TGFβ2 mediates the effects of Hedgehog on hypertrophic differentiation and PTHrP expression

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SUMMARY

The development of endochondral bones requires the coordination of signals from several cell types within the cartilage rudiment. A signaling cascade involving Indian hedgehog (Ihh) and parathyroid hormone related peptide (PTHrP) has been described in which hypertrophic differentiation is limited by a signal secreted from chondrocytes as they become committed to hypertrophy. In this negative-feedback loop, Ihh inhibits hypertrophic differentiation by regulating the expression of Pthrp, which in turn acts directly on chondrocytes in the growth plate that express the PTH/PTHrP receptor. Previously, we have shown that PTHrP also acts downstream of transforming growth factor β (TGFβ) in a common signaling cascade to regulate hypertrophic differentiation in embryonic mouse metatarsal organ cultures. As members of the TGFβ superfamily have been shown to mediate the effects of Hedgehog in several developmental systems, we proposed a model where TGFβ acts downstream of Ihh and upstream of PTHrP in a cascade of signals that regulate hypertrophic differentiation in the growth plate. This report tests the hypothesis that TGFβ signaling is required for the effects of Hedgehog on hypertrophic differentiation and expression of Pthrp. We show that Sonic hedgehog (Shh), a functional substitute for Ihh, stimulates expression of Tgfb2 and Tgfb3 mRNA in the perichondrium of embryonic mouse metatarsal bones grown in organ cultures and that TGFβ signaling in the perichondrium is required for inhibition of differentiation and regulation of Pthrp expression by Shh. The effects of Shh are specifically dependent on TGFβ2, as cultures from Tgfb3-null embryos respond to Shh but cultures from Tgfb2-null embryos do not. Taken together, these data suggest that TGFβ2 acts as a signal relay between Ihh and PTHrP in the regulation of cartilage hypertrophic differentiation.

Key words: Endochondral bone, Cartilage, Perichondrium, TGFβ, Hedgehog, PTHrP, Mouse

INTRODUCTION

Long bones develop by a process called endochondral bone formation, in which a cartilage model is generated and then is replaced with bone (Erlebacher et al., 1995; Cancedda et al., 1995). Endochondral development begins with condensation of undifferentiated mesenchymal cells that prefigure the future skeletal elements. In the core of these condensations, cells differentiate into chondroblasts, which secrete cartilage matrix. Mesenchymal cells surrounding the cartilage rudiment form the perichondrium. At a specific stage, which is unique to each element, chondrocytes progress through a program of cell proliferation, maturation and hypertrophy. Changes in the composition and properties of the cartilage matrix in the hypertrophic zone allow invasion by capillaries and the ultimate replacement of cartilage by the trabecular bone matrix secreted by invading osteoblasts. The rate of chondrocyte differentiation must be carefully regulated so that the proper shape and length of the bone is achieved and maintained.

Several factors have been implicated in the regulation of endochondral bone formation. Parathyroid hormone-related peptide (PTHrP) is a secreted peptide expressed in a wide variety of adult and embryonic cell types, including osteoblasts and chondrocytes (Suva et al., 1987; Broadus and Stewart, 1994). The PTH/PTHrP receptor (PTHR) is also expressed in a wide range of cell types including a population of prehypertrophic chondrocytes in the growth plate (Karperien et al., 1994; Lee et al., 1995). The importance of PTHrP in endochondral bone formation is demonstrated in mice homozygous for a targeted disruption of the Pthrp gene (Pthlh – Mouse Genome Informatics). Pthrp-null mice demonstrate accelerated maturation of chondrocytes leading to excessive endochondral bone formation (Karaplis et al., 1994; Amizuka et al., 1994). Mice with targeted deletion of the Pthhr demonstrated a similar phenotype (Lanske et al., 1996). Conversely, overexpression of PTHrP in chondrocytes leads to a delay in chondrocyte maturation and bone formation such that mice are born with a completely cartilaginous skeleton (Weir et al., 1996). Furthermore, mutations resulting in either a constitutively active or an inactive PTHrP in humans result in...
skeletal dysplasias: Jansen metaphyseal chondrodysplasia, which is characterized by delayed endochondral bone formation (Schipani et al., 1995; Schipani et al., 1997); and Blomstrand chondrodysplasia, which is characterized by extreme advanced endochondral bone maturation (Zhang et al., 1998; Jobert et al., 1998), respectively.

Another factor involved in endochondral bone formation, Indian hedgehog (Ihh), belongs to a family of morphogens involved in embryonic patterning and limb bud development (reviewed by Hammerschmidt et al., 1997). Ihh is initially expressed in chondrocytes of the early cartilaginous skeletal elements (Bitgood and McMahon, 1995). On maturation, expression becomes progressively restricted to postmitotic prehypertrophic chondrocytes adjacent to the PTHR-expression becomes progressively restricted to postmitotic prehypertrophic chondrocytes, which is characterized by extreme advanced endochondral bone maturation (Zhang et al., 1998; Jobert et al., 1998), respectively.

Furthermore, dominant-negative interference of TGFβ signaling in the perichondrium of transgenic mice results in increased hypertrophic differentiation and expression of Ihh in the growth plate (Serra et al., 1997). As members of the TGFβ superfamily act downstream of Hedgehog proteins in several developmental systems, (Heberlein et al., 1993; Laufer et al., 1994; Ingham and Fietz, 1995; Roberts et al., 1995), we proposed a model where TGFβ1 would act downstream of Ihh to mediate expression of PTHrP and hypertrophic differentiation. In this study, we used mouse embryonic metatarsal organ cultures to test the hypothesis that TGFβ signaling in the perichondrium is required for the effects of Shh, a functional substitute for Ihh (Vortkamp et al., 1996; Yang et al., 1998; Zhang et al., 2001), on hypertrophic differentiation. Our results not only indicate that the perichondrium is essential for the effects of Shh on chondrocyte differentiation but that TGFβ2 specifically is required for this effect.

