

Bone abnormalities in latent TGF- β binding protein (Ltbp)-3–null mice indicate a role for Ltbp-3 in modulating TGF- β bioavailability

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The TGF- β s are multifunctional proteins whose activities are believed to be controlled by interaction with the latent TGF- β binding proteins (LTBPs). In spite of substantial effort, the precise *in vivo* significance of this interaction remains unknown. To examine the role of the Ltbp-3, we made an *Ltbp-3*–null mutation in the mouse by gene targeting. Homozygous mutant animals develop cranio-facial malformations by day 10. At 2 mo, there is a pronounced rounding of the cranial vault, extension of the mandible beyond the maxilla, and kyphosis. Histological examination of the skulls from null animals revealed ossification

of the synchondroses within 2 wk of birth, in contrast to the wild-type synchondroses, which never ossify. Between 6 and 9 mo of age, mutant animals also develop osteosclerosis and osteoarthritis. The pathological changes of the *Ltbp-3*–null mice are consistent with perturbed TGF- β signaling in the skull and long bones. These observations give support to the notion that LTBP-3 is important for the control of TGF- β action. Moreover, the results provide the first *in vivo* indication for a role of LTBP in modulating TGF- β bioavailability.

Introduction

The latent TGF- β binding proteins (LTBPs)*-1–4 are matrix molecules composed of multiple (14–20) EGF-like domains and four domains containing eight cysteines (8-cys) that are specific for the LTBPs and the fibrillins (for review see Handford et al., 2000; Koli et al., 2001). The modular structure of Ltbp-3 is shown in Fig. 1 A (Yin et al., 1995). LTBP-1, 3, and 4 covalently bind latent TGF- β (Koli et al., 2001). The TGF- β s are 25-kd homodimeric cytokines derived by intracellular proteolytic processing of larger propeptides (Massague, 1998). However, once cleaved from the cy-

tokine, the TGF- β propeptide, called the latency-associated peptide (LAP), remains noncovalently associated with the mature TGF- β after secretion (Koli et al., 2001). This complex of TGF- β and LAP, the small latent complex, is inactive, and the dissociation of TGF- β from LAP is a crucial regulatory step in TGF- β action. The small latent complex can form a large latent complex by the bonding of cysteines in the LAP with a pair of cysteines in the third 8-cys domain of LTBP (Gleizes et al., 1996; Saharinen et al., 1996). All three TGF- β isoforms bind to LTBPs-1, 3, or 4; however, neither LTBP-2 nor the fibrillins bind TGF- β (Saharinen and Keski-Oja, 2000).

The LTBPs have been proposed to have two functions: as structural components of the extracellular matrix (Dallas et al., 1995) and as modulators of TGF- β bioavailability (Koli et al., 2001). Experiments in culture have shown that the association of LTBP with latent TGF- β is important for at least two aspects of TGF- β biology. First, the binding of LTBP-1 to the small latent complex facilitates its folding and secretion (Miyazono et al., 1991). Second, LTBP-1 modulates latent TGF- β activation (Flaumenhaft et al., 1993; Nakajima et al., 1997; Gualandris et al., 2000). However, these studies have not provided direct proof for the physiological role of LTBP in modulating TGF- β activity.

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*Abbreviations used in this paper: Bsp, bone sialoprotein; Ihh, Indian hedgehog; LAP, latency-associated peptide; LTBP, latent TGF- β binding protein; PTHrP, parathyroid hormone–related protein; PTHrP-R, PTHrP receptor; RIIDN, dominant negative receptor II; TGF- β , transforming growth factor- β .

Key words: latent TGF- β binding protein; TGF- β ; osteoarthritis; osteosclerosis; synchondroses

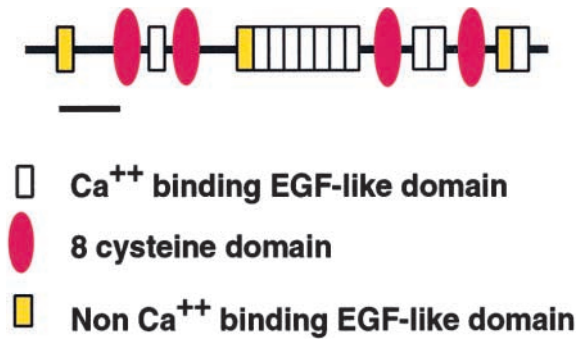


Figure 1. ***Ltbp-3* targeting strategy.** LTBP-3 protein structure. The region deleted in the targeted locus is underlined.

