β signaling pathway, the results suggest that TGF-β signaling is active in articular cartilage and that it varies in the postnatal growth plate as cells differentiate, becoming more active as cells approach hypertrophy.

DISCUSSION

Embryonic metatarsal bones grown in organ culture were used to test the hypothesis that the effects of TGF-β on chondrocyte growth and differentiation are mediated by the intracellular signaling molecules Smad2 and Smad3. The Smads are the most well-characterized mediators of signaling by members of the

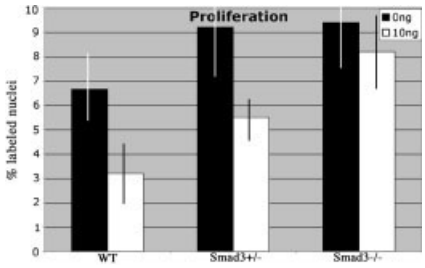

Fig. 6. Chondrocyte proliferation in Smad3-null bones. Cultures were untreated or treated with 10 ng/ml transforming growth factor-beta1 (TGF-β1) /ml for 24 hr followed by treatment with bromodeoxyuridine for 2.5 hr. Labeled cells were detected using immunofluorescence, and the percentage of labeled nuclei were determined. The averages from five to nine slides is shown, and the vertical lines represent the standard deviation. $P > 0.05$ for wild-type (WT) untreated relative to Smad3$^{+/-}$ or Smad3$^{-/-}$ untreated and Smad3$^{-/-}$ untreated compared with treated. Significance values are $P < 0.05$ for WT untreated compared with treated and Smad3$^{+/-}$ untreated compared with treated.

