TGF-β signaling is essential for joint morphogenesis

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Despite its clinical significance, joint morphogenesis is still an obscure process. In this study, we determine the role of transforming growth factor β (TGF-β) signaling in mice lacking the TGF-β type II receptor gene (Tgfbr2) in their limbs (Tgfbr2PRX-1KO). In Tgfbr2PRX-1KO mice, the loss of TGF-β responsiveness resulted in the absence of interphalangeal joints. The Tgfbr2PRX-1KO joint phenotype is similar to that in patients with symphalangism (SYM1-OMIM185800). By generating a Tgfbr2–green fluorescent protein–β–GEO–bacterial artificial chromosome β-galactosidase reporter transgenic mouse and by in situ hybridization and immunofluorescence, we determined that Tgfbr2 is highly and specifically expressed in developing joints. We demonstrated that in Tgfbr2PRX-1KO mice, the failure of joint interzone development resulted from an aberrant persistence of differentiated chondrocytes and failure of Jagged-1 expression. We found that TGF-β receptor II signaling regulates Noggin, Wnt9a, and growth and differentiation factor-5 joint morphogenic gene expressions. In Tgfbr2PRX-1KO growth plates adjacent to interphalangeal joints, Indian hedgehog expression is increased, whereas Collagen 10 expression decreased. We propose a model for joint development in which TGF-β signaling represents a means of entry to initiate the process.

Introduction

In industrialized countries, osteoarthritis affects more than one third of the adult population. Despite their clinical importance, the molecular mechanisms of joint morphogenesis are still unclear. The appendicular skeleton arises from the condensation of chondroprogenitor cells that undergo chondrocyte template formation that is subsequently replaced by bone to form the adult skeletal elements separated by cartilaginous joints. The synovial joints of the long bone elements form through segmentation of the continuous cartilaginous template with loss at the sites of the developing joints, loss of differentiated chondrocytes, and emergence of a nonchondrocytic joint-forming cell population that undergoes condensation, flattens, and develops an interzone that then cavitates to form the joint space within the articular cartilage (for review see Archer et al., 2003). There is limited information on the mechanisms that regulate the complex multistep process that leads to joint interzone formation. In fact, very few genes have been reported to be necessary and/or sufficient to initiate the joint formation process (namely Noggin, growth and differentiation factor-5 [Gdf-5], and Wnt9a [previously known as Wnt14]; Storm et al., 1994; Brunet et al., 1998; Hartmann and Tabin, 2001). The factors that induce the expression of these joint morphogenic molecules are undefined; furthermore, the mechanisms that determine the emergence of joint interzone cells within the chondrogenic condensates are unclear.

TGF-βs elicit their signal binding to TGF-β type II receptor (TβRII) that leads to the phosphorylation of TβRI and TβRII–TβRI complex formation, which then activates the signaling cascade through R-Smad–dependent (Smad-2,-3,-4) and Smad-independent pathways. In human and mouse embryonic cartilage, TGF-βs are expressed in the endochondral template with high expression in the perichondrium (Millan et al., 1991; Pelton et al., 1991a,b; Lawler et al., 1994; Serra and Chang, 2003). TβRI and TβRII have been reported to be expressed in the perichondrium and proliferative and differentiated chondrocytes (Serra and Chang, 2003).

Genetic manipulation of the TGF-β system genes have revealed their critical but still undefined roles in skeletogenesis (Serra et al., 1997; Ito et al., 2003; Baffi et al., 2004; for review see Dunker and Krieglstein, 2000). Targeted germine deletion of the Tgfb2 gene in mice results in perinatal lethality, and mice...
ablation will allow studies of TGF-β signaling that avoid the functional redundancy of the ligands and signaling pathways. Unfortunately, mice that are germline null for Tgfb2 exhibit early embryonic lethality that makes it impossible to evaluate the role of TGF-β1 signaling in skeletogenesis (Oshima et al., 1996). We have previously reported that in transgenic mice, overexpression of a dominant-negative Tgfb2 (DNIR) results in adult osteoarthritis (Serra et al., 1997). However, the phenotype was only observed in a few lines, most likely because expression was inconsistent and lacked tissue-specific targeting (Serra et al., 1997). Furthermore, the TGF-β cell targets and the temporal window of essential function during the endochondral process are not well defined. Conditional inactivation of Tgfb2 in differentiated chondrocytes results in mice without any long bone defects, leading to the conclusion that TGF-β signaling is not needed in the limb endochondral process (Baffi et al., 2004).

However, implanted TGF-β induces extra digit formation (Ganan et al., 1996). To circumvent the embryonic lethality of Tgfb2 systemic ablation and to determine the role of TGF-β signaling in early limb bud development, we generated mice in which the TβRII signaling is conditionally inactivated in limb buds and in a subset of other mesenchyme tissues starting at E9.5 (Tgfb2Prx1KO).

We show that in Tgfb2Prx1KO mice, TGF-β signaling ablation results in the following; (1) lack of interphalangeal joint development; (2) failure of joint interzone formation with a lack of Jagged-1 expression and aberrant survival of differentiated chondrocytes that leads to the absence of segmentation within the chondrogenic condensates; (3) failure of joint morphogenetic marker expression, including Notch, and increased bone morphogenetic protein (BMP) activity in limb bud cultures; (4) a selective defect on the endochondral growth plate process adjacent to the interphalangeal joints at early and late chondrogenesis with an increase of prehypertrophic chondrocyte markers and a decrease of terminally differentiated chondrocyte marker expression; and (5) midline defects and zeugopod and stylopod chondrodysplasia. Furthermore, using a TβRII reporting mouse and in situ and immunohistochemistry analyses, we have demonstrated that TβRII is highly and specifically expressed in developing joints.

