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# The Perichondrium Plays an Important Role in Mediating the Effects of TGF-β1 on Endochondral Bone Formation

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***ABSTRACT*** **Endochondral bone formation is complex and requires the coordination of signals from several factors and multiple cell types. Thus, chondrocyte differentiation is regulated by factors synthesized by both chondrocytes and cells in the perichondrium. Previously, we showed that expression of a dominant-negative form of the transforming growth factor β (TGF-β) type II receptor in perichondrium/periosteum resulted in increased hypertrophic differentiation in growth plate chondrocytes, suggesting a role for TGF-β signaling to the perichondrium in limiting terminal differentiation in vivo. Using an organ culture model, we later demonstrated that TGF-β1 inhibits chondrocyte proliferation and hypertrophic differentiation by two separate mechanisms. Inhibition of hypertrophic differentiation was shown to be dependent on Parathyroid hormone-related peptide (PTHrP) and expression of PTHrP mRNA was stimulated in the perichondrium after treatment with TGF-β1. In this report, the hypothesis that the perichondrium is required for the effects of TGF-β1 on growth and/or hypertrophic differentiation in mouse metatarsal organ cultures is tested. Treatment with TGF-β1 inhibited expression of type X collagen mRNA in metatarsal cultures with the perichondrium intact. In contrast, hypertrophic differentiation as measured by expression of Type X collagen was not inhibited by TGF-β1 in perichondrium-free cultures. TGF-β1 added to intact cultures inhibited BrdU incorporation in chondrocytes and increased incorporation in the perichondrium; however, TGF-β1 treatment stimulated chondrocyte proliferation in metatarsals from which the perichondrium had been enzymatically removed. These results suggest that the TGF-β1-mediated regulation of both chondrocyte proliferation and hypertrophic differentiation is dependent upon the perichondrium. Thus, one or several factors from the perichondrium might mediate the way chondrocytes respond to TGF-β1.**


**Key words: cartilage; perichondrium; TGF-β; chondrocyte differentiation; growth**

## INTRODUCTION

Endochondral bone develops through a complex process whereby a cartilage model is replaced with bone (Cancedda et al., 1995; Erlebacher et al., 1995; Hill and Logan, 1992). Early in skeletal development, the cartilage template is formed from undifferentiated mesenchymal cells, which condense and differentiate into chondrocytes forming the initial shape of the bone. Spindle-shaped cells at the periphery of the condensation form a sheath around the cartilage and become the perichondrium. Shortly after the condensations form, chondrocytes progress through a program of cell proliferation, maturation, and hypertrophy. At this last stage, the cells exhibit a number of changes (Nurminskaya and Linsenmayer, 1996) including synthesis of Type X collagen (Schmid and Conrad, 1982; Schmid and Linsenmayer, 1983). These events change the composition and, conceivably, the properties of the cartilage matrix in this zone (Chen et al., 1992) allowing the invasion of blood vessels and the ultimate replacement of cartilage matrix by bone and a marrow cavity. The rate of chondrocyte differentiation has to be strictly regulated both temporally and spatially by a variety of molecules so that the proper shape and length of the bone is achieved and maintained.

The perichondrium that surrounds the cartilage rudiment is thought to synthesize essential factors that regulate endochondral bone formation. Previous findings using chick tibiotarsus cultures have shown that the perichondrium can elaborate signals that negatively regulate both chondrocyte proliferation and differentiation (Long and Linsenmayer, 1998). Similarly, Haaijman et al., (1999) demonstrated that removal of the periarticular region of mouse metatarsal cultures resulted in increased hypertrophic differentiation. Furthermore, work by Vortkamp et al. (1996) support the role of Ihh signaling to the perichondrium in regulating chondrocyte hypertrophy.

Control of skeletal development and maintenance is complex and likely involves coordination of signals from several factors. Among these bone-forming regulatory factors, the transforming growth factor-β (TGF-β) superfamily has raised considerable interest. Members of the TGF-β superfamily are secreted sig-

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naling molecules that regulate many aspects of growth and differentiation (Massague, 1998; Moses and Serra, 1996; Roberts and Sporn, 1990). This family includes several TGF-β isoforms, growth and differentiation factors (GDFs), the activin and inhibins, and the bone morphogenetic proteins (BMPs). TGF-β1, 2, and 3 mRNA are synthesized in the mouse perichondrium and periosteum from 13.5d post-coitum (p.c.) until after birth (Gatherer et al., 1990; Pelton et al., 1990; Millan et al., 1991; Sandberg et al., 1988). In addition, TGF-β receptor proteins have been detected in the perichondrium (Serra et al., 1999).

Previously, we demonstrated that dominant-negative interference of TGF-β signaling in the periosteum/perichondrium, articular cartilage, lower hypertrophic zone of the growth plate, and synovium in transgenic mice result in increased hypertrophic differentiation in both growth plate and articular chondrocytes (Serra et al., 1997). This result indicated that TGF-β is one of the factors that prevents hypertrophic differentiation of chondrocytes in vivo and suggested a role for TGF-β signaling to the perichondrium in this regulation (Serra et al., 1997). Recently, we demonstrated that TGF-β1 inhibits both growth and hypertrophic differentiation in mouse embryonic metatarsal organ cultures and that TGF-β1 acts upstream of PTHrP to regulate hypertrophic differentiation (Serra et al., 1999). Furthermore, we showed that TGF-β1 induced expression of PTHrP mRNA primarily in the perichondrium, suggesting that the effect of TGF-β1 on hypertrophic differentiation was indirect. The question of whether the effect of TGF-β1 on chondrocyte growth was direct or indirect and mediated through the perichondrium was not addressed.

In this study, we have experimentally examined the role of the perichondrium on TGF-β1-mediated regulation during cartilage growth and hypertrophic differentiation. To this end, embryonic metatarsals in which the perichondrium had been removed were placed in organ culture and treated with TGF-β1. Growth and hypertrophic differentiation were compared in intact and perichondrium-free cultures. Our results indicate that TGF-β1-mediated effects are critically dependent upon the perichondrium.