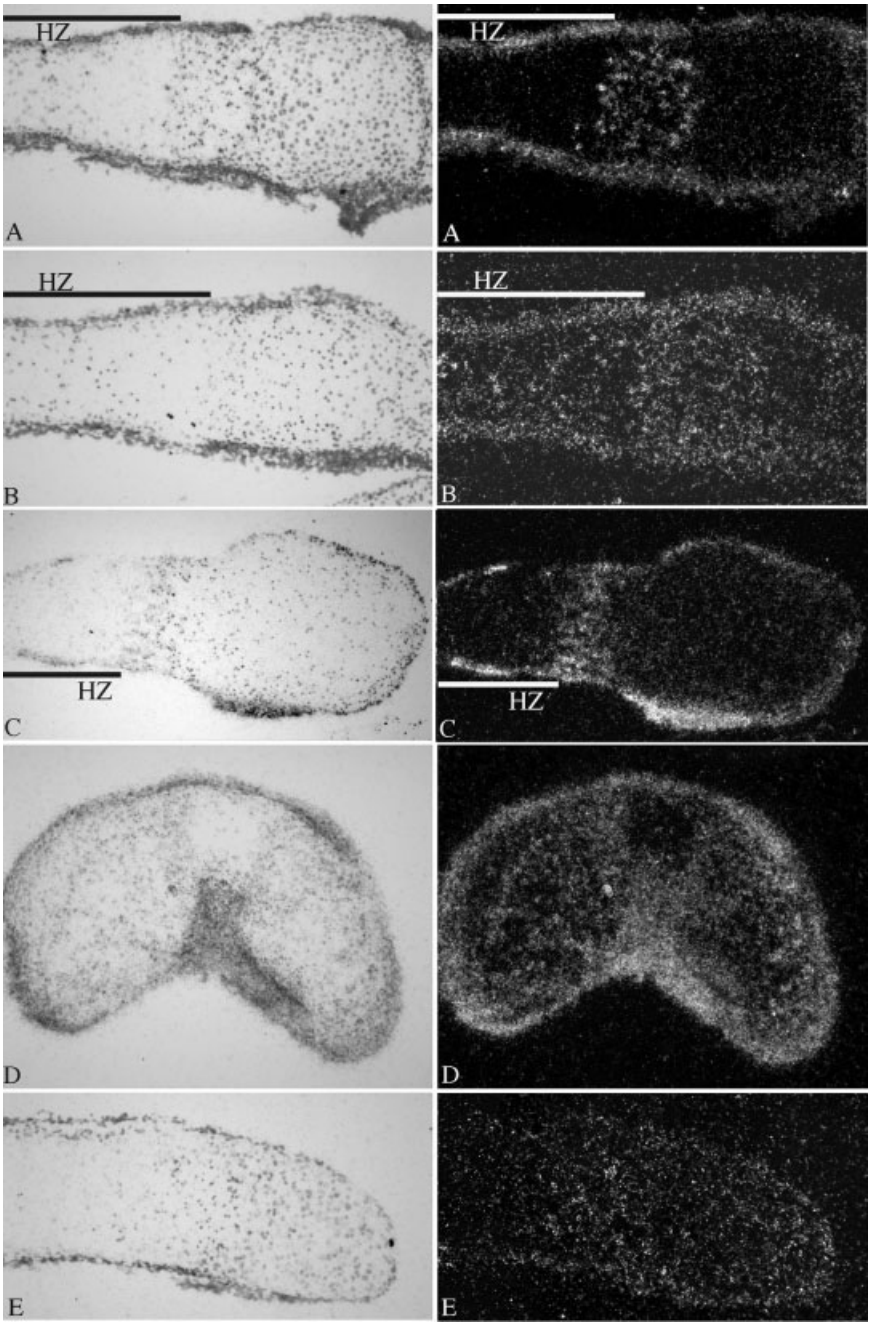

Fig. 7. Smad expression domains. Smad2 and Smad3 mRNA were localized in sections from metatarsals by in situ hybridization. **A–E:** Sections from bones that were either untreated (A–C,E) or treated (D) with transforming growth factor-beta1 (TGF-β1) were hybridized to ^{35}S-labeled antisense riboprobes for Smad2 (A,C,D) or Smad3 (B,E). Bright and darkfield images are shown. Hybridization is seen as white grains in the darkfield images. Hybridization to wild-type bones with sense probes (not shown) and hybridization of the Smad3 antisense probe to sections from Smad3-null bones (E) were used as negative controls to determine the level of background hybridization. Smad2 and Smad3 mRNA were detected in prehypertrophic chondrocytes and in the perichondrium. Smad2 mRNA was detected in sections from Smad3-null bones. All of the round cells in the culture expressed Smad2 and Smad3 after treatment with TGF-β1 for 5 days. HZ, hypertrophic zone.

TGF-β superfamily (Derynck and Zhang, 1996; Massague, 1998; Shi and Massague, 2003). Smads are specific in their signaling in that Smad2 and Smad3 mediate signaling by TGF-β and activin, while Smad1, Smad5, and Smad8 mediate BMP signals. Infection of the perichondrium with a dominant-negative form of Smad2, Ad-Smad2-3SA, which can block signaling through both Smad2 and Smad3 (Abdollah et al., 1997), prevented inhibition of hypertrophic differentiation and inhibition of proliferation normally observed after treatment with TGF-β1, suggesting the effects of TGF-β1 on long bone development are mediated through TGF-β/activin restricted Smads in the perichondrium. Infection of the perichondrium with a distinct dominant-negative form of Smad3, Ad-Smad3ΔC, which is specific for Smad3 (Wu et al., 1997, Ferguson et al., 2000), did not prevent inhibition of hypertrophic differentiation in response to 10 ng/ml TGF-β1. The same results were seen in cultures from Smad3-null mice, that is, 10 ng of TGF-β1/ml inhibited hypertrophic differentiation in Smad3$^{ex8/ex8}$ cultures to the same extent as in wild-type cultures. The results together suggest that Smad2 can compensate for the loss of Smad3 in the perichondrium with regard to regulation of hypertrophic differentiation. In wild-type, Smad-positive cultures, TGF-β1 inhibits incorporation of BrdU into chondrocytes by 50% to 60%. In cultures from Smad3-null mice, chondrocyte proliferation was not significantly reduced after treatment with TGF-β1. The results sug-

gest that, in the absence of Smad3, Smad2 cannot compensate to mediate inhibition of proliferation. The expression pattern of Smad2 and Smad3 overlap significantly and cannot account for the differences observed.