Results

TGF-β signaling is needed for joint development and to regulate midline and limb skeletogenesis

To conditionally inactivate the TGF-β signaling in limb buds, we crossed Tgfb2flox/flox homozygous females with Prx1-Cre(Cre+)Tgfb2DNIIR double heterozygous males (Cre+Tgfb2flox/−) to generate Tgfb2Prx1KO mice (homozygous knockouts). Newborn Tgfb2Prx1KO mice showed abnormal forelimbs and hindlimbs (Fig. 1 A), which were confirmed by microcomputed tomography (micro-CT) imaging and Alizarin red/Alician blue staining (Fig. 1, B and C). Tgfb2Prx1KO autopods lacked interphalangeal joint development, and, between the ossification centers of the phalanges, at the site where the joints should have been formed, there was a continuous pattern of cells with some bending,
short with signs of chondrodysplasia (Fig. 2, A and B). The humerus lacked the deltoid tuberosity and, similar to the femur, had dysplastic widened, flaring, and poorly mineralized metaphyses (Fig. 2, C and D).

The morphometric parameters of newborn $Tgbr2^{Prx1KO}$ mutants are summarized in Table I. Compared with control Cre$^+$ siblings, mutants are shorter, and their length is more affected than their weight, as demonstrated by the higher ponderal index. This finding indicates that in $Tgbr2^{Prx1KO}$ mutants, the skeletal growth is impaired by a primary defect on skeletogenesis and is not the result of a global intrauterine nutritional defect.

$Tgbr2^{Prx1KO}$ mice had several midline defects: they lacked sternum formation, had hypoplastic incisors, and lacked the parietal and interparietal bones, whereas the frontal and squamosal bones were reduced in size (Fig. 3, A–J). They were capable of suckling, but they experienced massive and progressively visible intracranial bleeding (although still alive) that, at the necropsy exam, occupied most of the brain and likely was the primary cause of death. Although it is possible that a respiratory insufficiency caused by the lack of the sternum may be a concomitant cause of death, it is unlikely to be the primary cause considering the severity of the intracranial bleeding. Lack of parietal and interparietal bone development was confirmed by micro-CT analyses of living newborn $Tgbr2^{Prx1KO}$ mice, indicating that loss was not caused by accidental removal of the vault during the Alizarin red/Alcian blue staining procedure (Fig. 3, I and J). The pelvic bones of $Tgbr2^{Prx1KO}$ mice were smaller and poorly mineralized with signs of chondrodysplasia (Fig. 3, E and F).

Because we observed skeletal defects in segments unexpected for the reported Prx-1–mediated Cre recombination, we decided to evaluate the Prx1-Cre expression pattern in $Tgbr2^{Prx1KO}$ mice by crossing females doubly homozygous for $Tgbr2^{lox/lox}$ and R26R loci ($Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$) with males heterozygous for Prx-1-Cre to generate $Tgbr2^{Prx1KO};R26R$ mice. In the R26R mice, the ROSA26 locus is targeted by gene trapping

![Figure 2](image-url)

**Figure 2.** TGF-β signaling regulates the morphological features of limb stylopods, zeugopods, and autopods. (A–D) Alizarin red/Alcian blue limb skeletons were prepared from newborn $Tgbr2^{Prx1KO}$ mutants and $Tgbr2^{lox/lox}$ control mice. Mutants (left) showed smaller forelimbs (A) and hindlimbs (B) with bended zeugopods and autopods. $Tgbr2^{Prx1KO}$ humerus and femur stylopods (C and D) were shorter, had a middle concavity, and showed dysplastic poorly mineralized metaphyses. Humerus (C) lacked the deltoid tuberosity.

### Table I. Newborn morphometric parameters in $Tgbr2^{Prx1KO}$ and control Cre$^+$ siblings ($Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$)

| Genotype                  | n  | Mean ± SD | P-value |
|---------------------------|----|-----------|---------|
| Body length (cm)          |    |           |         |
| $Tgbr2^{Prx1KO}$          | 9  | 2.52 ± 0.17 | 0.0039  |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 17 | 3.00 ± 0.28 |         |
| Body weight (g)           |    |           |         |
| $Tgbr2^{Prx1KO}$          | 9  | 1.15 ± 0.08 | 0.0362  |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 17 | 1.52 ± 0.37 |         |
| Ponderal index (g/cm$^3$) |    |           |         |
| $Tgbr2^{Prx1KO}$          | 9  | 7.37 ± 1.84 | 0.0086  |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 17 | 5.70 ± 1.14 |         |
| Humerus length (cm)       |    |           |         |
| $Tgbr2^{Prx1KO}$          | 4  | 0.28 ± 0.03 | 0.004   |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 8  | 0.51 ± 0.02 |         |
| Radius length (cm)        |    |           |         |
| $Tgbr2^{Prx1KO}$          | 4  | 0.15 ± 0.02 | <0.0001 |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 8  | 0.39 ± 0.01 |         |
| Ulna length (cm)          |    |           |         |
| $Tgbr2^{Prx1KO}$          | 4  | 0.22 ± 0.008 | 0.004  |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 8  | 0.49 ± 0.007 |         |
| Femur length (cm)         |    |           |         |
| $Tgbr2^{Prx1KO}$          | 4  | 0.33 ± 0.01 | <0.0001 |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 8  | 0.45 ± 0.03 |         |
| Tibia length (cm)         |    |           |         |
| $Tgbr2^{Prx1KO}$          | 4  | 0.25 ± 0.005 | 0.004  |
| $Tgbr2^{lox/lox};Tgfbr2^{lox/lox}$ | 8  | 0.45 ± 0.02 |         |

Body lengths (from the tip of the nose to the anus) were measured on anesthetized animals stretched on top of a ruler. Ponderal indexes were calculated using the formula of Rohrer as weight (grams)/[length (centimeters)]$^3$ × 100 (Rohrer, 1921). Stylopod and zeugopod lengths were measured in Alizarin red/Alcian blue-stained elements.
so that Cre recombination results in LacZ expression (Soriano, 1999). We found that in Tgfbr2 Prx1KO-R26R whole mount embryos (E10.5), X-galactosidase staining was evident in the developing forelimbs and hindlimbs as well as in the skull and in the anterior midline region of the trunk (Fig. 4 A). In sections of E15.5 Tgfbr2 Prx1KO-R26R embryos, X-galactosidase staining was visualized in the skull, limbs, and in the oral, midline, and pelvic regions, which are areas where the Tgfbr2 Prx1KO newborn mutants showed substantial skeletal abnormalities (Fig. 4 B).