## RESULTS

### Expression of Cartilage Differentiation Markers in TGF-β1 and PTHrP-Treated Metatarsal Cultures

Previously, we showed that PTHrP is required for the effects of TGF-β1 on terminal chondrocyte differentiation and we proposed that TGF-β1 and PTHrP act at the same point to regulate endochondral bone formation (Serra et al., 1999). To test this hypothesis in more detail, we studied the effect of TGF-β1 and PTHrP on the expression of three markers of cartilage differentiation. In situ hybridization experiments were performed using sections from embryonic metatarsal bone rudiments grown in organ culture and treated with TGF-β1 and PTHrP (Fig. 1). Type X collagen transcripts were detected in hypertrophic chondrocytes, but not in proliferating and resting chondrocytes or in the perichondrium. Less hybridization was detected in the center of the bone rudiment. The chondrocytes are most terminally differentiated and the matrix begins to mineralize in this area of the bone. Although Type X collagen mRNA is only detected in a subset of histologically hypertrophic cells, we and others (Schmid and Linsenmayer 1987; Serra et al., 1999) have shown that Type X collagen protein is detected throughout the hypertrophic zone. In contrast to untreated cultures, expression of Type X collagen in rudiments treated with TGF-β1 (Fig.1B) or PTHrP (Fig.1C) was restricted to cartilage in the center of the metatarsal rudiments. In these samples, the distance between the edges of Type X collagen mRNA expression was less than in controls, indicating a reduction in the hypertrophic zone.

We also examined the expression pattern of bone morphogenetic protein-6 (BMP-6). BMP-6 mRNA expression was detected in a few prehypertrophic chondrocytes at the border of the prehypertrophic and hypertrophic zones. Most of the BMP-6 mRNA detected was localized to the beginning part of the hypertrophic zone (Fig. 1D). In TGF-β1 and PTHrP-treated cultures, BMP-6 mRNA was expressed through the center of the rudiment, suggesting this area contains cells that are in the early stages of hypertrophic differentiation (Figs. 1E and F).

Next, we looked at the expression domain of a marker for prehypertrophic cells. PTH receptor expression was observed in prehypertrophic chondrocytes in control rudiments (Fig. 1G). Metatarsals treated with TGF-β1 and PTHrP (Fig. 1H,I) demonstrated an increase in the expression domain for PTH receptor. Hybridization was increased in the middle diaphyseal area of the rudiments indicating presence of a large pool of prehypertrophic chondrocytes and suggesting a delay in the conversion from the prehypertrophic to the hypertrophic phenotype in both TGF-β1- and PTHrP-treated cultures. Taken together, the results observed for all three markers suggest that both TGF-β1 and PTHrP have similar effects blocking the terminal differentiation of chondrocytes at the same point. The data also support our hypothesis that TGF-β1 and PTHrP act in a common signaling cascade.

### Effects of TGF-β1 on the Morphology and Histology of Intact and Perichondrium-Free Metatarsals

Endochondral bone formation during vertebrate embryogenesis is a highly regulated process resulting in increased bone length. Furthermore, any alteration of the appropriate regulation of cell differentiation like those induced by TGF-β1 can result in defects in bone development. Previously, we showed that PTHrP was stimulated by TGF-β1 in the perichondrium (Serra et al., 1999). In the previous study, mouse tissue was