**MATERIALS AND METHODS**

**Embryonic metatarsal rudiment organ cultures**

The three central metatarsal rudiments were isolated from each hind limb of 15.5 days post coitum ICR/B6D2 mouse embryos, embryos from crosses of Tgfb2+/− mice (Sanford et al., 1997) or embryos from crosses of Tgbβ3−/− mice (Proetzel et al., 1995). Noon on the day of the vaginal plug is 0.5 days post coitum. Three metatarsals were placed into each well of a 24-well plate containing 1 ml of organ culture medium: α-MEM (Gibco-BRL) supplemented with 0.005 mg/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 (Dieudeonne et al., 1994; Serra et al., 1999). Explants were grown at 37°C in a humidified 5% CO2 incubator. TGFβ1 (R&D Systems; 1 or 10 μg/ml) in 4 mM HCl or Shh-N (R&D Systems; 2 μg/ml) in 0.1% BSA in phosphate-buffered saline (PBS) or serum free Shh-conditioned medium (ShhN) (Zeng et al., 2001) were added to cultures 14-16 hours after dissection. Cultures treated with vehicle alone or with serum-free conditioned media from cells transfected with a GFP reporter construct were used as controls. Cultures were observed and photographed with an Olympus SZH 12 dissecting microscope after 5 days of treatment.

For perichondrium experiments, the perichondrium was removed from metatarsals from one limb, while the metatarsals from the contralateral limb were left intact. Perichondrium was removed enzymatically by incubating the bone for 3 minutes at room temperature in 1mg/ml collagenase type 2 (Worthington Biochemical) in PBS, as previously reported (Thesingh and Burger, 1983; Haujiman et al., 1999). The enzyme activity was stopped by transferring the rudiments to 10% FCS in PBS and the remaining perichondrium was removed mechanically by rolling the bone rudiments over a plastic surface (Thesingh and Burger, 1983; Haujiman et al., 1999).

For adenosine experiments, metatarsal bones from ICR/B6D2 mice were cultured overnight in 300 μl of conditioned media from 293 cells (DMEM +10% FCS) infected with adenosine-containing either a β-galactosidase reporter or the dominant-negative mutation of
the TGB-β type II receptor (DNIIR) (Chen et al., 1993). Bones were then placed into the normal organ culture media described above (Diedoule et al., 1994; Serra et al., 1999) then treated with either TGFβ1 or Shh. Bone rudiments infected with an adenovirus that expressed the β-galactosidase protein were used as controls. Adenoviruses were constructed as described elsewhere (Becker et al., 1994).

**Mouse genotyping**

DNA isolated from the tail and forelimbs of each Tgfb2 or Tgfb3 mouse embryo was used for genotyping. DNA was extracted using the standard proteinase K digestion procedure. An aliquot of the DNA was used for PCR genotyping. To identify Tgfb3-null mice, an upstream primer 5'-TGG GAG TCA TGG CGT TAA CT-3' in intron 5 and a downstream primer 5'-CAC TCA CAC TGG CAA GTA GT-3' in intron 6 were used to amplify fragments of 400 bp and 1.3 kb from the wild-type and null alleles, respectively (Proetzel et al., 1995). The amplification conditions were 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. For Tgfb2 mice the following primers were used: forward, AAATGTCAGGATA- TTGGCTG; reverse, AAACCCATAGATAGGGCATGC; and Neo-primer, GCCGGAAGGATACATCAT. The Neo/Reverse primer combination yielded a 600 bp band in null and heterozygous mice. The forward/reverse primer combination gave a 300 bp band in wild-type mice, 300 bp and 1500 bp bands in heterozygous mice, and a 1500 bp band in mice with the disruption in both alleles (Sanford et al., 1997). The amplification conditions were 35 cycles at 95°C for 30 seconds, 57°C for 50 seconds and 72°C for 1.5 minutes.

**Histology**

Metatarsal rudiments were fixed overnight at 4°C by immersion in 4% formaldehyde (PFA) in PBS, then dehydrated through a series of ethanols, cleared in xylene and embedded in paraffin. Sections (5 μm) were cut at a thickness of 5 μm and a downstream primer 5'-CAC TCA CAC TGG CAA GTA GT-3' in intron 6 were used to amplify fragments of 400 bp and 1.3 kb from the wild-type and null alleles, respectively (Proetzel et al., 1995). The amplification conditions were 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute. For Tgfb2 mice the following primers were used: forward, AAATGTCAGGATA-TTGGCTG; reverse, AAACCCATAGATAGGGCATGC; and Neo-primer, GCCGGAAGGATACATCAT. The Neo/Reverse primer combination yielded a 600 bp band in null and heterozygous mice. The forward/reverse primer combination gave a 300 bp band in wild-type mice, 300 bp and 1500 bp bands in heterozygous mice, and a 1500 bp band in mice with the disruption in both alleles (Sanford et al., 1997). The amplification conditions were 35 cycles at 95°C for 30 seconds, 57°C for 50 seconds and 72°C for 1.5 minutes.