**TβRII is highly and specifically expressed in developing joints**

Because the Tgfbr2 Prx1KO autopods lacked the interphalangeal joints, we decided to investigate the TβRII expression in developing joints. To this purpose, we modified bacterial artificial chromosomes (BACs) to generate a Tgfbr2-GFP-β–GEO-BAC mouse reporter transgene containing both GFP and IRES-β–GEO (LacZ/Neo) reporter genes. We found that in E12.5 (Fig. 5 A, arrows) and 16.5 (Fig. 5 B) whole mount and E16.5 phalangeal sections (Fig. 5 C) of Tgfbr2-GFP-β–GEO-BAC embryos, Tgfbr2 is highly expressed in the interphalangeal joints. We have also noted that Tgfbr2 is highly expressed in the shoulder and elbow joints (Fig. 5 B) as well as in the knee and hip joints (not depicted). We have established five independent transgenic Tgfbr2-GFP-β–GEO-BAC lines that demonstrate Tgfbr2 joint expression. Tgfbr2 expression was similar in hindlimb and forelimb interphalangeal joints (unpublished data).

The Tgfbr2 joint expression pattern in Tgfbr2-GFP-β–GEO-BAC mice was directly comparable with endogenous expression. In fact, in situ hybridization studies revealed that in E16.5 Tgfbr2 Prx1KO-R26R embryos, Tgfbr2 is highly expressed in the cells marking the interphalangeal joint interzone and in the phalangeal prehypertrophic chondrocytes (Fig. 5 D, middle). Immunofluorescence studies confirmed TβRII joint expression (Fig. 5 D, top). Furthermore, there was an intense staining of phosphorylated Smad-2 in the interzone cell nuclei (Fig. 5 D, bottom). In Tgfbr2 Prx1KO mutants, the lack of joints was accompanied by...
content/full/jcb.200611031/DC1). Furthermore, quantitative real-time PCR of genomic DNA extracted from Tgfbr2Prx1KO and Tgfbr2flox/flox forelimb- and hindlimb-dissected digit bones and interphalangeal joints after removal of the skin and surrounding tissues showed that the efficiency of deletion of the Tgfbr2 exon 2 was 92 ± 3.0% (n = 3 mice for each group); considering the heterogeneity of the sample, efficiency is considerable.

**TGF-β signaling initiates joint interzone formation, determining chondrocyte segmentation and interzone cell survival**

Initiation of the joint interzone is demarked by segmentation of the cartilaginous continuity across the future joint location (for review see Archer et al., 2003). In Tgfbr2Prx1KO mutants at E16.5, we found a persistence of Collagen 2–expressing chondrocytes along the whole digit, including the potential joint site, whereas in control animals at the same age, Collagen 2 expression was confined to the endochondral templates and absent in the fully demarked joint (Fig. 6 A). Several components of the Notch system are expressed in articular cartilage, and a Notch-1–positive population of progenitor joint cells has been recently isolated from articular cartilage. This leads to the hypothesis that Notch signaling within the articular cartilage blocks chondrocyte differentiation, maintaining clonality and proliferation of the progenitor joint-forming cells (Hayes et al., 2003; Dowthwaite et al., 2004). In mice, interzone develops at E12.5–13.5. We found that E13.5 Tgfbr2Prx1KO mutants failed to form the interzone and lacked Jagged-1 expression, whereas in control mice, interzone cells highly expressed Jagged-1 (Fig. 6 B). It has been postulated that apoptosis may play a role in determining the fate of differentiated chondrocytes within the developing joint (for review see Archer et al., 2003). An intense positive TUNEL staining for apoptotic nuclei was observed in E13.5 control forming joints, whereas Tgfbr2Prx1KO E13.5 mutants lacked cell apoptosis within the presumptive joint region (Fig. 6 C).

**TGF-β signaling is required for joint morphogenic gene expression**

The activation of Noggin transcription is critical for joint formation, although its regulation is unknown. Mice that are null mutants for Noggin lack joints, and Noggin heterozygous loss of function mutations are found in some of the patients with proximal symphalangism (SYM1-OMIM185800) that lack proximal and medial interphalangeal joints, whereas the distal interphalangeal joint is never affected (Brunet et al., 1998; Gong et al., 2000; Takahashi et al., 2001). Analysis of Noggin expression at E13.5 and 16.5 by in situ hybridization and immunofluorescence revealed a complete down-regulation in the joints of Tgfbr2Prx1KO embryos (Fig. 7, A and B). Gdf-5 is one of the earliest markers expressed in developing joints, and Gdf-5 is mutated in the brachypodism mouse, which has interphalangeal joint defects (Storm et al., 1994). Furthermore, a Gdf-5 mutation with a gain of aberrant BMP-2–like function was reported in a family with SYM1 (Seemann et al., 2005). It has been hypothesized that in early chondrogenesis, Gdf-5 inhibits joint formation and induces cartilage development, whereas its role in late chondrogenesis