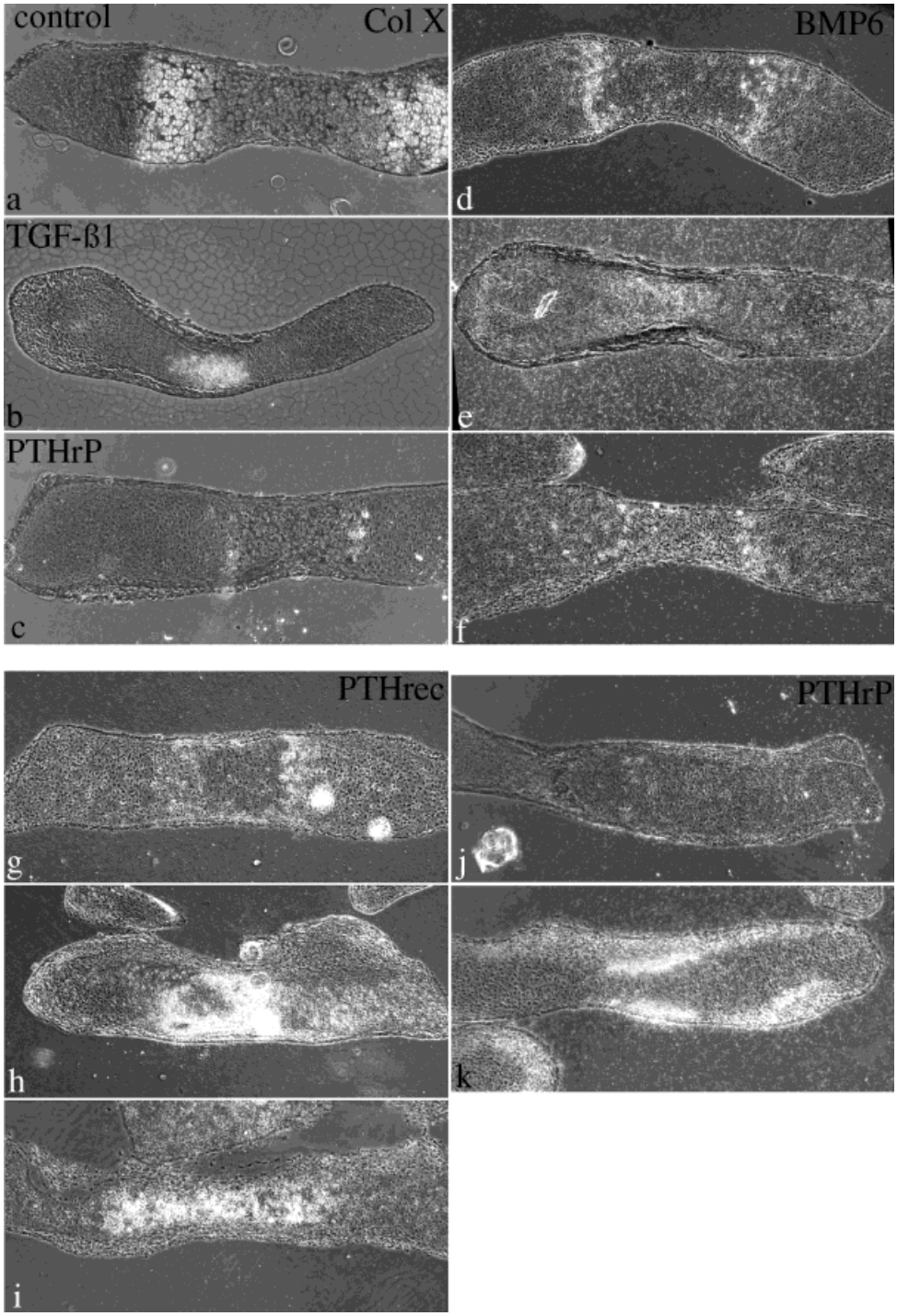


Fig. 1. Effects of TGF-β1 and PTHrP on Col X, BMP6, PTH receptor, and PTHrP expression patterns. In situ hybridization of control metatarsal rudiments grown in the absence of growth factors (a,d,g,j), or in the presence of 10 ng TGF-β1 /ml (b,e,h,k) or $10^{-5}$ M PTHrP (c,f,i) are shown. Images were captured using a SPOT digital camera. Phase contrast and bright field images were superimposed using Photoshop software. Hybridization is seen as bright white grains on the gray phase contrast background. **a–c:** In situ hybridization with Type X collagen antisense probe. Treatment with TGF-β1 and PTHrP resulted in a decreased in the domain of cartilage synthesizing Type X collagen mRNA when compared to untreated controls. **d–f:** In situ hybridization with BMP6 antisense probe. BMP6 mRNA was localized in untreated cultures to a few prehypertrophic chondrocytes at the transition between the prehypertrophic and hypertrophic zones. Most BMP6 mRNA was localized to early hypertrophic cells at the beginning of the histologically hypertrophic zone. BMP6 mRNA was restricted to the center of TGF-β1 and PTHrP treated bones. **g–i:** In situ hybridization with PTH receptor antisense probe. PTH receptor mRNA was localized to prehypertrophic chondrocytes in untreated cultures. Treatment with TGF-β1 and PTHrP resulted in an expansion in the expression domain of PTH receptor expression so that it was expressed across the center of the bone rudiment. **j,k:** In situ hybridization with PTHrP antisense probe. Weak PTHrP expression was detected in the perichondrial region and a few nonhypertrophic chondrocytes in untreated rudiments. TGF-β1-treated cultures showed a dramatic increase of PTHrP expression in the perichondrium.

hybridized to a riboprobe made from a rat cDNA. To confirm the localization of PTHrP mRNA, in situ hybridization was performed on sections from untreated and TGF-β1 treated bone using a riboprobe made from mouse PTHrP cDNA (obtained from Tom Clemens, University of Cincinnati). The expression of PTHrP was detected only weakly in perichondrial cells and in chondrocytes in control samples (Fig. 1J). By contrast, a high level of PTHrP expression was found in rudiments treated with TGF-β1. In these samples, expression of PTHrP was mainly located in the perichondrium, being especially marked at the diaphyseal zone (Fig. 1K).

Since TGF-β1 requires PTHrP for its effects on chondrocyte differentiation (Serra et al., 1999) and PTHrP is expressed primarily in the perichondrium, the hypothesis that the perichondrium is required to mediate the effects of TGF-β1 on development of the metatarsal cultures was tested. To examine the role of perichondrium on the signaling pathway of TGF-β1, the perichondrium was stripped off the entire length of the metatarsal rudiments before culturing. Metatarsals with intact perichondrium were used as controls. There were no statistically significant differences in overall length of control or perichondrium-free rudiments after 5 days in culture (Fig. 2B). In addition, there were no significant differences in the length of the hypertrophic zone as indicated by the length of the "clear" area in the center of the bone rudiments (described in Dieudonne et al., 1994; Fig. 2C). Treatment with TGF-β1 for 5 days normally results in a decrease in the overall length of the metatarsal bone and a decrease in the "clear" area in the center of the bone that represents the hypertrophic zone when compared to untreated cultures (Dieudonne et al., 1994; Serra et al., 1999). We examined the morphology of intact and perichondrium-free cultures to compare the effects of TGF-β1 on the development of the metatarsal cultures in each condition (Fig. 2A). As expected, perichondrium-intact rudiments treated with TGF-β1 for 5 days exhibited considerably less longitudinal growth than untreated controls (Fig. 2B). Treatment with TGF-β1 also resulted in a decrease in the length of the hypertrophic zone (Fig. 2C). Moreover, the effects of TGF-β1 were dose dependent (Figs. 2A,a–c). By contrast, the perichondrium-free cultures did not show statistically significant alterations in overall length or in the length of the hypertrophic zone when they were treated with TGF-β1 at 1 or 10 ng/ml for 5 days (Fig. 2A,d–f; B,C). These results suggest that the effects of TGF-β1 on both longitudinal growth and hypertrophic differentiation require the perichondrium.

Because it is difficult to measure the hypertrophic zone ("clear" area) in intact metatarsal cultures treated with TGF-β1, the effects of TGF-β1 on the histology of intact and perichondrium-free cultures was characterized. As previously shown (Dieudonne et al., 1994; Serra et al., 1999), in addition to their decreased total length, the perichondrium-intact rudiments treated with TGF-β1 showed a considerable decrease in the area of the total cartilage containing histologically hypertrophic cells as compared to untreated controls (Fig. 3A,a,b; B). In addition, no statistically significant differences in the area of histologically hypertrophic cartilage were observed between intact and perichondrium-free cultures (Fig. 3A,a,c; B). In metatarsal rudiments where perichondrium was removed (Fig. 3A, c,d), treatment with 10 ng/ml TGF-β1 did not have a marked effect on the amount of hypertrophic cartilage in the metatarsal bone. It was noted, however, that perichondrium-free cultures were wider than the intact cultures and the cells within the tissue were less tightly packed (Fig. 3C). The perichondrium may provide physical support to the organ culture that is lost when it is removed, resulting in the loose histology observed. In addition, TGF-β1 treatment in perichondrium-free cultures resulted in a decrease of the degree of structural anisotropy. As a result, boundaries between resting, proliferating, and hypertrophic zones appeared poorly defined and were very difficult to distinguish (Fig. 3D). Nevertheless, there was not a significant decrease in the amount of histologically hypertrophic cartilage observed in TGF-β1-treated perichondrium-free cultures relative to the untreated culture. Therefore, these results suggest that the perichondrium is required for TGF-β1's effect on hypertrophic differentiation.

### Expression of Type X Collagen in Perichondrium-Free Cultures

Since TGF-β1 treatment of perichondrium-free metatarsal cultures did not result in a dramatic decrease in hypertrophic cartilage observed in hematoxylin and eosin stained sections, expression of Type X collagen, a marker for hypertrophic differentiation, was used to more clearly define the hypertrophic zone (Fig. 4). As described above, Type X collagen transcripts were detected in hypertrophic chondrocytes but less hybridization was detected in the center of the bone (Fig. 4A,a). Therefore, the hypertrophic zone was measured as the area between the two outer boundaries of the Type X collagen mRNA expression domain. No significant differences in the area of hypertrophic cartilage in intact and perichondrium-free cultures were detected (Fig. 4A,a,c; B). Treatment with TGF-β1 resulted in a dramatic decrease in the expression level of Type X collagen, which was restricted to the very center of the bone rudiment (Fig. 4A,a,b). Unlike intact rudiments, the expression of Type X collagen in perichondrium-free rudiments was not significantly modified by TGF-β1 treatment (Fig. 4A,c,d; B). In contrast to what was observed in hematoxylin and eosin stained sections, the boundaries of Type X collagen mRNA expression were clearly demarcated in the TGF-β1-treated, perichondrium-free cultures. These data support the hypothesis that the perichondrium is required for TGF-β1's effects on terminal differentiation.