**In situ hybridization**

In situ hybridization was performed as described (Pelton et al., 1990). Metatarsals were fixed overnight in paraformaldehyde (PFA) at 4°C, then dehydrated in ethanol and embedded in paraffin. Sections (5 μm) were hybridized to 35S-labeled antisense riboprobes. Slides were exposed to photographic emulsion at 4°C for 4 days (Col10a1) to 2 weeks (others), then developed, fixed and cleared. Sections were counterstained with 0.02% Toluidine Blue. Sections hybridized with a labeled-sense Col10a1 riboprobe were used as negative controls. No positive hybridization signal was found in negative controls. Bright field and dark field images were superimposed using Photoshop software so that the bright grains of hybridization could be seen on the gray background. Probes used were as follows. The mouse Type X collagen (Col10a1) probe (a gift from Dr Bjorn Olsen, Harvard Medical School, Boston, MA) was a 650 bp HindIII fragment containing 360 bp of non-collagenous (NC1) domain and 260 bp of 3'-untranslated sequence of the mouse Col10a1 gene in pBluescript (Apte et al., 1992). The Pthrp probe (a generous gift from Dr Tom Clemens, University of Cincinnati Medical School) consisted of a 280 bp fragment of the mouse Pthrp cDNA cloned into pGEM. Tgfb1 (974 bp), Tgfb2 (442 bp) and Tgfb3 (610 bp) probes (a kind gift of Dr Harold Moses, Vanderbilt University School of Medicine, Nashville, TN) have been described elsewhere (Pelton et al., 1990).

**X-gal staining**

Metatarsal bone rudiments from Ptc lacZ/+ mice (Goodrich et al., 1997) or those infected with adenovirus expressing the β-galactosidase protein were fixed at 4°C in fresh 4% PFA in PBS for 30 minutes, washed in PBS, incubated for 15 minutes in permeabilizing solution (2 mM MgCl2, 0.01% sodium deoxycholate and 0.02% NP-40 in PBS), then washed again in PBS and incubated overnight in stain solution (2 mM MgCl2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal in PBS) at room temperature. Metatarsals were then fixed in 4% PFA at 4°C for 2 hours and cleared overnight in 80% glycerol at room temperature. Stained rudiments were observed and photographed with an Olympus SZH 12 dissecting microscope. Some samples were also cryosectioned after staining, and observed and photographed with an Olympus BX-60 upright microscope.

**Whole-mount immunocytochemistry**

Metatarsals that were infected with adenovirus expressing β-gal or DNIIR were fixed at 4°C in fresh 4% PFA in PBS for 30 minutes. The tissues were washed in PBS containing 0.1% Tween-20 (Fisher Scientific, New Jersey; PBST) for 10 minutes, then placed in 3% normal goat serum (Vector Laboratories, Burlingame, CA) in PBST for an additional 30 minutes. This was followed by incubation at room temperature for 2-3 hours in primary goat anti adenovirus FITC-conjugated antibody (Fitzgerald Industries International, Concord, MA; catalog number 60-A01) diluted 1:100 into PBST. After this incubation, the rudiments were washed three times at room temperature in PBST for 15 minutes each wash. The metatarsal bones were mounted in 70% glycerol and viewed under epifluorescence using an Olympus BX-60 upright microscope with a SPOT digital camera.

**BrdU labeling**

Metatarsal rudiments were treated with 100% Shh-conditioned medium or 100% control conditioned medium for 24 hours followed by treatment with 10 μM BrdU (Boehringer Mannheim) for 2.5 hours. Bone rudiments processed for detection of BrdU-labeled cells were washed twice in PBS at 37°C, fixed overnight by immersion in 4% PFA in PBS at 4°C, dehydrated through a graded ethanol series, cleared in xylene and embedded in paraffin. Sections (5 μm) were obtained and mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Blocks were cut parallel to the bone vertical axis. Sections were processed essentially as described previously (Serra et al., 1999; Alvarez 2001).

**RESULTS**

**Shh inhibits hypertrophic differentiation in embryonic mouse metatarsal bone rudiments grown in organ culture**

Previously, it was shown that misexpression of Ihh in developing chick long bones resulted in a delay in hypertrophic differentiation and an increase in expression of Pthrp mRNA (Vortkamp et al., 1996). In addition, mice with a targeted deletion of the Ihh gene demonstrated accelerated endochondral bone formation and no Pthrp expression in cartilage rudiments (St-Jacques et al., 1999), indicating that Ihh acts as a negative regulator of chondrocyte differentiation in vivo. Shh, has biological properties that are similar to those of Ihh and has previously been used as a functional substitute for Ihh in many reports (Vortkamp et al., 1996; Yang et al., 1998; Zhang et al., 2001). Organ cultures allows for the study of complex biological processes in a three-dimensional structure in the context of native cell-cell and cell-extracellular matrix interactions but are easier to manipulate than whole animals. To determine if embryonic mouse metatarsal bones grown in organ culture could be used as a model to study signaling
pathways downstream of Hedgehog, the effects of Shh on development of embryonic metatarsal organ cultures was determined. First, bones were treated with varying concentrations of serum-free media conditioned by cells that had been transfected with an expression plasmid containing the soluble active N-terminal domain of Shh (ShhN) (Zeng et al., 2001) and mixed with standard organ culture media (ratio of conditioned media to organ culture media used: 100%, 1:1 and 1:10). This treatment resulted in a dose-dependent decrease in the area of hypertrophic cartilage in each culture but no alterations were detected in the length of the bone after 5 days of treatment when compared with bones treated with control conditioned media (data not shown). Next, metatarsals in standard organ culture media were treated with varying concentrations of recombinant Shh-N terminal peptide (R&D Systems). After 5 days in culture, untreated metatarsals had grown longitudinally and each stage of chondrocyte differentiation was visible (Dieudonne et al., 1994; Serra et al., 1999). A clear area representing hypertrophic cartilage was observed surrounding the dark area of mineralized cartilage at the center of the bone rudiment. Treatment with 2 µg Shh/ml resulted in inhibition of matrix mineralization and a decrease in the length of the clear or hypertrophic area observed in the bone rudiment (Fig. 1A,B). There was no detectable difference in the overall length of untreated and Shh-treated rudiments. Consistent with this observation, we did not detect a difference between control and Shh treated cultures in the rate of chondrocyte proliferation within the proliferative zones; however, the proliferating zone was larger in the Shh-treated cultures (Fig. 1C,D and data not shown). The decrease in the area of hypertrophic cartilage in treated bones relative to untreated bones was confirmed histologically and by localization of a marker for hypertrophic chondrocytes, Type X Collagen (Col10a1) mRNA (Fig. 1). Zones of periarticular round proliferating (RP), flat proliferating (FP) and hypertrophic (HZ) cartilage were easily detected in sections from untreated and treated cultures. Bone rudiments treated with Shh showed a considerable decrease in the area of the total cartilage containing histologically hypertrophic cells when compared with controls (Fig. 1C,D). Treatment with Shh also resulted in a dramatic decrease in the expression level of Col10a1 mRNA, which was restricted to cartilage in the very center of the bone rudiment (Fig. 1E,F), indicating that Shh inhibits hypertrophic differentiation in mouse metatarsal organ cultures.