the lack of Tgfbr2 mRNA and protein expression as well as a decrease of cell nuclei positive for phosphorylated Smad-2, indicating the effective Prx1–mediated Cre recombination of Tgfbr2 (Fig. 5 D). Regarding the Tgfbr2 expression in the growth plate adjacent to the joints, we found that it was expressed at a much lower level than joints by cells that morphologically resemble prehypertrophic chondrocytes (Fig. 5 D). PCR amplification of genomic DNA extracted by laser capture microdissection (LCM) from paraffin sections of E16.5 joint cells and cells outlining the joint mesoderm demonstrated a specific Tgfbr2 recombination and subsequent loss of the floxed alleles (Tgfbr2 exon 2) in Tgfbr2Prx1KO joints compared with Tgfbr2flox/flox (Fig. S1, available at http://www.jcb.org/cgi/
is to maintain joint formation (Storm and Kingsley, 1999). In Tgfbr2Prx1KO mutants, Gdf-5 expression was increased at E13.5, whereas it was abrogated in E16.5 (Fig. 7, A and B). Wnt9a is expressed in developing joints, and its misexpression in chicken digit rays induces ectopic joint formation, whereas the loss of Wnt9a in mice results in synovial chondromatosis (Hartmann and Tabin, 2001; Spater et al., 2006). In Tgfbr2Prx1KO mutant joints, Wnt9a expression is down-regulated in E13.5 (not depicted) and 16.5 (Fig. 7 B). These results indicate that TGF-β signaling in developing limbs is mandatory for joint formation and to regulate Noggin, Gdf-5, and Wnt9a expressions.

TGF-β signaling regulates Noggin expression and BMP activity in limb bud micromass cultures

It is difficult to infer from the results found in Tgfbr2Prx1KO mutants whether TGF-β signaling directly regulates Noggin transcription or whether TGF-β sustains the limb bud growth, ensuring an adequate environment for the joints to develop and Noggin to be expressed. Therefore, we decided to evaluate the role of TGF-β signaling in Noggin expression in limb bud micromass cultures. In CreTgfbr2floxed cultures, Tgfbr2 expression was conditionally inactivated, and the lack of TβRII binding expression was verified by a 125I–TGF-β1 affinity cross-linking cell surface–binding assay (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200611031/DC1). In control
MMP\(^+\)Tgfr2\(^{fl ox/fox}\) cultures, the T\(\beta\)RI, T\(\beta\)RII, and T\(\beta\)RIII were identified, whereas in Cre\(^+\)Tgfr2\(^{KO}\) cultures, \(^{125}\)I–TGF-\(\beta\)1 binding to T\(\beta\)RII was greatly reduced (Fig. S2 A). The specificity of \(^{125}\)I–TGF-\(\beta\)1 binding was confirmed by the fact that labeled bands were displaced by cold TGF-\(\beta\)1 in excess (Fig. S2 A). Cre recombination was also confirmed by an intensely positive X-galactosidase staining in Cre\(^+\)Tgfr2\(^{KO}\).R26R that was negative in control MMP\(^+\)Tgfr2\(^{fl ox/fox}\).R26R cultures (Fig. S2 B).

We found that in MMP\(^+\)Tgfr2\(^{fl ox/fox}\) cultures, TGF-\(\beta\) treatment induced Noggin mRNA and protein expression as determined by quantitative RT-PCR and Western immunoblotting (WIB) analyses (Fig. 8, A and B). Similar results were found when wild-type micromass cultures were treated with TGF-\(\beta\) (7.8 ± 2.1-fold compared with untreated control [1.2 ± 0.3-fold]; \(P < 0.05; n = 3\)). Noggin binds to BMPs, preventing BMP receptor activation and, therefore, signaling; the canonical BMP signal is through the phosphorylation cascade of Smad-1, -5, and -8 that complex and induce transcription. Therefore, in accordance with the increase of Noggin expression, we found that in MMP\(^+\)Tgfr2\(^{fl ox/fox}\) cultures, TGF-\(\beta\) decreased BMP activity, as indicated by a decrease of phosphorylated Smad-1, -5, and -8 (Fig. 8 B). Knocking out the TGF-\(\beta\) signaling in Cre\(^+\)Tgfr2\(^{KO}\) cultures resulted in the abrogation of TGF-\(\beta\) effects on Noggin expression and Smad-1, -5, and -8 phosphorylation (Fig. 8, A and B). Notably, a decrease of Smad-1, -5, and -8 phosphorylation was found in untreated Cre\(^+\)Tgfr2\(^{KO}\) compared with MMP\(^+\)Tgfr2\(^{fl ox/fox}\) cultures, possibly as a result of the unresponsiveness of Cre\(^+\)Tgfr2\(^{KO}\) cells to endogenous TGF-\(\beta\).