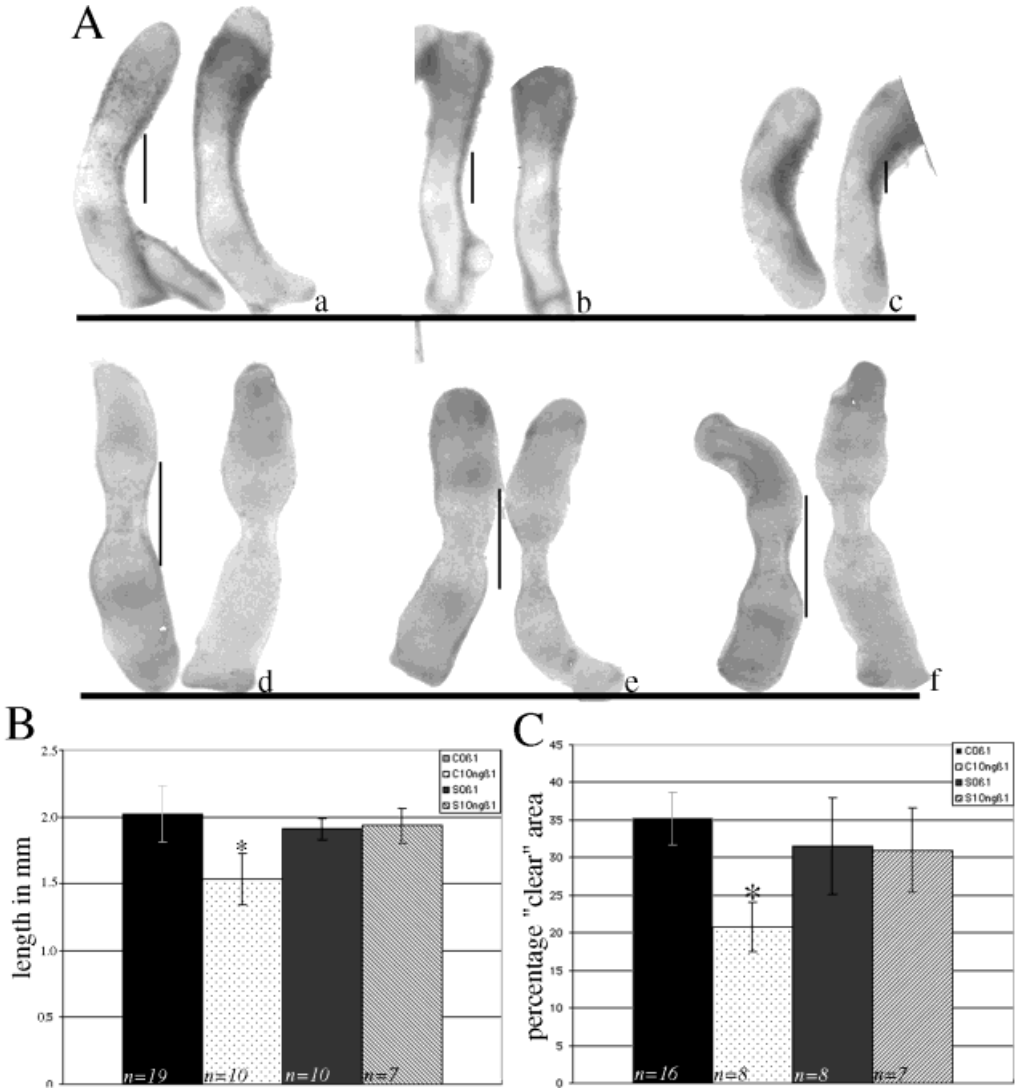


Fig. 2. Effects of TGF-β1 on perichondrium-intact and perichondrium-free metatarsals. **A:** Morphology of metatarsal cultures. Perichondrium-intact rudiments were either untreated (**a**) or treated with 1ng TGF-β1 /ml (**b**) or 10 ng TGF-β1 /ml (**c**) for 5 days. Perichondrium-free rudiments treated with 1 ng/ml TGF-β1 (**e**) or 10 ng/ml TGF-β1 (**f**) were compared to untreated perichondrium-free rudiments (**d**). Lines represent the approximate length of the "clear" area representing the hypertrophic zone in several of the rudiments pictured. **B:** Metatarsal length. The length of each bone rudiment was measured in mm. Each column represents the mean of several samples (n=x) indicated at the bottom of each column. Error bars represent the standard deviation. Significance was calculated using Student's *t*-test as a $P < 0.05$. There was a statistically significant decrease in bone length in intact cultures treated with TGF-β1 (C10ngβ1) when compared to untreated intact cultures (C0β1). No significant difference in length was detected in untreated perichondrium-free cultures (S0β1) when compared to the C0β1 group. No significant difference in length was detected in TGF-β1 treated perichondrium-free cultures (S10ngβ1) when compared to the S0β1 group. **C:** Hypertrophic zone. The percentage "clear" area in each bone rudiment was used as a measure of the amount of hypertrophic cartilage. The percent "clear" area was calculated as (the length of the "clear" area/the total length of the bone) × 100. Each column represents the mean percent of several samples (n=x) indicated at the bottom of each column. Error bars represent the standard deviation. Significance was calculated using a Student's *t*-test as a $P < 0.05$. There was a statistically significant decrease in the amount of hypertrophic cartilage in intact cultures treated with TGF-β1 (C10ngβ1) when compared to untreated intact cultures (C0β1). No significant difference in the amount of hypertrophic cartilage was detected in untreated perichondrium-free cultures (S0β1) when compared to the C0β1 group. No significant difference in the amount of hypertrophic cartilage was detected in TGF-β1 treated perichondrium-free cultures (S10ngβ1) when compared to the S0β1 group.