The effects of Shh on development of mouse metatarsal cultures were very similar to those previously reported for PTHrP treatment (Serra et al., 1999). In addition, it has been shown that Ihh stimulates expression of Pthrp in chick limbs (Vortkamp et al., 1996) and that Ihh is required for expression of Pthrp in mouse (St-Jacques et al., 1999). To determine if Pthrp mRNA expression was stimulated in the mouse metatarsal cultures treated with Shh, Pthrp mRNA was localized by in situ hybridization in sections from metatarsal cultures that were either untreated or treated with Shh for 5 days (Fig. 1G,H). In the absence of Shh, little to no Pthrp mRNA was detected. After treatment with Shh, hybridization to the PTHrP probe was detected in the periarticular regions of the bone as well as the perichondrium, similar to what has been observed after treatment with TGFβ1 (Serra et al., 1999; Alvarez et al., 2001). Occasionally, Pthrp mRNA was also detected in a subset of hypertrophic cells at the very center of the bone rudiment (see Figs 6, 7).

The above data are consistent with previously published data in chick regarding the effects of Ihh on endochondral bone

![Fig. 1. Effects of Shh treatment on embryonic mouse metatarsal bone rudiments. Mouse embryonic metatarsal explants were either untreated (A,C,E,G) or treated with 2 µg/ml recombinant Shh (B,D,F,H) for 5 days.](image-url)
formation (Vortkamp et al., 1996) and suggest that the mouse metatarsal organ culture is a suitable model to study signaling pathways used by Hedgehog proteins to regulate hypertrophic differentiation and expression of Pthrp mRNA. Similar results were observed for all of the above experiments with conditioned media and recombinant protein, suggesting that the effects of the conditioned media are due to Shh. The advantages of using the recombinant protein include: (1) the fact that the protein can be placed into the standard organ culture medium that contains factors not present in the conditioned media that enhance mineralization; and (2) that there was little to no variability in the activity of the recombinant protein, whereas the activity of the conditioned media varied dramatically from batch to batch. Recombinant protein was used in the experiments described below unless otherwise indicated.

Shh stimulates Tgfb2 and Tgfb3 mRNA

It has previously been shown that the effects of Ihh on hypertrophic differentiation are mediated through PTHrP (Vortkamp et al., 1996; Lanske et al., 1996; Karp et al., 2000). It has also been demonstrated that the effects of TGFβ1 on hypertrophic differentiation are mediated by PTHrP (Serra et al., 1999). Members of the TGFβ superfamily act downstream of Hedgehog signaling in several biological systems (Heberlein et al., 1993; Lauber et al., 1994; Ingham and Fietz, 1995; Roberts et al., 1995), so we proposed that TGFβ acted downstream of Hedgehog to regulate the rate of chondrocyte hypertrophic differentiation. If this hypothesis is correct, we might predict that Shh regulates expression of Tgfβ mRNA in metatarsal cultures. In situ hybridization was performed to localize the expression of the three Tgfβ isoforms in sections from untreated and Shh-treated metatarsal rudiments (Fig. 2). Tgfβ1 mRNA was localized to a subset of prehypertrophic and hypertrophic cells as well as perichondrial cells along the diaphysis in both untreated and Shh-treated rudiments (Fig. 2A,B). Hybridization intensity appeared similar in both untreated and treated samples. A low level of hybridization to the Tgfβ2 probe was observed in the perichondrium and prehypertrophic chondrocytes in untreated cultures (Fig. 2C). After treatment with Shh, there was a dramatic increase in hybridization to the Tgfβ2 probe in both the inner and outer layers of the perichondrium (Fig. 2D). Expression was detected in both the inner and outer layers of the perichondrium. The highest expression level was observed in inner perichondrial cells located adjacent to terminally differentiated chondrocytes. Tgfβ2 mRNA was also detected in both prehypertrophic and hypertrophic chondrocytes after treatment with Shh. In untreated cultures, a low level of hybridization to the Tgfβ3 probe was detected in the perichondrium and in prehypertrophic and hypertrophic chondrocytes (Fig. 2E). Tgfβ3 mRNA levels were increased dramatically in the outer layer of the perichondrium after treatment with Shh (Fig. 2F). Tgfβ3 expression was also detected in prehypertrophic and hypertrophic chondrocytes. Shh stimulated expression of Tgfβ2 and Tgfβ3 with the most striking expression observed in the perichondrium.