We previously showed that removal of the perichondrium or expression of a dominant-negative form of the TGF-β type II receptor in the perichondrium blocks the effects of TGF-β1 on metatarsal cultures (Alvarez et al., 2001, 2002). The data suggested that the perichondrium mediates the effects of TGF-β1 on both the exit of chondrocytes from the cell cycle through an unknown secondary factor and inhibition of hypertrophic differentiation most likely through PTHrP (Serra et al., 1999). In this study, we show that dominant-negative interference of Smads in the perichondrium by Ad-Smad2-3SA also blocks the effects of TGF-β on both proliferation and differentiation. However, the nuclear localization of Smads in the chondrocytes after treatment with TGF-β suggest chondrocytes can respond directly to TGF-β. Previously, it was shown that, in the presence of the perichondrium, chondrocyte proliferation is inhibited. In the absence of the perichondrium, there was a statistically significant increase in proliferation (Alvarez et al., 2001). We propose that this increase is due to direct stimulation of chondrocytes by TGF-β and that, in the intact cultures, a balance of factors results in a net decrease in proliferation.

Cultures infected with Ad-Smad3ΔC demonstrated a partial block to inhibition of proliferation by TGF-β. In contrast, TGF-β did not inhibit proliferation in cultures from $Smad3^{ex8/ex8}$ mice. The differences in the dominant-negative approach and the use of cultures from Smad3-null mice present two possibilities for the discrepancy. First, Smad3 function is required in chondrocytes as well as the perichondrium to mediate inhibition by TGF-β. Second, despite the observation that all three viruses infected the perichondrium to the same level and high levels of dnSmad3 protein were detected by Western blot (not shown), it is a formal possibility that the level of dnSmad3 expression was not sufficient to achieve a complete dominant-negative effect, resulting in a partial block to TGF-β signaling. Previously, infection with adenovirus containing a dominant-negative mutation of TβRII (Alvarez et al., 2002) and presently AdSmad2dn blocked inhibition of proliferation and hypertrophic differentiation by TGF-β1, suggesting levels of infection and gene expression from these adenovirus vectors were sufficient to achieve the dominant-negative effect. Similar results with regard to inhibition of hypertrophic differentiation were obtained with Ad-Smad3ΔC–infected cultures and cultures from $Smad3^{ex8/ex8}$ mice, but, based on results with cultures from $Smad3^{+/ex8}$ mice, the effects of TGF-β1 on hypertrophic differentiation are likely to be very sensitive to the amount of Smad3 available (see below). Nevertheless, because complete lack of Smad3 prevented inhibition of proliferation by TGF-β1, we can conclude that Smad2 cannot compensate for this function.

Smad3 demonstrates a dose-dependent effect with regard to TGF-β signaling in metatarsal development. Proliferation was not inhibited in cultures from $Smad3^{ex8/ex8}$ mice, but cultures from $Smad3^{+/ex8}$ mice demonstrated an intermediate level of inhibition relative to the wild-type cultures. Disruption of one or both alleles of Smad3 altered the dose response of TGF-β on inhibition of differentiation. In wild-type mice, treatment with TGF-β1 resulted in a dose-dependent decrease in hypertrophic differentiation, so that 22% of the cartilage in the cultures was hypertrophic after treatment with 1 ng of TGF-β1 /ml, and 12% was hypertrophic in cultures treated with 10 ng of TGF-β1/ml compared with untreated cultures in which 31% of the cartilage was hypertrophic after 5 days. In cultures from $Smad3^{+/ex8}$ and $Smad3^{ex8/ex8}$ mice, inhibition of hypertrophic differentiation was not detected after treatment with the lower dose of TGF-β1; however, at the higher dose of TGF-β1, hypertrophic differentiation was inhibited to the same extent as in wild-type mice. This finding suggests that Smad3 exhibits haploinsufficiency with regard to regulating hypertrophic differentiation at lower doses of TGF-β1. The data also suggest that responsiveness to TGF-β may be very sensitive to the levels of Smad3 available.