**TGF-\(\beta\) signaling in autopod endochondral cartilage development**

It has been hypothesized that the developing joints act as signaling centers to control the adjacent endochondral template development (for review see Archer et al., 2003). Because the Tgfr2\(^{Prx1KO}\) mutant lacks the interphalangeal joints, it represents an ideal model to test this hypothesis. Therefore, we performed a systematic evaluation of cartilage marker expressions in growth plates adjacent to the presumptive interphalangeal joints at E13.5, E16.5, and postnatal day (P) 0 in Tgfr2\(^{Prx1KO}\) mutants and control Tgfr2\(^{fl ox/fox}\) siblings (Fig. 9, A–C). We found that Tgfr2\(^{Prx1KO}\) growth plates presented several remarkable and selective abnormalities; Collagen 10 expression is consistently decreased at E13.5, E16.5, and P0 compared with Tgfr2\(^{fl ox/fox}\) controls, indicating a dramatic delay in chondrocyte hypertrophy in the mutants (Fig. 9, A–C). Conversely, in Tgfr2\(^{Prx1KO}\) growth plates, Indian hedgehog (Ihh) was increased and more widely expressed from the proliferative zone to the canonical prehypertrophic chondrocyte zone compared with Tgfr2\(^{fl ox/fox}\) controls; this finding was consistent at E13.5, E16.5, and P0 (Fig. 9, A–C). In Tgfr2\(^{Prx1KO}\) mutants at E16.5 and P0, parathyroid hormone–related protein (PTH-\(rP\)) expression is increased and more diffuse in the prehypertrophic/upper proliferative zone and in the perichondrium; at E13.5, PTH-\(rP\) expression is similar to the control (Fig. 9, A–C). Collagen 2 and Sox-9 expressions are similar to the control in Tgfr2\(^{Prx1KO}\) but Collagen 2–expressing cells at P0 display a less organized columnar distribution than controls (Fig. 9, A–C). This disorganization was also observed in the hematoxylin and eosin staining that also showed that hypertrophic chondrocytes are larger but show a decreased expression of Collagen 10 (Fig. 9 C).

**Discussion**

The role of TGF-\(\beta\) signaling in skeletogenesis is not well determined, and contradictory data have been reported (for review see Dunker and Krieglstein, 2000). We have generated the Tgfr2\(^{Prx1KO}\) mutant mice in which the Tgfr2 is conditionally inactivated in limb buds and a subset of mesenchyme tissues starting at very early embryonic limb development. The Tgfr2\(^{Prx1KO}\)
mouse allowed us to determine that TGF-β signaling is essential for interphalangeal joint development. TGF-β signaling initiates the joint interzone formation by regulating interzone cell survival and chondrocyte segmentation. TGF-β elicits these effects operating upstream of Noggin, GDF-5, and Wnt9a expressions. Our results uncover a novel step regulated by TGF-β signaling that is required for establishing the correct initiation of joint development.

**TβRII and signaling are expressed in the limb joints**

Although a role of TGF-β signaling in joints has been speculated, TβRII expression in joints has never been clearly reported. To incontrovertibly define that TβRII is expressed in developing interphalangeal joints, we generated the Tgfbr2<sup>Prx1KO</sup> mouse reporter transgene and used immunofluorescence and in situ hybridization studies. Furthermore, we found a high staining of phosphorylated Smad-2 in the interzone cell nuclei, corroborating the finding that TβRII is expressed in the cells, demarking the interzone and signaling through the R-Smad pathway. The Tgfbr2-GFP-β—GEO-BAC mouse allowed us to observe that Tgfbr2 is also highly expressed in the proximal limb joints. The Tgfbr2<sup>Prx1KO</sup> mutants lack only the interphalangeal joints, whereas the proximal limb joints are formed. It may be possible that the Prx-1–mediated Cre recombination expression varies within the limb and is present in the interphalangeal joints, whereas it is lacking or less effective in the proximal limb joints. Another possibility is that TGF-β signaling is essential for interphalangeal joint development, whereas it is dispensable for development of the proximal limb joints. The efficiency and specificity of the Prx-1–mediated Cre recombination of TβRII in the joints was supported by the in situ hybridization and immunofluorescence studies and by the PCR amplification analyses of joint cell DNA obtained by LCM of joint cells or from digit bones and interphalangeal joints.
TGF-β signaling is essential for interzone formation

Joints in Tgfbr2Prx1KO mutants appear to be arrested in their development at about the time at which interzone starts to develop: differentiated chondrocytes extend with a continuous pattern across the phalanges without any sign of apoptosis, and interzone cells lack Jagged-1 expression and any sign of condensation. The molecular mechanisms underlying interzone formation are not yet well understood. Although Wnt9a was reported as a potential joint inducer, the recent report that Wnt9a-null mice have joints has redefined its role more as a joint keeper (Hartmann and Tabin, 2001; Spater et al., 2006). GDF-5 is highly expressed in developing joints. It has been hypothesized that in early chondrogenesis (E14.5), Gdf-5 inhibits joint formation and induces cartilage development, whereas at a later stage of chondrogenesis (E15.5), it regulates joint structure formation or maintenance (Storm and Kingsley, 1999). Our data indicate that TGF-β signaling down-regulates joint GDF-5 expression at an early chondrogenesis stage, whereas it up-regulates its joint expression at a later stage, suggesting that it operates upstream of GDF-5.

Another conundrum in understanding joint interzone development is the fate of differentiated chondrocytes and in the meantime emergence of the interzone cells. It has been recently reported that a distinct mesenchymal cell population takes part in the interzone and articular layer formation (Pacifi ci et al., 2006). Furthermore, a Notch-1–positive progenitor cell population has been recently isolated within the articular cells. These Notch-1 progenitors are capable of engrafting in vivo into articular structures and have been hypothesized to maintain articular cartilage integrity, preserving interzone cell clonality and proliferation while preventing differentiation into chondrocytes (Dowthwaite et al., 2004). To corroborate this hypothesis, a derangement of the Notch signaling has been reported in articular synoviocytes from patients with rheumatoid arthritis that is characterized by aberrant synoviocyte proliferation (Ando et al., 2003). A direct cross talk between the Notch and TGF-β signaling pathways comes from a recent study in which a direct interaction between Notch intracellular domain and Smad-3 was demonstrated, and activation of Smad-3 by TGF-β led to an enhancement of Notch-induced Hes1 gene transcription (Blokzijl et al., 2003). We hypothesize that TGF-β serves as an essential joint signaling center and that activating Notch signaling forces progenitor interzone cells to remain in an undifferentiated state while regulating the apoptosis of differentiated chondrocytes. Our hypothesis needs further investigation, and the possibility that down-regulation of Jagged-1 in the Tgfbr2Prx1KO mutants is consequent to the lack of interzone formation should also be considered. In bone marrow–derived mesenchymal stem cells, we have previously reported that TGF-β induces chondrogenesis by exerting similar dichotomous effects (Longobardi et al., 2006). Deciphering the role of TGF-β signaling in joint development can provide substantial insight to identify the interzone cells and to define the role of chondrocyte-programmed cell death and progenitor interzone cell survival in joint formation.