The perichondrium is required to mediate the effects of Shh on hypertrophic differentiation

Misexpression of Ihh in chick limbs stimulated expression of Patched (Ptc1) and Gli1, two components of Hedgehog signaling, in perichondrial cells adjacent to the Ihh-expressing zone suggesting that the effect of Ihh on chondrocyte differentiation was indirect and mediated by the perichondrium (Vortkamp et al., 1996). More recently, we and others have detected Ptc1 mRNA and protein in prehypertrophic chondrocytes (St-Jacques et al., 1999) (data not shown), raising the possibility that Ihh may act directly on chondrocytes to regulate growth and/or differentiation. To determine which cells were responding to Shh, metatarsal bones from mice in which the lacZ gene was inserted into the Ptc1 locus (Ptc lacZ/+) (Goodrich et al., 1997) were cultured in the presence or absence of Shh (Fig. 3). In untreated cultures, β-gal activity was detected by X-gal staining in the perichondrium near the previously described Ihh expression domain. Low levels of staining were also detected in prehypertrophic chondrocytes of this region. In Shh-treated cultures, intense staining was seen in the perichondrium surrounding the entire bone rudiment. Variable and patchy staining was detected in chondrocytes. The staining pattern indicated that treatment with Shh activates
Ptc1 expression in the perichondrium; however, activation in chondrocytes was variable. To address this issue more directly, the perichondrium was stripped off the entire length of metatarsal rudiments, which were either left untreated or treated with Shh (Fig. 4). Metatarsals with perichondrium intact were used as controls. First, the morphology of intact and perichondrium-free cultures untreated or treated with Shh were examined (Fig. 4A-D). As expected, intact rudiments treated with Shh showed a reduction in the length of the clear hypertrophic area when compared with untreated controls (Fig. 4A,B). By contrast, in perichondrium-free rudiments, Shh treatment had no effect on the length of the hypertrophic zone (Fig. 4C,D). As previously observed (Alvarez et al., 2001), perichondrium-free cultures demonstrated a degree of disorganization where each zone of cartilage was difficult to distinguish (data not shown), suggesting that the perichondrium may provide physical support to the organ culture that is lost when it is removed. Next, in situ hybridization for Col10a1 mRNA was performed to more clearly define the hypertrophic zone (Fig. 4E-H). Cultures treated with Shh in which the perichondrium was intact showed a decrease in the amount of cartilage expressing Col10a1 mRNA when compared with untreated controls (Fig. 4E,F). Unlike intact rudiments, the expression of Col10a1 in perichondrium-free cultures was not altered by treatment with Shh (Fig. 4G,H). These results indicate that the perichondrium is crucially important for mediating the effects of Shh on terminal differentiation. Furthermore, the data suggest that a factor from the perichondrium may mediate this response of chondrocytes to Shh.

**The effects of Shh on hypertrophic differentiation require TGFβ signaling in the perichondrium**

As Shh stimulated expression of mRNA for Tgfβ genes in the perichondrium and the perichondrium is essential for Shh-mediated inhibition of hypertrophic differentiation, we tested the hypothesis that TGFβ signaling in the perichondrium is required for inhibition of hypertrophic differentiation by Shh. To block signaling by TGFβs in the perichondrium, metatarsal bone rudiments were infected with adenoviruses that express a dominant-negative mutation of the TGFβ type II receptor (DNIIR). This DNIIR has been shown to block signaling by all three TGFβ isoforms (Chen et al., 1993; Brand et al., 1993; Brand and Schneider, 1995). Metatarsal bones infected with adenoviruses that express the β-galactosidase reporter gene (β-gal) were used as controls. As the adenoviruses used are replication defective, only the outer layer of cells, the perichondrium, is infected after soaking the rudiments in virus conditioned medium overnight (Fig. 5E,F). The level and location of infection was visualized by two methods. First, rudiments infected with the control β-gal virus were stained with X-gal so that cells expressing the β-galactosidase reporter stained blue (Fig. 5E). Approximately 65% to 75% of the cells in the perichondrium were stained with X-gal and no staining was seen in areas were the perichondrium was stripped away after the infection (data not shown). Second, whole-mount immunofluorescence with an antibody directed to the adenovirus coat protein was used to visualize cells infected with both the control and DNIIR-containing virus (Fig. 5F). Similar results were obtained with both techniques and similar infection efficiency was observed with the control and DNIIR viruses.

Previously, we have shown that the perichondrium is required for the effects of TGFβ on hypertrophic differentiation and on chondrocyte proliferation (Alvarez et al., 2001). To test if DNIIR expression from the adenovirus vector was sufficient to block TGFβ signaling, metatarsals infected with adenoviruses containing the β-gal reporter or DNIIR were treated with 0, 1 or 10 ng TGFβ1/ml (Fig. 5A,B). Cultures infected with the control β-gal virus responded to TGFβ as expected. There was a dose-dependent decrease in the length of the bone rudiments and a decrease in the area of the hypertrophic zone (Fig. 5A). By contrast, treatment with TGFβ1 had no effect on bone rudiments infected with the DNIIR adenovirus (Fig. 5B), indicating that DNIIR expression from the adenovirus is sufficient to block TGFβ signaling.

Next, to test the hypothesis that TGFβ signaling is required for the effects of Shh on hypertrophic differentiation, bone rudiments that were either infected with β-gal or DNIIR virus were treated with 0 or 2 µg/ml of Shh (Fig. 5C,D). Shh treatment resulted in a reduction of the area of hypertrophic cartilage in the rudiments infected with the β-gal control virus (Fig. 5C); however, bones infected with the DNIIR virus did not appear to respond to Shh treatment (Fig. 5D). To identify the hypertrophic zone more clearly, Col10a1 mRNA was localized in sections from cultures infected with either β-gal or
DNIIR virus, and treated with either 0 or 2 μg/ml Shh (Fig. 6A-D). In bone rudiments infected with β-gal virus, treatment with Shh resulted in a dramatic reduction in the expression domain for Col10a1 (Fig. 6A,B). The expression domain of Col10a1 was not altered by Shh treatment in bone rudiments infected with DNIIR virus, suggesting that TGFβ signaling is required for Shh-mediated inhibition of hypertrophic differentiation (Fig. 6C,D).