To address this point, we have generated *Ltbp-3*-null mice. These mice have an altered skull shape caused by the premature ossification of the cranial base synchondroses. In addition, there is enhanced accumulation of trabecular bone in the long bones and vertebrae and degeneration of the articular cartilage as the animals age. These phenotypic abnormalities are consistent with postulated roles of TGF- β in bone formation and homeostasis. As such, this report represents the first indication for physiological control of TGF- β activity by an LTBP.

Results and discussion

Generation of *Ltbp-3*-null mice

Ltbp-3-null mice were produced by gene targeting using a targeting vector to replace two exons containing a nonunit number of codons (Joyner, 1995), including bases 278–807 of the ORF with the neo^r selectable marker (see online supplemental material, available at <http://www.jcb.org/cgi/content/full/200111080/DC1>, for details). These two exons code for the first EGF-like repeat, the pro-gly-rich region, and the beginning of the first 8-cys domain (Fig. 1). This deletion causes a frameshift in the ORF and premature termination of translation. Three clones (17, 22, and 25) with homologous recombination in the *Ltbp-3* gene were detected by Southern blot analysis (unpublished data), and two clones were used to produce chimeric animals. *Ltbp-3*-null animals were obtained by crossing heterozygous progeny of chimeric mice. Northern blot hybridization of lung

Figure 2. ***Ltbp-3*-null mouse.** (A) Detection of *Ltbp-3* mRNA in total lung RNA by Northern blot hybridization. (Top) Northern blot hybridization. +/+, Wild-type; -/-, *Ltbp-3*-null animal. (Bottom) ethidium bromide image of RNA before transfer. (B) X-ray radiography of 2-mo-old wild-type and *Ltbp-3*-null mice.

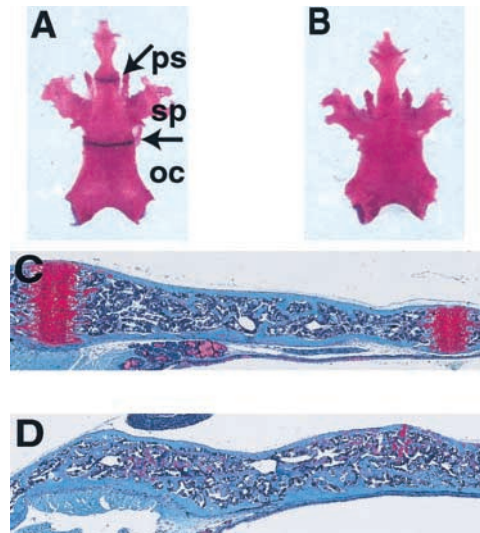
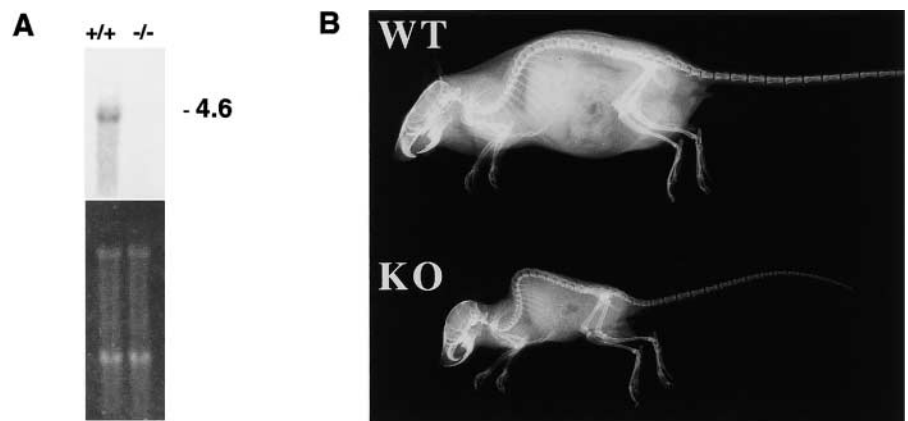


Figure 3. **Histology of wild-type and *Ltbp-3*-null skulls.** (A and B) Fusion of bones of the skull base in 3-wk-old *Ltbp-3* mice. Whole mount skulls of wild-type (A) and mutant (B) animals were stained with alcian blue for cartilage and Alizarin red S for calcified bone, and skull bases were dissected. Arrows point to synchondroses between occipital (oc), sphenoid (sp) and presphenoid (ps) bones. (C and D). Histology of the skull base of 3-wk-old wild-type (C) and *Ltbp-3*-null (D) animals. Cartilage is stained red and bone blue. The basooccipital-basosphenoid synchondrosis is on the left.