Smad2 and Smad3 mRNA were localized to overlapping expression domains in metatarsals in culture for 5 days. The highest levels of expression were detected in the perichondrium and in maturing chondrocytes close to the hypertrophic zone. Previously, it was shown that Smad3 mRNA is localized to the perichondrium of mouse embryonic day 16 ribs (Yang et al., 2001). In older mice, postnatal day 12, Smad3 mRNA was detected in the articular cartilage and trabecular bone (Yang et al., 2001). The expression pattern for Smad2 mRNA was similar to that of Smad3, and no changes in Smad2 expression were detected in $Smad3^{ex8/ex8}$ cartilage (Yang et al., 2001). Likewise, we did not detect differences in Smad2 expression in $Smad3^{ex8/ex8}$ metatarsals in culture for 5 days with or without TGF-β treatment. The expression pattern of Smad proteins was characterized previously using a variety of antibodies. Smad2 and Smad3 antibodies described by (Nakao et al., 1997) detected Smad2 and Smad3 protein in growth plates from 6-week-old rats (Sakou et al., 1999). Smad2 was strongly detected in proliferating chondrocytes, and Smad3 protein was primarily seen in maturing prehypertrophic cells. Flanders et al. (2001) developed a series of Smad antibodies that demonstrated a high level of specificity. By using these antibodies, both Smad2 and Smad3 were detected in the perichondrium of the femur from embryonic day 18 mice. A mix of cytoplasmic and nuclear staining was observed for both Smad2 and Smad3 protein in round and flat chondrocytes. A higher percentage of flat chondrocytes contained the Smad proteins relative to round chondrocytes. In the present study, we also observed more Smad staining in chondrocytes near the hypertrophic zone relative to round chondrocytes toward the end of the bone rudiment. In addition, predominantly cytoplasmic Smad staining was observed in untreated metatarsals in culture for 24 hr and for 5 days. After treatment with TGF-β1, Smad staining was increased and virtually all Smad protein was detected on the nucleus of both the perichondrium and chondrocytes.

Furthermore, Smad protein was detected as a mixture of cytoplasmic and nuclear staining in articular cartilage from mice at 4 weeks of age. Chondrocytes in the growth plate of mice at 4 weeks of age demonstrated variable staining depending on their location within the growth plate. Cells at the top of the growth plate had predominantly cytoplasmic staining, whereas cells closer to the hypertrophic zone demonstrated increasing nuclear staining. The results suggest differential TGF-β signaling within specific chondrocytes of the growth plate.

Distinct developmental defects have been observed in mice with targeted disruption of the Smad2 versus the Smad3 gene (Chang et al., 2002). Smad2 and Smad3 share a high degree of homology and can mediate signals from the same set of ligands (Shi and Massague, 2003). Mice that are null for Smad2 die before embryonic day 10 without mesoderm or an anterior to posterior axis (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998). The early lethality of the Smad2 mice makes is difficult to determine the specific role of Smad2 in skeletal development. Smad2 mice also exhibit haploinsufficiency with regard to craniofacial development (Nomura and Li, 1998). Mice null for Smad3 develop relatively normally but demonstrate defects in the immune system (Yang et al., 1999), an accelerated wound healing response (Ashcroft et al., 1999), and metastatic colon cancer (Zhu et al., 1998). Smad3-null mice also develop osteopenia and osteoarthritis-like condition that is most likely due to defects in the regulation of hypertrophic differentiation in adult mice (Borton et al., 2001; Yang et al., 2001). Differences in the expression patterns of Smad2 and Smad3 cannot account for the specific requirements of Smad2 or Smad3 in specific biological processes. It is possible that Smad2 and Smad3 activate different genes. For example, Smad3 can directly bind to a specific DNA sequence, CAGA, but Smad2 cannot (Dennler et al., 1998; Shi and Massague, 2003).

There is considerable evidence that TGF-β and the Smads play a role in regulating the cartilage phenotype. Smad2 and Smad3 have been shown to at least partially mediate regulation of differentiation by TGF-β in cultures of sternal chondrocytes (Ferguson et al., 2000). In addition, mice that express a dominant-negative mutation of the TGF-β type II receptor (MT-DNIIR) exhibit a progressive skeletal disorder that resembles osteoarthritis (Serra et al., 1997b). The defect is characterized by fibrillation and loss of articular cartilage that is preceded by decreased proteoglycan staining and increased expression of Col10a1. Continued hypertrophic differentiation is also seen in the growth plates of adult mice. The mice develop normally, and problems in the cartilage are not seen until the mice are 1 to 2 months of age. This finding may be due to the postnatal expression pattern of the transgene. Defects in the growth plate and in the maintenance of articular cartilage are also observed in $Smad3^{ex8/ex8}$ mice at approximately 1 month of age, suggesting that early skeletal development is not uniquely dependent on Smad3 (Yang et al., 2001). Mice with targeted deletion of the Ltbp3 gene demonstrate similar abnormalities later in life (Dabovic et al., 2002). It is possible that signaling by TGF-β directly in chondrocytes is more active in postnatal cartilage. This model is supported by the sparse nuclear localization of Smads in embryonic bones that are not treated with TGF-β and strong nuclear staining in the postnatal growth plate.