Fig. 4. Role of the perichondrium in signaling by Shh. Morphology of metatarsal cultures (A-D). Bone rudiments in which the perichondrium remained intact (A,B) or was stripped from the length of the rudiment (C,D) were either left untreated (A,C) or treated with 2 μg/ml Shh (B,D) for 5 days. A marked decrease in the length of the hypertrophic zone (black line) was observed in intact cultures treated with Shh when compared with untreated, intact controls. No difference in length of the hypertrophic zone was detected in Shh-treated, perichondrium-free cultures when compared with untreated, perichondrium-free cultures. (E-H) Col10a1 expression. Intact (E,F) and perichondrium-free rudiments (G,H) were either left untreated (E,G) or treated with 2 μg/ml Shh (F,H) for 5 days. The area of cartilage expressing Col10a1 was reduced in intact cultures treated with Shh when compared with the intact untreated controls. The area of cartilage expressing Col10a1 was not altered in stripped rudiments treated with Shh when compared with the untreated, stripped controls. Dark field images are shown. Hybridization is detected as bright white grains on the dark background. Scale bars: 360 μm in A-D; 250 μm in E-H.

Fig. 5. Infection of embryonic metatarsal bone rudiments with adenovirus. Metatarsal rudiments infected with adenovirus that express the β-gal reporter gene (A,C) or a dominant-negative mutation of the TGFβ type II receptor (B,D) in the perichondrium were untreated (A,C), treated with TGFβ1 (1 or 10 ng/ml; B) or Shh (2 μg/ml; D) for 5 days. Treatment with TGFβ1 in rudiments infected with β-gal virus resulted in a decrease in the overall length of the metatarsal bones and in a decrease in the length of the hypertrophic zone (black lines) when compared with untreated cultures. (A) The effects of TGFβ1 were dose dependent. (B) TGFβ1 treatment did not affect the overall length of the metatarsal or the length of the hypertrophic zone (black line) in rudiments previously infected with DNIIR virus. (C) Shh treatment of rudiments infected with the β-gal virus resulted in a decrease in the length of the hypertrophic zone (black line). (D) Bone rudiments infected with DNIIR virus and treated with Shh did not show any decrease of the length of the hypertrophic area when compared with untreated rudiments. Metatarsal bones infected with an adenovirus that express the β-gal reporter gene were used to determine the efficiency and location of infected cells. (E) Whole bone rudiments were stained for β-galactosidase activity with X-gal. Infected cells appear blue. (F) Adenovirus infection was also visualized by whole-mount immunocytochemistry with a fluorescence-conjugated antibody directed to the adenovirus coat protein. Infected cells appear green. Staining only in the plane of focus is visible resulting in dark areas at either end of the bone. Scale bars: 250 μm in A-D; 180 μm in E,F.
It has previously been shown that the effects of Ihh on hypertrophic differentiation are dependent on PTHrP (Vortkamp et al., 1996; Lanske et al., 1996). It has also been shown that the effects of TGFβ1 on hypertrophic differentiation are dependent on PTHrP (Serra et al., 1999). Therefore, we tested the hypothesis that regulation of Pthrp expression by Shh is dependent on TGFβ signaling. Pthrp mRNA was localized by in situ hybridization in sections of metatarsal cultures that were infected with β-gal or DNIIR viruses then treated with or without Shh (Fig. 6E-H). Little to no Pthrp was detected in the absence of Shh in bone rudiments infected with either virus (Fig. 6E,G). As expected, Pthrp expression was detected in the perichondrium, a subset of cells in the hypertrophic zone, and the periarticular region of bones infected with the control β-gal virus and treated with Shh (Fig. 6F); however, in bones infected with DNIIR virus and treated with Shh, Pthrp was not detected (Fig. 6H). The data indicate that TGFβ signaling in the perichondrium is required for Shh-mediated inhibition of hypertrophic differentiation and stimulation of Pthrp expression and suggest that TGFβ acts as a signal relay between Ihh and PTHrP to regulate the rate of chondrocyte differentiation.

**TGFβ2 is required for the effects of Shh on hypertrophic differentiation**

The DNIIR used in the above experiments blocks signaling by all three TGFβ isoforms (Chen et al., 1993; Brand et al., 1993; Brand and Schneider, 1995). As we had shown that treatment with Shh resulted in increased Tgfb2 and Tgfb3 mRNA levels in the perichondrium, we tested the hypothesis that these specific isoforms of TGFβ are required for Shh-mediated inhibition of hypertrophic differentiation. To this end, embryonic metatarsals from crosses of Tgfb2+/− or Tgfb3+/− mice were isolated and grown in organ culture. Metatarsals from Tgfb2- or Tgfb3-null mice were left untreated or treated with Shh for 5 days and compared with untreated or Shh-treated littermates with the TGFβ alleles intact (Fig. 7). The level of hypertrophic differentiation was measured as the area of cartilage expressing Col10a1 mRNA (Fig. 7A-H). In rudiments from embryos with wild-type Tgfb3 allele, treatment with Shh resulted in a decrease in the area of cartilage expressing Col10a1 (Fig. 7A,B). In rudiments from embryos with both Tgfb3 alleles disrupted, treatment with Shh also resulted in a decrease in the expression domain of Col10a1 (Fig. 7C,D), suggesting that TGFβ3 specifically is not required for Shh-mediated inhibition of hypertrophic differentiation. By contrast, the expression domain of Col10a1 was not reduced in cultures from Tgfb2-null embryos after treatment with Shh (Fig. 7G,H) while expression was reduced, as expected, in the cultures from Tgfb2-positive mice (Fig. 7E,F). This result suggests that TGFβ2 is required to mediate the effects of Shh on hypertrophic differentiation. As TGFβ2 specifically was required for Shh to inhibit hypertrophic differentiation, we tested the hypothesis that TGFβ2 is also required for Shh-mediated stimulation of Pthrp mRNA expression (Fig. 7I-L). In cultures from Tgfb2-positive embryos, treatment with Shh resulted in increased Pthrp mRNA levels in the perichondrium, the periarticular cartilage, and in a subset of hypertrophic cells when compared with untreated Tgfb2-positive rudiments (Fig. 7I,J). PTHrP mRNA was not detected in sections from metatarsals from Tgfb2-null mice either treated or untreated with Shh.
Hedgehog and TGFβ signaling in cartilage