### TGF-β1 Has Perichondrium Dependent Effects on Chondrocyte Proliferation in Metatarsal Cultures

Previously, we showed that TGF-β1 inhibits DNA synthesis in chondrocytes of metatarsal bone rudiments grown in organ culture (Serra et al., 1999). We proposed that the decrease in cell proliferation was at least partially responsible for the decreased length of the bone rudiments observed after 5 days of treatment. We also proposed that the effects of TGF-β1 on growth

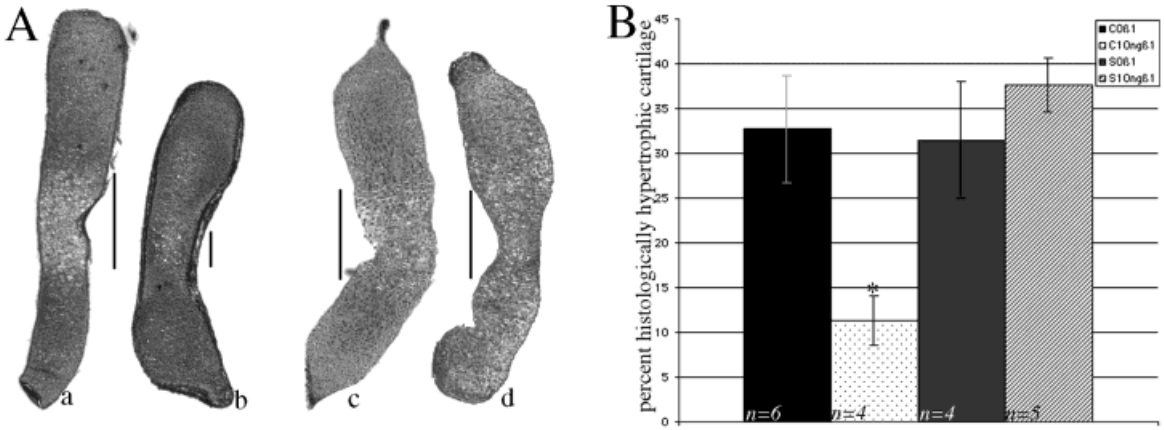


Fig. 3. Effects of TGF-β1 on perichondrium-intact and perichondrium-free metatarsal histology. **A:** Histology. Hematoxylin and eosin-stained sections of intact (**a,b**) and perichondrium-free rudiments (**c,d**) grown in the absence (a,c) or in the presence (b,d) of 10 ng TGF-β1/ml. Distinction between proliferating and hypertrophic cartilage was easily detected in control rudiments (a). In samples without the perichondrium, the demarcation between proliferating and hypertrophic zones was observed but the bone rudiment was wider and the cells appeared less tightly packed together as in controls (c). The cartilage appeared more disorganized in TGF-β1 treated perichondrium-free cultures (d) than in untreated perichondrium-free cultures. Lines represent the approximate length of the histologically hypertrophic zone. **B:** Percentage of histologically hypertrophic cartilage. The percent of cartilage that was histologically hypertrophic was calculated as (length of histologically hypertrophic cartilage/total length of section) × 100. Each column represents the mean and error bars represent the standard deviation. Significance was determined using the Student *t*-test as described above. TGF-β1 treatment of intact metatarsals (C10ngβ1) resulted in a significant ($*P < 0.05$) decrease in the fraction of histologically hypertrophic cartilage when compared to untreated intact controls (C0β1). No significant differences in the percent of hypertrophic cartilage were observed in TGF-β1 treated perichondrium-free cultures (S10β1) when compared to untreated perichondrium-free controls (S0β1) or in C0β1 cultures compared to S0β1 cultures.

were independent on PTHrP since longitudinal growth was inhibited by TGF-β1 in PTHrP-null cultures. Since the length of perichondrium-free bone cultures was not altered by treatment with TGF-β1, the hypothesis that the perichondrium is required for the effects of TGF-β1 on DNA synthesis in metatarsal organ cultures was tested. To test this hypothesis, intact and perichondrium-free cultures were left untreated or were treated with 10 ng TGF-β1/ml for 24 hr followed by an additional 2.5 hr of incubation with Bromo-deoxyUridine (BrdU). BrdU incorporation into DNA was detected by immunofluorescence (Fig. 5). In control cultures, BrdU-labeled cells were detected throughout the zones of histologically resting and proliferating cartilage as expected in embryonic skeletal tissue. Some labeled cells were also seen scattered in the perichondrium (Fig. 5A, arrowhead). TGF-β1 treatment of perichondrium-intact rudiments reduced chondrocyte proliferation as can be seen by the low number of BrdU-labeled cells in cartilage (Fig. 5B). Proliferation in cartilage was reduced by 2.5-fold in intact, TGF-β1 treated rudiments (Table 1). By contrast, these TGF-β1-treated rudiments showed a marked increase in the number of BrdU-labeled cells in the perichondrium compared with control cultures (Fig. 5A and B, arrowheads). Perichondrium-intact rudiments treated with TGF-β1 showed a 4-fold increase of the proliferation rate in the perichondrium (Table 1). There was no statistically significant alteration in BrdU incorporation in untreated chondrocytes from intact cultures when compared to perichondrium-free cultures (Fig. 5A,C; Table 1). After treatment with TGF-β1, perichondrium-free cultures demonstrated a statistically significant increase in BrdU incorporation (Fig. 5D; Table 1). These data suggest that the perichondrium is required for TGF-β1 to inhibit chondrocyte proliferation. Furthermore, the data suggest that a factor from the perichondrium can mediate the way chondrocytes respond to TGF-β1. In the absence of perichondrium, chondrocytes respond to TGF-β with increased DNA synthesis but in the presence of perichondrium DNA synthesis is inhibited.

## DISCUSSION

Embryonic metatarsal bone rudiments grown in organ culture were used to test the hypothesis that the perichondrium plays a key role in TGF-β1-mediated regulation of endochondral bone formation. Removal the perichondrium resulted in the inability of TGF-β1 to affect chondrocyte differentiation or proliferation. These data suggest that factors from the perichondrium can mediate the response to TGF-β1. Previous studies have provided evidence that the perichondrium participates in regulatory pathways that control the differentiation of chondrocytes. Removal of the perichondrium from chicken embryonic tibiotarsi cultures resulted in an extended zone of cartilage expressing Type X collagen, suggesting that a factor from the perichondrium regulates chondrocyte differentiation in a negative manner (Long and Linsenmayer, 1998). Addition of PTH to the stripped cultures reduced the area of cartilage expressing Type X collagen and raised the