The TGF-β ligand expression pattern in developing cartilage has been previously reported, including by our group (Pelton et al., 1991a,b; Lawler et al., 1994). TGF-β1 is expressed in the digit perichondrium at E13.5, and, by E16.5, TGF-β2 and -β3 are also expressed in the perichondrium, including in the digit perichondrium (Millan et al., 1991; Pelton et al., 1991a,b; Lawler et al., 1994; Serra and Chang, 2003). It has been previously reported that in cartilage development, TGF-β exerts their actions in a paracrine fashion (Lawler et al., 1994). We hypothesize that a similar mechanism occurs during digit joint development. Future studies are needed to evaluate TGF-β ligand delivery to the interphalangeal joints.

TβRⅡ operates upstream of joint gene expression, induces Noggin expression, and modulates BMP activities in vitro

Our data indicate that TGF-β signaling in the joints functions as a master regulator for expression of the key joint morphogenic genes GDF-5, Noggin, and Wnt9a. We propose a working hypothesis model for joint development in which TGF-β signaling is essential in inducing the joint interzone formation, and it operates early in joint development to regulate the expression of critical joint morphogenic genes such as Noggin to modulate BMP activities (Fig. 10).

The joint phenotype observed in the Tgfbr2Prx1KO mouse is similar to that in patients with proximal symphalangism (SYM1-OMIM118800) in which the proximal and medial interphalangeal joints are lacking, whereas the distal interphalangeal joint is not affected (Gong et al., 1999; Takahashi et al., 2001). Although functionally the distal interphalangeal joint is indistinguishable from the other interphalangeal joints, the observation of phenotypical abnormalities only affecting the proximal and medial interphalangeal joints in patients with SYM1 and now in the Tgfbr2Prx1KO mouse indicates a distinct development for the distal joint. In patients with SYM1, heterozygous mutations of Noggin as well as a heterozygous mutation of GDF-5 with an aberrant BMP-like gain of function have been reported (Gong et al., 1999; Seemann et al., 2005). Furthermore, Noggin-null mutant mice lack joint development (Brunet et al., 1998). These findings clearly indicate that lack of Noggin function or increase in BMP activities result in the failure of joint development. The regulatory factors that determine Noggin expression within the joints are unknown. Our data indicate that Noggin expression is down-regulated in the Tgfbr2Prx1KO mouse, and, in limb bud cultures, TGF-β induces Noggin expression and reduces BMP signaling. Gazzerro et al. (1998) have previously reported that TGF-β1 induces Noggin mRNA in cultured rat osteoblasts with unclear function. Our working hypothesis is that TGF-β signaling induces joint development by regulating Noggin expression and, therefore, BMP activities. However, considering the multiple and diverse limb abnormalities found in the Tgfbr2Prx1KO phenotype, it seems likely that more than one mechanism would be involved. We have noted that in E13.5 mutants compared with controls, Noggin mRNA was increased in the surrounding tissue but was not increased at E16.5; on the other hand, at E16.5, we noted an increase of protein expression. We hypothesize that at E13.5, the lack of Noggin joint expression leads to a compensatory response in the surrounding tissue that is associated with an increase of protein expression still detectable at
E16.5 (probably as a result of a prolonged protein half-life). However, at E16.5, this mRNA compensatory response seems to be defective. Future studies are needed to identify the mechanisms responsible for this compensatory response. In the controls, Noggin expression in the surrounding tissue is greater at E16.5 than at 13.5; the significance of this increase is also unclear, and further studies outside of the scope of the present experiments are needed to determine this.

**TGF-β signaling in cartilage development: lack of joint development is associated with selective effects on the adjacent chondrogenesis**

We have found that in Tgfbr2Prx1KO mutants, the growth plates adjacent to the interphalangeal joints present an increase of Ihh expression and a decrease of Collagen 10 expression at early as well as late chondrogenesis; PTH-rP expression is increased in late chondrogenesis. GDF-5–releasing beads implanted in the interdigital space of developing mouse limbs resulted in an increase of Ihh expression that has also been noted in Noggin-null mutant mice. Up-regulation of Ihh signaling in Patched-1−/−; Collagen 2a1-Cre mice resulted in joint fusion (Brunet et al., 1998; Storm and Kingsley, 1999; Mak et al., 2006). We hypothesize that TGF-β signaling within the joints plays a central role in orchestrating the interplay between joint formation and adjacent endochondral template development by controlling Ihh expression that induces PTH-rP and, thus, repressing the rate of chondrocyte hypertrophy. Interestingly, in Tgfbr2Prx1KO mutants, PTH-rP expression is increased in the perichondrium but more remarkably in the prehypertrophic/upper proliferative cells. Using a PTH-rP–LacZ reporter mouse, Chen et al. (2006) have recently reported that PTH-rP is expressed in this subpopulation of cells. The role of these PTH-rP–expressing cells is still not clearly defined, but they may contribute to the PTH-rP inhibitory effect on hypertrophy (Chen et al., 2006). Hematoxylin and eosin morphometric analysis showed that hypertrophic chondrocytes seem to be larger in P0 Tgfbr2Prx1KO mutants, but Collagen 10 expression was clearly decreased at any stage, indicating a functional derangement. These findings are consistent with our hypothesis that TGF-β signaling is required for the appropriate progression of the prehypertrophic to hypertrophic chondrocytes, and its abolishment is associated with a derangement of the pre- and hypertrophic growth plate zones.