DISCUSSION

Metatarsal organ culture model

The hypothesis that TGFβ signaling in the perichondrium is required to mediate the effects of Hedgehog on hypertrophic differentiation was tested using an embryonic mouse metatarsal organ culture model. Although Ihh is expressed in the developing cartilage, Shh was used in these experiments as a functional substitute for Ihh. Shh has biological properties that are similar to those of Ihh and has previously been used as a substitute for Ihh in many studies (Vortkamp et al., 1996; Yang et al., 1998; Zhang et al., 2001; Pathi et al., 2001). Recently, a comparison of the biological response to Shh, Ihh and desert hedgehog showed that all three proteins could inhibit hypertrophic differentiation in hind limb organ cultures (Pathi et al., 2001). Differences were observed in the potency of each protein with Shh being the most potent suppressor of hypertrophic differentiation. In this report, we show that, as expected, Shh inhibits hypertrophic differentiation and stimulates Pthrp expression, a role normally played by Ihh in endochondral bone formation (Vortkamp et al., 1996; St-Jacques et al., 1999).

Fig. 7. Col10a1 and Pthrp expression in Tgfb2- and Tgfb3-null cultures. Col10a1 (A-H) expression was localized by in situ hybridization to sections from metatarsal rudiments from Tgfb3-positive (A,B) and Tgfb3-null (C,D) litters or Tgfb2-positive (E,F) and Tgfb2-null (G,H) litters that were untreated (A,C,E,G) or treated with 2 μg/ml Shh (B,D,F,H) for 5 days. Col10a1 expression was reduced in Tgfb3-positive and Tgfb3-null cultures after treatment with Shh (A-D). Shh treatment also reduced the expression domain of Col10a1 in Tgfb2-positive bone rudiments; however, there was no reduction in Col10a1 expression in bone rudiments from Tgfb-null mice after treatment with Shh (E-H). Only dark field images are shown for Col10a1 in situ hybridization. Pthrp (I-L) expression was localized using in situ hybridization to sections from metatarsal cultures derived from Tgfb3-positive (I,J) and Tgfb2-null (K,L) embryos. Cultures were either left untreated (I,K) or were treated with 2 μg/ml Shh (J,L) for 5 days. As observed previously, Pthrp mRNA was detected in the periarticular region, the perichondrium and a subset of hypertrophic cells in Tgfb2-positive cultures that had been treated with Shh (J). Little to no hybridization was detected in untreated cultures from Tgfb2-positive and -null embryos (I,K), as well as in cultures from Tgfb2-null embryos treated with Shh (L). Both bright field (I-L) and dark field (I-L’) images are shown for PTHrP in situ hybridization. Scale bar: 330 μm.

(Tig. 7K,L). This result suggests that TGFβ2 is required for Shh-mediated regulation of PTHrP expression.
on hypertrophic differentiation are dependent on PTHrP, while the effects of Ihh on chondrocyte proliferation are independent of PTHrP (Karp et al., 2000). In addition, TGFβ has PTHrP-dependent and -independent effects on endochondral bone formation (Serra et al., 1999). TGFβ-mediated inhibition of hypertrophic differentiation requires PTHrP but regulation of proliferation is independent of PTHrP. In light of the complexity of endochondral bone formation, it is not surprising that secreted signaling proteins would regulate several aspects of skeletal development, and participate in several independent and interdependent signaling cascades.

**Role of the perichondrium**

Removal of the perichondrium resulted in the inability of Shh to inhibit hypertrophic differentiation indicating an important role for the perichondrium in this response. Previously, it has been shown that both cartilage proliferation and differentiation are regulated by the perichondrium in chick tibiotarsus cultures (Long and Linsenmayer, 1998). Addition of PTH to the cultures reversed the effects of removing the perichondrium on hypertrophic differentiation but not on proliferation, suggesting two independent signaling pathways regulating growth and differentiation. Expression of Ptc1 and Gli1, two downstream targets of Hedgehog signaling, are stimulated in the perichondrium of chick limbs infected with an Ihh-expressing retrovirus, suggesting that the effects of Ihh are indirect and mediated by the perichondrium (Vortkamp et al., 1996). Recently, Ptc1 was detected in prehypertrophic chondrocytes and it was suggested that these immature chondrocytes could be the direct targets of Ihh action (St-Jacques et al., 1999). Our data support the model in which the perichondrium mediates the effects of Hedgehog on hypertrophic differentiation, but do not exclude the possibility that Ihh acts directly on chondrocytes to regulate proliferation.