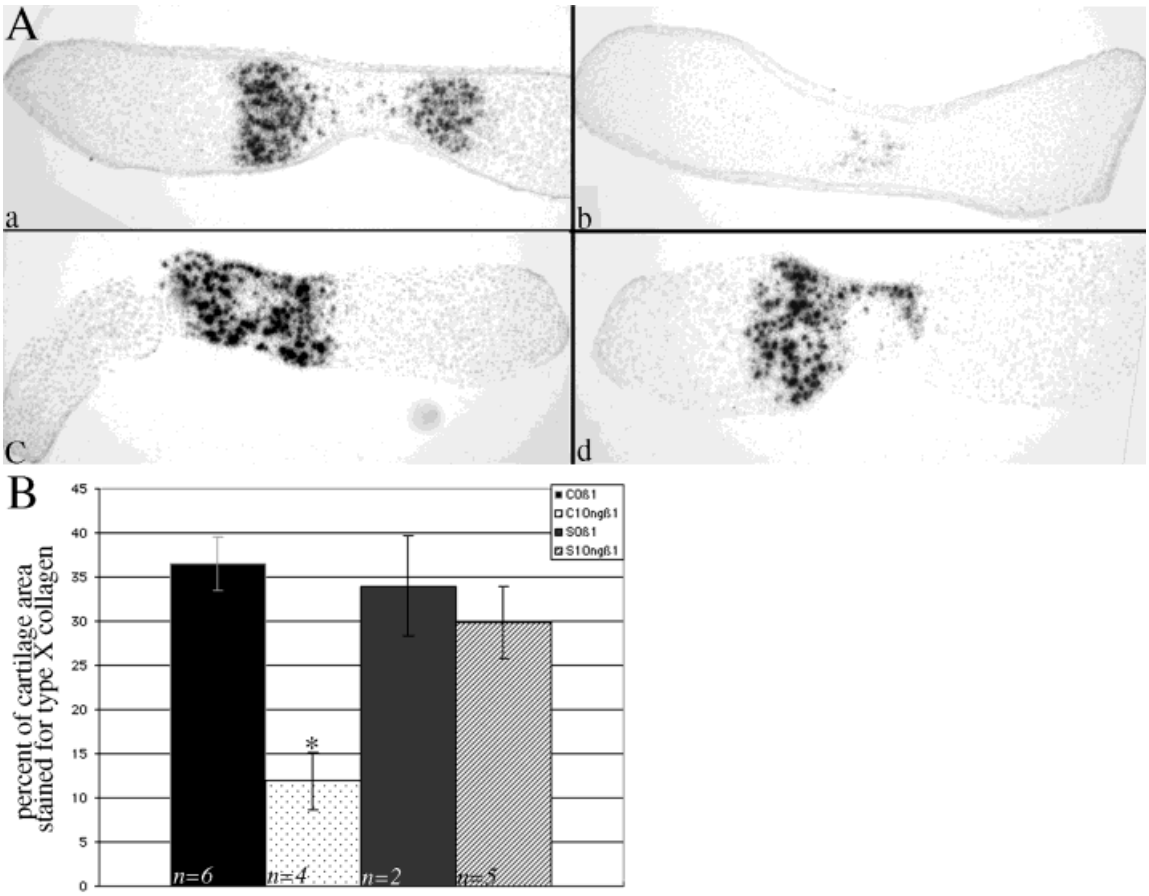


Fig. 4. Effects of TGF-β1 on type X collagen expression in intact and perichondrium-free cultures. **A:** In situ hybridization. Intact (**a,b**) and perichondrium-free rudiments (**c,d**) were untreated (a,c) or treated with 10 ng TGF-β1/ml (b,d). Sections were hybridized to a $^{35}$S-labeled riboprobe to Type X collagen. Type X collagen mRNA was detected in the hypertrophic chondrocytes at the beginning of the zone of histologically hypertrophic cartilage. Its expression was decreased in the central part of the hypertrophic zone (a). Sections from intact TGF-β1 treated demonstrated very little hybridization to the Type X collagen probe. Hybridization was restricted to the very center of the bone rudiment (b). Type X collagen mRNA was detected throughout the zone of histologically hypertrophic cartilage in both untreated and TGF-β1 treated perichondrium-free cultures (c,d). Bright field images are shown. **B:** Percentage of cartilage expressing Type X collagen. The percentage of cartilage expressing Type X collagen mRNA was calculated as (length between the outermost domains of type X collagen hybridization/total length of section) ×100. Each column represents the mean and standard deviations are shown. Significance was calculated using Student's *t*-test as described above. Treatment with TGF-β1 in perichondrium-intact metatarsals resulted in a significant ($*P < 0.05$) decrease in the percentage of cartilage expressing Type X collagen mRNA (C10ngβ1) when compared to untreated intact controls (C0β1). Removal of perichondrium did not significantly affect the expression pattern of type X collagen (C0β1 vs. S0β1). Treatment of perichondrium-free rudiments with TGF-β1 (S10ngβ1) showed no significant changes in the percent of cartilage that expressed Type X collagen mRNA when compared with untreated perichondrium-free rudiments (S0β1).

possibility that PTHrP could be one of the factors from the perichondrium that regulates hypertrophic differentiation (Long and Linsenmayer, 1998). In the current study, measurements of the area corresponding to the hypertrophic cartilage were systematically performed to determine the effect of removing the perichondrium on chondrocyte differentiation in embryonic mouse metatarsal cultures. No significant alteration in the extension of the morphologically defined area of hypertrophic cartilage or in the extension of the expression domain of Type X collagen were observed after removal of the perichondrium. Disparities between studies could be attributable to differences in the structure of the cartilage during prenatal ossification in mammalian and avian systems and to differences in the developmental stage of both types of cultures.

Removal of the perichondrium also resulted in an extended zone of chondrocytes incorporating BrdU in chicken embryonic tibiotarsi, indicating that the perichondrium negatively regulates the proliferation of chondrocytes as well as differentiation (Long and Linsenmayer, 1998). In the present study, changes in the percentage of chondrocytes incorporating BrdU were not observed in perichondrium-free rudiments compared with intact controls. As described above, addition of PTH to perichondrium-free tibiotarsus cultures reversed the expansion of the Type X collagen positive domain (Long and Linsenmayer, 1998). PTH, however,