Our data are only apparently in contradiction with the findings observed in Tgfbr2fl ox/fl ox Collagen 2a-cre mice in which Tgfbr2 was conditionally inactivated in Collagen 2a–expressing cells. In the Tgfbr2fl ox/fl ox Collagen 2a-cre mouse, chondrocyte differentiation and development of long bones are normal. We hypothesize

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### Table 1

| Normal | Absence of TGF-β signaling |
|--------|---------------------------|
|        | Prechondrogenic mesenchyme | Prechondrogenic mesenchyme |
|        | Chondrogenic differentiation | Chondrogenic differentiation |
|        | Joint initiation: condensation | Failure of joint condensation |
|        | Joint interzone development | Lack of interzone |
|        | Joint space development | Persistence of chondrocytes in the joint space |
|        | **TgfR2** | **FgfR2** |
|        | **Jagged-1** | **FgfR2** |
|        | **Apoptosis** | **FgfR2** |
|        | **Noggin** | **FgfR2** |
|        | **Chondrocytes** | **FgfR2** |

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**Figure 10.** TGF-β is a master signaling center within the joint interzone. Proposed model for the role of TβRII signaling in interphalangeal joint development. TβRII is specifically expressed by the joint-developing cells, and lack of TGF-β signaling results in the failure of interzone formation by lack of the survival of interzone-forming cells, persistence of differentiated chondrocytes in the joint region, and derangement of joint morphogenic gene expressions.
that in the endochondral growth process, TGF-β signaling is required to control the rate of differentiation of prehypertrophic chondrocytes to hypertrophic chondrocytes, whereas it has relatively scarce effect on collagen 2–expressing chondrocytes. An increase of Ihh expression was found in the growth plates of newborn DNIIR, and, by 1–2 mo of age, these mice develop progressive osteoarthritis with replacement of the articular cartilage with Collagen X–expressing cells (Serra et al., 1997). We speculate that over time, DNIIR mice develop a compensatory mechanism that overrides the Ihh inhibitory effect on hypertrophy.

**TGF-β signaling regulates calvaria development**

The Tgfb2Prx1KO mouse lacks the parietal and interparietal bones. Interestingly, a similar phenotype has been reported in patients with familial parietal foramina (PFM-1, OMIM168500 PFM-2, and OMIM609597) that have symmetrical, oval defects in the parietal bones. Some of these patients have been reported to have haploinsufficiency either in the homeobox gene Alx4 (OMIM609597) or in the Msx2 gene (OMIM168500; Wuys et al., 2000a,b). In mice, Msx2, Twist, and Alx4 cooperate in controlling the migration and differentiation of crest-derived skeletal mesenchyme in the vault (Ishi et al., 2003; Antonopoulou et al., 2004). Craniofacial conditional inactivation of Tgfb2 in the Tgfb2flox/;Wnt1-cre mouse also resulted in calvaria defects (Ito et al., 2003). We have noted that in the Tgfb2Prx1KO, R26R mouse, the LacZ-stained cells are arrested below the parietal and interparietal bones (Fig. 5, A and B), whereas in controls, LacZ-stained cells cover the vault (not depicted). The flat bones of the skull vault develop from two migratory mesenchymal cell populations, the cranial neural crest, and paraxial mesoderm. The possibility that a defect in migration of the Tgfb2Prx1KO skeletogenic mesenchyme cells within the vault can lead to the vault bones and the interplay between TGF-β signaling with Mxs2, Twist, and Alx4 genes is under investigation, and further studies are needed. In conclusion, the Tgfb2Prx1KO mouse model has unraveled critical information on skeletal development and opened novel potential therapeutic approaches to treat degenerative joint diseases such as osteoarthritis.

**Materials and methods**

**Generation of Tgfb2Prx1KO, Tgfb2-GFP–β–GEO-BAC, and Tgfb2Prx1KO;R26R mice**

To generate Tgfb2Prx1KO mutants, male Tgfb2flox/;R26R homozygous mice were mated with Prx1-Cre and Tgfb2flox/;R26R heterozygous males (Chytil et al., 2002; Logan et al., 2002). As previously reported, we have generated the Tgfb2flox/Cre mice by flanking with loxP sites the exon 2 of Tgfb2 transcripts for the TGF-β–binding domain (Chytil et al., 2002). In the Prx1-Cre mouse, the lacZ-stained cells are arrested by the parietal and interparietal bones (Fig. 5, A and B), whereas in controls, lacZ-stained cells cover the vault (not depicted). The flat bones of the skull vault develop from two migratory mesenchymal cell populations, the cranial neural crest, and paraxial mesoderm. The possibility that a defect in migration of the Tgfb2Prx1KO skeletogenic mesenchyme cells within the vault can lead to the vault bones and the interplay between TGF-β signaling with Mxs2, Twist, and Alx4 genes is under investigation, and further studies are needed. In conclusion, the Tgfb2Prx1KO mouse model has unraveled critical information on skeletal development and opened novel potential therapeutic approaches to treat degenerative joint diseases such as osteoarthritis.