Treatment with Shh stimulated expression of Tgfb2 and Tgfb3 mRNAs in the perichondrium. Previously we have shown that the effects of TGFβ1 on both proliferation and hypertrophic differentiation are dependent on the perichondrium (Alvarez et al., 2001). We have also demonstrated that TGFβ1 stimulates Pthrp mRNA in the perichondrium and that TGFβ-mediated inhibition of hypertrophic differentiation requires PTHrP, presumably synthesized from the perichondrium (St-Jacques et al., 1999). Our data support the model in which the perichondrium mediates the effects of Hedgehog on hypertrophic differentiation, but do not exclude the possibility that Ihh acts directly on chondrocytes to regulate proliferation.

Members of the TGFβ superfamily as signal relays downstream of hedgehog signaling

Previously, it was reported that misexpression of an activated BMP type IA receptor in chick limbs resulted in a delay in hypertrophic differentiation and increased PTHrP expression (Zhou et al., 1997). It was proposed that BMP could act as the signal relay downstream of Ihh signaling. In support of this model, it was subsequently shown that Bmp2 and Bmp4 expression were induced in the perichondrium by Ihh (Pathi et al., 1999). By contrast, several groups using exogenous BMPs, dominant-negative receptors and BMP antagonists in various model systems have suggested that BMPs act to promote hypertrophic differentiation (Leboy et al., 1997; Enomoto-Iwamoto et al., 1998; Pathi et al., 1999) most likely through BMP receptor type IB (Volk et al., 2000). Furthermore, Haaijman et al. have reported that BMP7 does not regulate Pthrp expression in mouse metatarsal organ cultures, and that the effects of BMP7 on hypertrophic differentiation are independent of PTHrP (Haaijman et al., 1999). More recently, it has been shown that noggin, and antagonist of BMP activity, cannot override the effects of Ihh on hypertrophic differentiation in limbs from transgenic mice that misexpress Ihh under the control of the type II collagen promoter (Minina et al., 2001). The later data taken together suggest BMP does not act as a secondary signal in hedgehog-mediated regulation of differentiation and PTHrP expression. The discrepancies may be due to the methods used. In tissue infected with activated BMP receptor IA, signaling is initiated in cells that do not normally respond to BMP or do not normally respond through the type IA receptor. In cultures treated with exogenous ligand, BMP antagonists or dominant-negative receptor, only cells that normally express BMP receptors and signaling components are affected. In addition, there are differences in the timing of treatment. Chick limbs are infected with the active receptor and essentially treated with BMP at an earlier stage of endochondral bone formation than mouse organ cultures or chick sternal chondrocytes. Our data support a model where TGFβ2 acts as at least one of the signals downstream of Ihh and upstream of PTHrP to regulate hypertrophic differentiation. First, TGFβ stimulates Pthrp expression and PTHrP is required for the effects of TGFβ on chondrocyte differentiation (Serra et al., 1999). Second, Shh stimulates expression of Tgfb2 and Tgfb3 mRNA. Third, intact TGFβ signaling is required for Shh to inhibit hypertrophic differentiation and stimulate Pthrp expression. Finally, in the absence of TGFβ2, differentiation is not inhibited by Shh.

Mice with targeted deletion of the Tgfb2 gene demonstrate several skeletal defects, including shortened long bones (Sanford et al., 1997). Skeletal defects were not observed in newborn Tgfb1- or Tgfb3-null mice (Shull et al., 1992; Kulkarni et al., 1993; Proetzel et al., 1995; Kaartinen et al., 1995). We did not detect any differences in the overall length or area of hypertrophic cartilage in metatarsal bones from E15.5 day wild-type and Tgfb2- or Tgfb3-null mice. This suggests that the effect of losing TGFβ2 on the length of the long bones occurs later in gestation. Alternatively, the effect of losing TGFβ2 in vivo may be specific for certain skeletal elements as the length of the metatarsal was not specifically examined in the previous study (Sanford et al., 1997). The etiology of shortened bones in the Tgfb2-null mice was not determined and may be due to alterations in growth, differentiation or both. Previously, we have demonstrated that dominant-negative interference of TGFβ signaling in mouse perichondrium resulted in increased hypertrophic
differentiation in mice after birth (Serra et al., 1997). As the dominant-negative receptor used in that study blocks all three isoforms of TGFβ, we were not able to determine the specific ligand involved. Based on our experiments in organ culture, we speculate that loss of TGFβ2 signaling at least partially disrupts the normal feedback loop that regulates hypertrophic differentiation.

The skeletal phenotype of Tgfβ2-null mice is far less severe than that observed for Ihh- or Pthrp-null mice (Karaplis et al., 1994; St-Jacques et al., 1999). This observation suggests that TGFβ2 is not the only downstream target of Ihh and that other factors can regulate expression of PTHRp. Ihh has been shown to regulate several steps in endochondral bone formation and it is likely to have both TGFβ-dependent and TGFβ-independent effects. It is also possible that other factors can compensate for TGFβ2 in the null mice. However, it has previously been shown that targeted disruption of Tgfβ1 in the skin resulted in delayed rather than increased expression of the other TGFβ isoforms after wounding (Crowe et al., 2000). Because the other isoforms were not able to compensate for loss of TGFβ2 in the organ culture experiments presented here, we would predict that no compensation occurs. However, if there is any compensation in bone, it would occur later in development.

Endochondral bone formation requires several complex signaling cascades. Crosstalk within and between these cascades is likely and would allow coordination of growth and differentiation required to form the correct bone shape and length. Elucidation of the interactions between different differentiation required to form the correct bone shape and length. Elucidation of the interactions between different signaling pathways and cell types within the skeleton will ultimately improve our understanding of skeletal disease and our ability to treat it.

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