The findings in this study that Smad3 is not required to mediate hypertrophic differentiation but is required to mediate proliferation, appear at variance with results reported by Yang et al. (2001). The previous study did not assay proliferation directly, the increase in bone length from the beginning to the end of the culture was measured. A reduction in the total length of the bone was interpreted as inhibition of proliferation. The total length of the bone is the result of the combination of proliferation, hypertrophy, and matrix deposition. In the present study, incorporation of BrdU was not inhibited by TGF-β in the absence of Smad3, although the total length of the bone was inhibited. The previous results suggested that, in the absence of Smad3, inhibition of hypertrophic differentiation by TGF-β does not occur. The present study shows that hypertrophic differentiation is inhibited in Smad3-null cultures after treatment with 10 ng of TGF-β/ml but not after treatment with 1 ng of TGF-β/ml. There are several explanations for this apparent discrepancy. First, variations in the activity of TGF-β used in each study could exist. Differences in the activity of TGF-β fit with the observation from this study that hypertrophic differentiation is not inhibited in bones from $Smad3^{ex8/ex8}$ mice treated with 1 ng of TGF-β /ml. If only one dose (1 ng/ml) was assayed in this study, we would have made the same conclusion reported previously. Likewise, if more than one dose had been assayed in the previous study, a different conclusion may have resulted. Second, Yang et al. used cultures from 17.5-day embryos, and cultures for the present study are derived from 15.5-day embryos. At 17.5 days, chondrocyte differentiation and hypertrophy are well under way. At 15.5 days, there are very few hypertrophic chondrocytes. It is known that the response to TGF-β is often dependent on the differentiation status of the cells; thus, the difference in the age of the cultures could also affect the result (Moses and Serra, 1996).

In summary, we propose that Smad2 and Smad3 have unique and redundant roles in skeletal development. Smads, likely in the perichondrium, are required to mediate the effects of TGF-β1 on long bone development in organ culture. Smad2 can compensate for the loss of Smad3 in regulation hypertrophic differentiation in the embryonic tissue. The role of Smad3 in regulating chondrocyte growth and differentiation is complex and responsiveness to TGF-β1 is sensitive to the levels of Smad3 available. A clearer understanding of the mechanism of TGF-β action in embryonic skeletal development should provide a basis for understanding degenerative diseases of the cartilage and potential means of repair or regeneration.

EXPERIMENTAL PROCEDURES

Mouse Embryonic Metatarsal Organ Cultures

The three central developing metatarsal bones were isolated from each hind limb of embryonic day (E) 15.5 ICR/B6D2 mouse embryos or embryos from crosses of Smad3$^{ex8/+}$ mice (Yang et al., 2001). Noon on the day of finding the vaginal plug was considered day 0.5 of gestation. All bones were cultured in 24-well plates with three bones in 1 ml of medium containing alpha-MEM (Gibco-βRL) supplemented with 0.05 ng/ml ascorbic acid, 0.3 mg/ml L-glutamine, 0.05 mg/ml gentamicin, 1 mM β-glycerophosphate, and 0.2% bovine serum albumin (BSA) as previously described (Dieudonne et al., 1994; Serra et al., 1999). Explants were grown at 37°C in humidified 5% CO_2 incubator. TGF-β1 (R&D Systems; 1 or 10 ng/ml) in 4 mM HCl was added to cultures 12–16 hr after dissection. Cultures were observed and photographed with an Olympus SZH12 dissecting microscope after 5 days of treatment.

For adenovirus experiments (Alvarez et al. 2002), metatarsal bones from mice were cultured overnight in 300 μl of conditioned medium from 293 cells (DMEM +10% FCS) infected with adenovirus containing either a β-galactosidase reporter (Ad-β-gal) or the dominant-negative mutation of Smad2 or Smad3 (Ad-Smad2-3SA, Ad-Smad3ΔC; Zhang et al., 1996; Abdollah et al., 1997; Wu et al., 1997). Ad-Smad2-3SA and Ad-Smad3ΔC were constructed by the method of (Becker et al., 1994) by Maureen McDonnell and Brian Law, Vanderbilt University (McDonnell et al., 2001). Bones were then placed into the normal organ culture medium described above (Dieudonne et al., 1994; Serra et al., 1999) then treated with TGF-β1.