To generate the Tgfb2GFP–β–GEO-BAC mouse, the BAC clone RP24-317C21 containing Tgfb2 was obtained from the Children's Hospital Oakland Research Institute. As schematically presented in Fig. S3 (available at http://www.jcb.org/cgi/content/full/jcb.200611031/DC1), a GFP-IRES-β–GEO-BAC cassette was inserted into Tgfb2-BAC at the endogenous Tgfb2 translational start site using the homologous recombination technique of Warmsing et al. (2003), Lee et al. (2001), and as previously reported (Morlock et al., 2003; Deal et al., 2006) as follows: the plasmid pBGFtTet was generated by ligating the IRES-β–GEO-SV40A cassette from pGTL.1 and an FRT-flanked tetracycline resistance cassette into a modified pBluescript II SK+ backbone (Mountford et al., 1994). An eGFP open reading frame (CLONTECH Laboratories, Inc.) was then inserted upstream of IRES-β–GEO-SV40A to create pGFPBGFtTet. The combination cassette was constructed by subcloning 50- and 3′-recombination arms (both containing part of the Tgfb2 exon 1) into pGFPBGFtTet such that the recombination arms flanked the GFP-IRES-β–GEO-FRT-Tet-FRT cassette. The forward strand (relative to Tgfb2) sequences of the 50-bp homology arms were as follows: for the 5′ arm, CCGTTCGGTG-GCGGACGGCGCGATGGAGCGAGCGGCGGCTCC and for the 3′ arm, ATGGGCGGGGGGCTGCTCCGGGGGGCGTGGGCGGCTGCATTACGTCCGTG. Both recombination arms were created by annealing PAGE-purified oligonucleotides designed to allow direct ligation to pGFPBGFtTet. The final cassette with recombination arms was digested from the vector, gel purified, and recombined with BAC as described previously (Lee et al., 2001). Successful recombients were selected by tetracycline resistance. The tetracycline resistance gene was then removed by FLPe recombinase excision (Lee et al., 2001; Morlock et al., 2003). The correctly modified BAC was verified by conventional and pulsed-field gel analysis of restriction digests to confirm expected banding patterns as well as direct BAC sequencing. Tgfb2BAC DNA was purified according to established techniques and was used for pronuclear injection of C57Bl/6J or DBA/2 F1 hybrid embryos (D'Ileone et al., 2000). Injections and oviduct transfers were performed by the Vanderbilt Transgenic Core Facility using standard techniques in accordance with protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee. All BACs were injected as uncultured DNAs.

To generate the Tgfb2Prx1KO;R26R mouse, the Tgfb2flox/ and R26R (obtained from P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA; Soriano, 1999) were first crossed to obtain the Tgfb2flox/;R26R female mice that were then crossed with Prx1-Cre heterozygous males. For timed pregnancies, noon of the day when evidence of a vaginal plug was found was considered E0.5.

**Skeletal analysis**

Alizarin red/Alican blue staining was performed as previously reported (Morlock et al., 1996). Images were taken using a stereo microscope (SZX12; Olympus) equipped with a digital camera (DP7; Olympus) and imported into Photoshop (Adobe). Living animal micro-CT imaging (Imtek MicroCATII-CT) was performed setting micro-CT slices at 0.1 mm from the stereotypically reconstructed in 3D arrays using the same thresholds.

**Histology, immunofluorescence, immunohistochemistry, TUNEL assay, in situ hybridization studies, and detection of β-galactosidase activity in whole mount embryos and cryosections**

Internal and dissected limb embryos or decalcified newborns were sectioned using standard procedures. For general morphology, sections were stained with hematoxylin and eosin using standard procedures.

For immunohistochemistry or immunofluorescence, either the Cytoma-A or Vectastatin Elite ABC Immunostaining kits (DakoCytomation) were used. The following primary antibodies were used: anti-IhH polyclonal (Santa Cruz Biotechnology, Inc.), anti-IhH (Cell Signaling), and anti-Noggin polyclonal (R&D Systems). Apoptotic nuclei were visualized using the DeadEnd Colorimetric TUNEL System (Promega).

In situ hybridization studies were performed as previously reported (Deal et al., 2006). Digoxigenin-UTP-riboprobes were synthesized (DIG-RNA-Labeling kit; Roche) from plasmids with insertion of Noggin (provided by J. Kralli, Harvard University, Cambridge, MA; provided by S.K. Dey, Vanderbilt University, Nashville, TN; mouse Collagen 2α1 and 2α5 (provided by D. Kingsley, Stanford University, Palo Alto, CA), mouse Ihh (provided by A. McMahon, Harvard University, Cambridge, MA), and PTHrP (provided by H. Kronenberg, Harvard University). Matsuura et al., 1991; Storm and Kingsley, 1996; Das et al., 1997). Whole RNA was made using a mMessage mMachine T7 Kit (Ambion clone #30435371; GenBank/EMBL/DDBJ accession no. BC066165); the plasmid
was linearized with XmnI and riboprobe synthesized with T7 polymerase. Collagen 10α1 and Sox9 probes were also made. The primers for Sox9 were forward (GACATGAAAAGAGTAAACAGTG) and reverse (AGG CTAAGGCACCTGTTGAACT); the primers for Collagen 10α1 were forward (GCGAGGTCTCAGGGCTCTCA) and reverse (GATCAGAGTAGTCCCTTGCT). PCR products were cloned into pGEMT-Easy and linearized with NcoI, and riboprobes were synthesized with T7 polymerase. Whole mount embryo X-galactosidase staining was performed as previously reported (Mortlock et al., 2003). Images were taken using an inverted microscope (IX71; Olympus) equipped with a digital camera (DP71; Olympus) and were imported into Photoshop (Adobe), where they were formatted without using any image enhancement. For cryosectioning, whole mount stained embryos were cryosembbed in optimal cutting temperature compound (Sakura). 50-μm sections were warm adhered on Superfrost Plus slides (Fisher Scientific), washed thoroughly, and mounted using Aqua-PolyMount (Polysciences). Section images were taken using the IX71 microscope with DP71 digital camera. Sections subjected to immunofluorescence were imaged using a microscope (Axioskop; Zeiss Microimaging, Inc.) with a camera (Micromax; Princeton Instruments), and images were imported into Photoshop for formatting.

Micromass cultures, in vitro conditional inactivation of TβRII, X-galactosidase histochemical staining, and affinity labeling with Noggin and TGF-β1 reporter construct. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200611031/DC1.

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