RNA from null animals with a probe mapping 3' from the deleted region showed the absence of the *Ltbp-3* transcript (Fig. 2 A). Therefore, we concluded that these mice were effectively *Ltbp-3*-null animals. *Ltbp-3*-null mice were born in the expected Mendelian ratio with no apparent defects. By day 10, null animals displayed a rounded head and shortened snout. X-ray radiography of 2-mo-old mutant mice revealed a domed skull, abnormal apposition of the upper and lower incisors, and curvature of the cervical and thoracic vertebrae (thoracic kyphosis) (Fig. 2 B).

Craniofacial abnormalities

The appropriate anatomical development of the skull requires the coordinated growth of the membranous and endochondral bones to accommodate the increasing size of the brain. Therefore, the sutures between the bones of the cranial vault as well as growth plates in the skull base remain

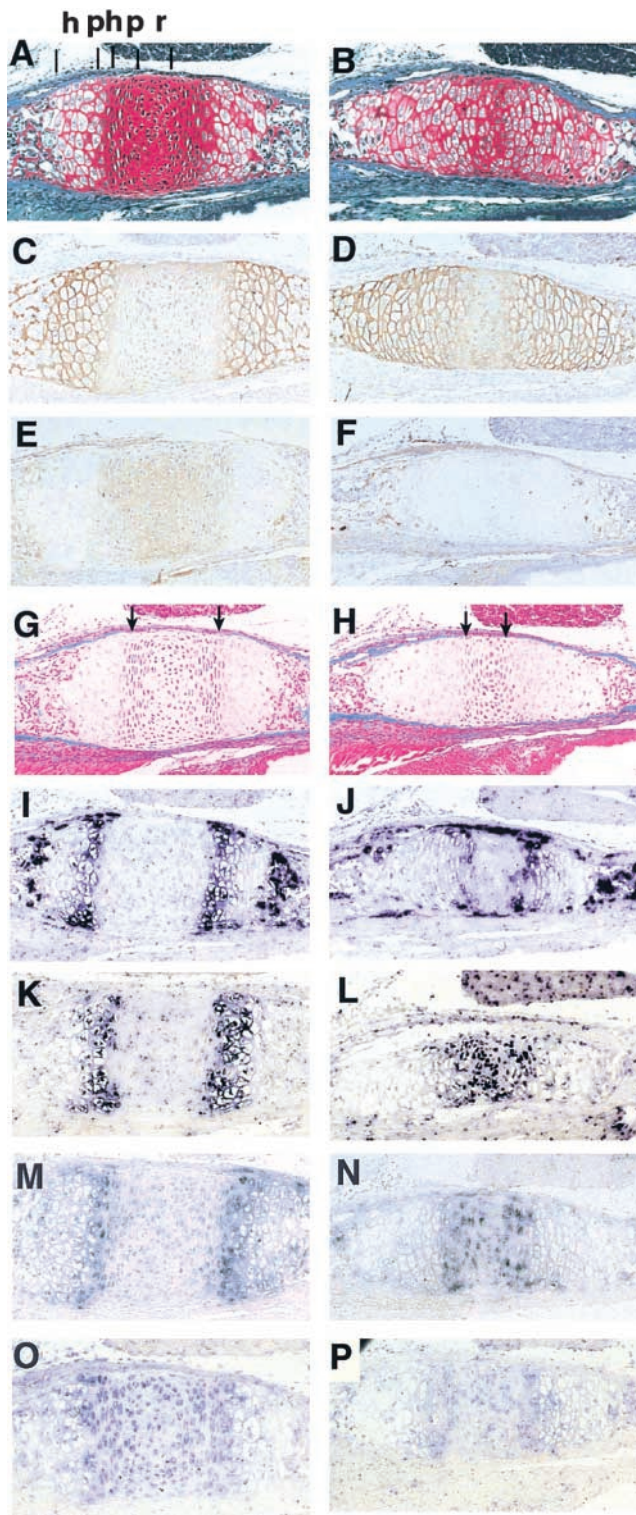


Figure 4. The histological changes in basooccipital-basosphenoïd synchondroses in 1.5-d-old wild-type and *Ltbp-3*-null animals.