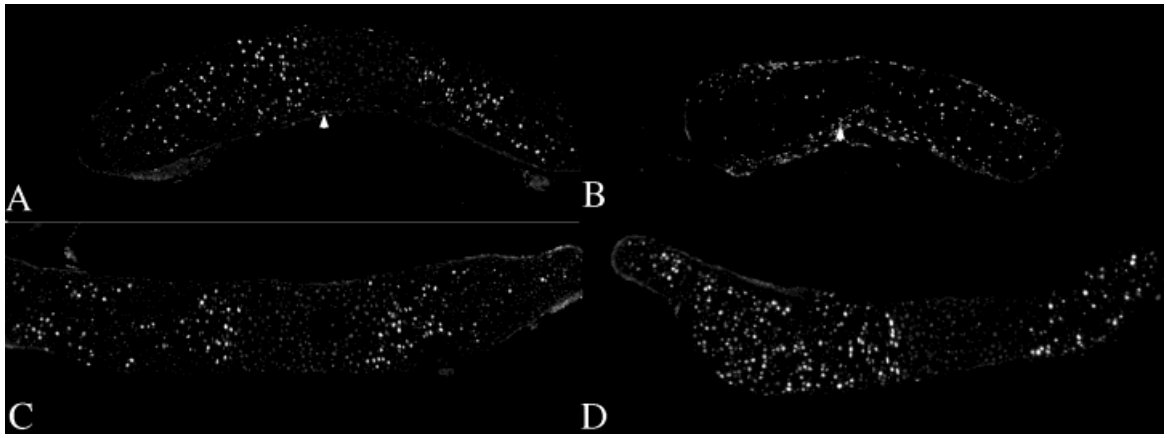


Fig. 5. BrdU labeling. Intact (**A,B**) and perichondrium-free (**C,D**) metatarsals either untreated (A,C) or treated with 10 ng TGF-β1/ml (B,D) for 24 hr were labeled with BrdU for an additional 2.5 hr. Labeled cells were detected using immunofluorescence. In untreated cultures, labeled cells were observed in the resting and proliferating cartilage and a few labeled cells were seen in the perichondrium (arrowhead, A). Few labeled cells were detected in the cartilage of TGF-β1 treated metatarsal cultures. However, many labeled cells could be detected throughout the perichondrium (arrowhead, B). There were no detectable differences in the number and distribution of labeled cells in cartilage between untreated intact and perichondrium-free metatarsal rudiments (A,C). Perichondrium-free samples treated with 10 ng/ml TGF-β1 exhibited an increased number of chondrocytes incorporating BrdU in cartilage, when compared to its untreated counterpart (D). See Table 1 for quantification of the results.

**TABLE 1. Percent BrdU Positive Cells[a]**

| | Cartilage | | Perichondrium | |
|---|---|---|---|---|
| | 0β | 10 ng/ml β1 | 0β | 10 ng/ml β1 |
| C | 17.97 ± 1.53 | 6.80 ± 1.70[b] | 12.96 ± 6.99 | 44.94 ± 6.92[e] |
| S | 17.48 ± 3.1[c] | 35.25 ± 4.14[d] | | |

[a]Each value is the mean of three or four samples ± standard deviation. C0β: intact untreated metatarsal rudiments. C10β: intact metatarsal rudiments treated with 10 ng TGF-β1/ml. S0β: stripped, perichondrium-free untreated metatarsal rudiments. S10β: stripped, perichondrium-free metatarsal rudiments treated with 10 ng TGF-β1/ml.
[b]C0β compared to C10β, $P = 0.0011$.
[c]C0β compared to S0β $P = 0.7952$.
[d]S0β compared to S10β $P = 0.0005$.
[e]$P < 0.05$ when compared to C0β.

failed to repress the expansion of the proliferating zone in perichondrium-free tibiotarsi. Thus, the regulation of chondrocyte differentiation by the perichondrium appears to be dependent upon the PTH receptor and the regulation of proliferation is likely independent of it. This is in agreement with the results observed in mouse metatarsal organ cultures where PTHrP inhibited hypertrophic differentiation but did not affect incorporation of BrdU (Serra et al., 1999).

The rate and extent of hypertrophic differentiation are thought to be regulated by Ihh and PTHrP, two secreted peptides (reviewed in Wallis, 1996). In this model, Ihh stimulates the expression of PTHrP in the periarticular region (Vortkamp et al., 1996). It was proposed that Ihh acted through the perichondrium since misexpression of Ihh resulted in induction of the expression of ptc and gli, two components of hedgehog signaling, in the perichondrium (Lanske et al., 1996; Vortkamp et al., 1996). Later it was proposed that Ihh acted to regulate PTHrP expression through an intermediary factor. Based on signaling cascades in other developmental systems (Heberlein et al., 1993; Ingham and Fietz, 1995; Laufer et al., 1994; Roberts et al., 1995) it was proposed that members of the TGF-β superfamily would act as a signal relay between Ihh and PTHrP to regulate endochondral bone formation. In support of this model, it was recently shown that misexpression of Ihh in chick limbs alters expression of specific bone morphogenetic proteins (BMPs) in the perichondrium (Pathi et al., 1999). Furthermore, misexpression of an activated BMP receptor in chick limbs resulted in induction of PTHrP mRNA and inhibition of hypertrophic differentiation (Zov et al., 1997). In contrast, Haaijman et al., (1999) observed that while treatment with BMP-7 inhibited hypertrophic differentiation, it did not stimulate expression of PTHrP mRNA in embryonic mouse metatarsal organ cultures. Furthermore, metatarsals from PTHrP-null mice were able to respond to BMP-7 indicating that PTHrP is not required for the BMP-7 effect. The authors went on to show that mouse metatarsals from which the perichondrium and/or periarticular region had been removed were not capable of responding to exogenous BMP-7, emphasizing the importance of these regions for the effects of BMP-7 on chondrocyte hypertrophy in vitro (Haaijman et al., 1999). Although both BMP-7 and TGF-β1 require the perichondrium for their effects on hypertrophic differentiation, the data suggest that TGF-β1 and BMP-7 regulate endochondral bone formation by different mechanisms (Haaijman et al., 1999; Serra et al., 1999). TGF-β1 requires PTHrP whereas BMP-7 does not. Whether or not TGF-β1 acts down-

stream of Ihh in this signaling cascade has not been determined.

Using mouse embryonic metatarsal bone rudiment cultures, we have shown that TGF-β1 inhibits not only terminal differentiation of chondrocytes but also chondrocyte proliferation (Serra et al., 1999). Here, we tested the hypothesis that the perichondrium is also required to mediate the action of TGF-β1 on this step of endochondral bone formation. Until now, it was unclear whether TGF-β1 acted directly or indirectly on chondrocytes to inhibit proliferation. The data suggest that TGF-β1 regulates growth indirectly through the perichondrium and that one or more unknown factors synthesized by perichondrial cells may be involved in this process. For example, fibroblast growth factor receptor 3 (FGFR3) is expressed in chondrocytes in the proliferating zone (Deng et al., 1996; Peters et al., 1993). FGFR3-null mice demonstrate enhanced and prolonged longitudinal bone growth (Deng et al., 1996). Conversely, overexpression of FGFR3 resulted in a decrease in endochondral bone growth; a result of a marked inhibition of chondrocyte proliferation (Naski et al., 1998). The results in the transgenic and "knock out" mice suggest that an undefined FGF ligand acting through this receptor inhibits chondrocyte proliferation in vivo. BMP4 and Patched signaling pathways, which are significantly downregulated in proliferating zone chondrocytes in response to activated FGFR3, must also be considered as potential regulators of chondrocyte proliferation in vivo (Naski et al., 1998). The relationship between the TGF-β and FGF signaling pathways in endochondral bone formation has not been determined although is known that TGF-β1 and -β2 in combination with bFGF exert synergistic effects on chondrogenesis in culture (Frenz et al., 1994).