The length of bones was calculated by measuring the length of each metatarsal in photographs taken with an Olympus SZH12 dissecting microscope with a Magnafire digital camera. The total length was measured in photographs that were all taken at the same magnification (×25). The length measured in the photographs was used in the graphs presented. To calculate the actual bone length, divide the measured length by the magnification indicated on the microscope and camera (×25). The mean and standard deviation were calculated by using Microsoft Excel. The significance of differences in bone length were determined using a Student's *t*-test also calculated with Microsoft Excel. Groups with a probability value less than 0.05% ($P < 0.05$) were considered significantly different.

The percentage of the bone rudiment containing hypertrophic cartilage was calculated by three separate methods: (1) measuring the total length of the bone rudiment and the length of the "clear" area on whole bones (Dieudonne et al., 1994; Alvarez et al., 2001), (2) measuring the histologically hypertrophic area in hematoxylin and eosin stained sections, and/or (3) measuring the area of the bone culture containing Type X collagen (Col10a1) mRNA after in situ hybridization. Measurements were taken from photographs from a SZH12 dissecting microscope or an Olympus BX-51 upright microscope. The percentage of the total cartilage that was hypertrophic was calculated as follows: (length of hypertrophic zone/total length) ×100. The mean and standard deviation was calculated for each group. Significant differences were determined using the Student's *t*-test. Significantly different results were indicated by $P < 0.05$.

Whole-Mount Immunocytochemistry

Metatarsal cultures that were infected with adenovirus were fixed at 4°C in fresh 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 30 min. The tissues were washed in PBS containing 0.1% Tween-20 (PBST; Fisher Scientific, NJ) for 10 min, then placed in 3% normal goat serum (Vector Laboratories, Burlingame, CA) in PBST for an additional 30 min. This procedure was followed by incubation at room temperature for 2–3 hr in primary goat anti adenovirus FITC-conjugated antibody (Fitzgerald Industries International, Inc., Concord, MA; catalog no. 60-A01) diluted 1:100 into PBST. After this incubation, the bones were washed three times at room temperature in PBST for 15 min each wash. They were mounted in 70% glycerol and viewed under epifluorescence by using an Olympus BX-51 upright microscope with a Magnafire digital camera.

Immunofluorescence

Immunofluorescent staining for β-gal was performed by using the rabbit anti-β-gal antibody from Abcam, Cambridge, MA. Immunofluorescent staining for Smad2 and Smad3 was performed by using two different antibodies: (1) Smad3 (I-20) polyclonal antibody that recognizes both Smad2 and Smad3 proteins obtained from Santa Cruz Biotechnology (Santa Cruz, CA; #sc6202) and (2) PS2 polyclonal antibody that specifically recognized Phospho-Smad2 (a kind gift from Dr. Carl-Henrik Heldin, Ludwig Institute). Sections were dewaxed, rehydrated, and treated with 0.05% Saponin in water for 30 min at room temperature. Saponin was removed by washing three times for 5 min each in Tris buffered saline with 0.1% Tween-20 at room temperature. Immunostaining was then performed by using components and directions from the Vectastain Elite staining kit; however, Cy3-conjugated avidin (Vector Laboratories) was substituted for the avidin–biotin–peroxidase complex. Excess Cy3-conjugated avidin was removed from the sections by washing three times for 10 min each in Tris buffered saline with 0.1% Tween-20 at room temperature. Sections were counterstained with YoPro, a green nuclear stain, and the sections were immediately mounted with Aquapoly mount (Poly Sciences, Warrington, PA). Fluorescence was observed and imaged by using an Olympus BX-51 microscope and a Magnafire digital camera.

Histology and In Situ Hybridization

Metatarsal cultures were fixed overnight at 4°C by immersion in 4% fresh PFA in PBS, then dehydrated through

a series of ethanols, cleared in xylene, and embedded in paraffin. Sections were cut at a thickness of 5 μm and mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Sections were stained with hematoxylin and eosin as noted using standard procedures. Photographs of the sections were taken by using an Olympus BX-51 upright microscope with a Magnafire digital camera.