In the present report, we have observed that treatment of perichondrium-free cultures with TGF-β1 enhanced chondrocyte proliferation approximately twofold. TGF-β1 has been shown to induce a dose-dependent increase in chondrocyte proliferation in Swarm rat chondrosarcoma cell cultures (Guerne and Lotz, 1991) and in primary human articular chondrocyte (Guerne et al., 1994). TGF-β1 was shown to be the most potent mitogen among a long list of chondrocyte regulatory factors. The presence of the perichondrium may account for some of the differences in TGF-β1 responses observed in chondrocyte cell cultures and in organ cultures. Our results suggest that a factor synthesized by cells in the perichondrium may modulate the effects of TGF-β1 on proliferation, enforcing the hypothesis that the perichondrium plays a key role in modulating the response to TGF-β1.

The present study suggests that the perichondrium mediates the effects of TGF-β1 on both the exit of chondrocytes from the cell cycle through an unknown factor and their subsequent differentiation through the PTHrP/PTH receptor signaling complex. Our results demonstrate that the perichondrium is required for TGF-β1 to inhibit both growth and hypertrophic differentiation. There are two possible models for the requirement of the perichondrium. The first is a linear pathway in which TGF-β1 regulates the expression of a factor in the perichondrium that, in turn, acts directly on chondrocytes. The second possibility is that parallel pathways exist in which TGF-β1 acts directly on chondrocytes but an additional factor from the perichondrium acting on chondrocytes is also required for the response. Future experiments will address these issues and provide further evidence of how signaling from several factors and multiple cell types are coordinated to build and maintain a functional skeletal system.

## EXPERIMENTAL PROCEDURES.

### Embryonic Metatarsal Rudiment Organ Cultures

The three central metatarsal rudiments were isolated from each hind limb of 15.5 d p.c. mouse embryos. 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 bones for 2 min at room temperature in 1 mg/ml collagenase type 2 (Worthington Biochemical Corp.) in phosphate-buffered saline (PBS), as previously reported (Haaijman et al., 1999; Thesingh and Burger, 1983). The enzyme activity was stopped by transferring the rudiments to 10% Fetal Bovine Serum in PBS and the remaining perichondrium was removed mechanically by rolling the bone rudiments over plastic (Haaijman et al., 1999; Thesingh and Burger, 1983). All rudiments were cultured in 24-well plates in 1 ml of medium containing alpha-MEM (Gibco-BRL) supplemented with 0.05 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 (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 or PTHrP (1-34) (Bachem) in 10 mM acetic acid containing 1%BSA was added to cultures 12–16 hr after dissection. Medium was changed on the third day of culture. Cultures were observed and photographed with an Olympus SZH12 dissecting microscope after 5 days of treatment. Metatarsal rudiments were fixed overnight at 4°C in fresh 4% paraformaldehyde and then dehydrated through a series of ethanols and xylene then embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin or used for immunodetection of BrdU incorporation and for in situ hybridization.

The length of bones was calculated by measuring the length of each metatarsal in photographs taken with an Olympus SZH12 dissecting microscope with camera attachment, then dividing the length measured in the photograph by the magnification indicated on the microscope and camera. The mean and standard deviation were calculated. The significance of differences in bone length was determined using Student's *t*-test.

Numbers with a probability value less than 0.05% ($P < 0.05$) were considered significantly different. The percent of the bone rudiment containing hypertrophic cartilage was calculated in a similar manner. Measurements of the total length of the bone rudiment and the length of the "clear" area on whole bones or the hypertrophic or Type X collagen containing areas in bone sections were taken from photographs from a SZH12 dissecting microscope or an Olympus BX-60 upright microscope. The percent of the total cartilage that was hypertrophic was calculated as: (length of hypertrophic zone/ total length) $\times 100$. Next the mean and standard deviation was calculated for each group. Significant differences were determined using Student's *t*-test. Significantly different results were indicated by $P < 0.05$.

### BrdU Labeling

Metatarsal rudiments were treated with 10 ng TGF-β1/ml for 24 hr followed by treatment with 10 μM BrdU (Boehringer Mannheim) for 2.5 hr. Metatarsal rudiments 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 2N 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 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 using Vectastain Elite staining kit (Vector Laboratories) as described by the manufacturer. A rat mAb 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, and the sections were immediately mounted with Aquapoly mount (Poly Sciences). Fluorescence was observed and imaged using an Olympus BX-60 upright microscope with a SPOT digital camera and Photoshop software. Labeled and unlabelled cells from sections of three to four separate bone rudiments were counted and the percent labeled nuclei was calculated for each group. Sagittal sections from near the center of the bone rudiment were selected for counting. Hypertrophic and periarticular cartilage were excluded from the area counted. The mean and standard deviation were calculated and significant differences between each group were calculated using Student's *t*-test. Results that were statistically different were determined by a $P$ value $< 0.05$.

### In Situ Hybridization

In situ hybridization was performed as described (Pelton et al., 1990). Metatarsal rudiments 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 (Type X collagen) to 2 weeks (others), then developed, fixed, and cleared. Sections were counterstained with 0.02% toluidine blue. Sections hybridized with a labeled-sense riboprobe were used as negative controls. No positive hybridization signal was found in negative controls. Phase contrast and dark field images were captured with a SPOT digital camera. Phase contrast and dark field images were superimposed using Photoshop software so that the bright grains of hybridization could be see on the gray phase contrast background.

Probes for in situ hybridization were as follows: The mouse Type X collagen probe (a gift from Dr. Bjorn Olsen, Harvard Medical School, Boston, MA) was a 650-bp Hind III fragment containing 360 bp of noncollagenous (NC1) domain and 260 bp of 3′-untranslated sequence of the mouse type X collagen gene, subcloned into the Hind III site of pBluescript (Apte et al., 1992). The mouse BMP-6 probe consisted of a 893-bp fragment of the BMP-6 gene, which was a gift from Dr. Brigid Hogan, (Vanderbilt University Medical School, Nashville, TN; Lyons et al., 1989). The mouse PTH receptor and PTHrP probes were the generous gift of Dr. Tom Clemens (University of Cincinnati Medical School). The PTHrec fragment used consisted of 366 bp from the 3′ end of the cDNA. The PTHrP probes was a 288-bp fragment of the cDNA.

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