In situ hybridization was performed as described (Pelton et al., 1990). Metatarsals were fixed overnight in paraformaldehyde at 4°C, then dehydrated in ethanol and embedded in paraffin. Sections (5 μm) were hybridized to ^{35}S-labeled antisense riboprobes. Slides were exposed to photographic emulsion at 4°C for 4 days (Col10a1) or 2 weeks (Smads), then developed, fixed, and cleared. Sections were counterstained with 0.02% toluidine blue. Sections hybridized with a labeled-sense Col10a1or Smad riboprobe were used as negative controls. Only background hybridization was detected with the sense probes. Brightfield and darkfield images were captured with a Magnafire digital camera. The mouse Type X collagen (*Col10a1*) probe was a gift from Dr. Bjorn Olsen, Harvard Medical School (Boston, MA). The probe generated was a 650-bp *Hin*dIII fragment containing 360 bp of noncollagenous (NC1) domain and 260 bp of 3′-untranslated sequence of the mouse *Col10a1* gene in pBluescript (Apte et al., 1992). Mouse Smad 2 and Smad3 cDNA were obtained from Dr. J. Yingling (Duke, NC; Yingling et al., 1996).

BrdU Labeling

Metatarsal bones were treated with 10 ng of TGF-β1/ml for 24 hr followed by treatment with 10 μM BrdU (Boehringer Mannheim) for 2.5 hr. Metatarsal were then washed twice in PBS at 37°C, fixed in paraformaldehyde at 4°C overnight, embedded in paraffin, and cut into 5-μm sections. Sections were deparaffinized, denatured in 2 N HCl for 20 min at 37°C, and neutralized in 1% boric acid/0.0285% sodium borate, pH 7.6. Next, the sections were treated with 0.005 mg of trypsin/ml 0.05 M Tris, pH 7.6, for 3 min at 37°C and washed three times in PBS. Immunostaining was then performed by using Vectastain Elite staining kit (Vector Laboratories) as described by the manufacturer. A rat monoclonal antibody directed to BrdU (Harlan) was used as the primary antibody at a 1:200 dilution. Cy3-conjugated avidin (Vector Laboratories) was substituted for the avidin–biotin–peroxidase complex in the Vector Elite kit. Excess Cy3-conjugated avidin was removed from the sections by washing three times for 10 min each in PBS at room temperature. Sections were counterstained with YOPRO, a nuclear stain that fluoresces green. The sections were then immediately mounted with Aquapoly mount (Poly Sciences). Fluorescence was observed by using an Olympus BX-51 upright microscope with a Magnafire digital camera and Photoshop software. Labeled and unlabeled cells from at least three separate bone rudiments were counted and the percentage labeled nuclei were calculated for each group. The mean and standard deviation were calculated and significant differences between each group were calculated by using a Student's *t*-test. Results that were statistically different were determined by a P value < 0.05.

Mouse Genotyping

DNA that was isolated by the standard proteinase K digestion procedure from the liver rudiment of each *Smad3* mouse embryo was used for genotyping. The wild-type Smad3 allele was detected by using primer 1 (5′-CCACTTCATTGCCAT-ATGCCC-TG-3′) and primer 2 (5′-CCCGAA-CAGTTGGATTCACACA-3′). The primer 1 is located 5′ to the deletion and the primer 2 is located within the deletion. This primer pair yields a fragment of ~400 bp from wild-type and Smad3$^{ex8/+}$ but not from Smad3$^{ex8/ex8}$ mice. DNA was also amplified by using the primer 1 and primer 3, which is located in the pLoxpneo (5′-CCAGACTGCCTTGG-GAAAAGC-3′) to detect the mutant Smad3 allele. In this case, a 250-bp fragment was detected in mice heterozygous or homozygous for the mutant Smad3 allele, while no signal was detected in wild-type mice (Yang et al., 2001). The amplification conditions were 32 cycles at 94°C for 30 sec, 58°C for 45 sec, and 72°C for 45 sec.

ACKNOWLEDGMENTS

We thank Philip Sohn and Jill Placey for excellent technical assistance. We thank Maureen McDonnel and Brian Law (Vanderbilt University) for construction of Ad-Smad2-3SA and Ad-Smad3ΔC. Smad2 and Smad3 cDNA probes were kindly provided by Dr. J. Yingling, Duke. Smad3$^{ex8/ex8}$ mice were generously provided by Dr. C. Deng, NIH. The PS2 antibody was kindly provided by Dr. Carl-Henrick Heldin, Ludwig Institute. This work is supported by AR45605 and AR46982 from NIAMS/NIH to Dr. Serra. Dr. Alvarez was supported by grant PF 00 10985154 from the Ministry of Education, Culture, and Sports, Spain.

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