(A and B) Weingart hematoxylin, Safranin O and Fast green staining. (A) Wild-type. (B) Mutant. Wider zone of hypertrophic chondrocytes in the mutant synchondrosis compared to the wild-type indicates more extensive differentiation. Cartilage is stained red. h, hypertrophic chondrocyte zone; r, resting chondrocyte zone; p, proliferating chondrocyte zone; ph, prehypertrophic chondrocyte zone; (C and D) Immunostaining for collagen X. (C) Wild-type. (D) Mutant. (E and F) Immunostaining for collagen II. (E) Wild-type. (F) Mutant. (G and H) Masson's trichrome staining for bone. More advanced bone

nonossified for an extensive period after birth. There are reports describing potential roles for TGF- β s in the differentiation of the membranous bones of the skull (Opperman et al., 1999), but there is no information concerning TGF- β function in the biology of the bones of the skull base. Histological studies of the sutures in wild-type and *Ltbp-3*-null animals revealed no pathological synostosis, i.e. premature fusion of one or more of the cranial vault sutures (unpublished data). However, differential staining for cartilage and bone in whole mount preparations, as well as histological analysis of the cranial base in 21 day old animals, revealed that the cartilaginous growth plates of the synchondroses were absent in mutant animals (Fig. 3, B and D), whereas the synchondroses were nonossified in wild-type littermates (Fig. 3, A and C).

Histologically, a synchondrosis resembles two opposed growth plates with a common zone of resting chondrocytes and separate zones of proliferating and hypertrophic chondrocytes. (Fig. 4 A). The earliest histological changes in the skull base of *Ltbp-3*-null animals were detected in the basooccipital-basosphenoïd synchondrosis 1–2 d after birth. The overall structure of the synchondrosis was altered, as no distinguishable columns of proliferating chondrocytes were visible in the mutant synchondrosis (Fig. 4, compare A and B), and the zones of hypertrophic chondrocytes were wider. Collagen X, a marker for hypertrophic chondrocytes (Elima et al., 1993), was restricted to the ends of the synchondrosis in wild-type animals (Fig. 4 C), but was detected almost throughout the synchondrosis in *Ltbp-3*-null animals (Fig. 4 D). In addition, collagen type II, a marker for nonhypertrophic chondrocytes (Swalla et al., 1988), was present in the central zone of wild-type synchondrosis, but was absent from that of *Ltbp-3*-null animals (Fig. 4, E and F). The distance between the cortical bone fronts was smaller in the null animals compared to wild-type littermates as visualized by Masson's trichrome staining, which stains bone blue (Fig. 4, G and H). The ectopic ossification in mutant synchondrosis was also clear from the expression of the bone sialoprotein (*Bsp-1*) gene, an osteoblast specific marker (Bianco et al., 1991), in the cells surrounding the synchondrosis (Fig. 4, I and J). Although the basooccipital-basosphenoïd synchondrosis was obliterated by 3–5 d after birth, the first changes in the basosphenoïd-presphenoïd synchondrosis in the null animals were not seen until days 3–10 (unpublished data).

fronts (blue) are apparent in mutant (H) versus wild-type (G) synchondrosis. Arrows point to fronts of the cortical basooccipital and basosphenoïd bones. (I and J). In situ hybridization for bone sialoprotein 1 (*Bsp-1*). (I) Wild-type. (J) Mutant. (K and L) In situ hybridization for *Ihh*. (K) Wild-type. (L) Mutant. The strong signal in the middle of the mutant mouse synchondrosis (L) suggests that these chondrocytes are already committed to hypertrophic differentiation. (M and N) In situ hybridization for *PTH/PTHrP-R*. Expression pattern is less defined in the mutant animal sample (N) compared to the wild-type (M), and the transcript is detected through the central region of the synchondrosis. The intensity of the signal is similar in wild-type and in *Ltbp-3*-null samples. (O and P). In situ hybridization for *PTHrP*. In wild-type synchondroses (O) expression is apparent in proliferating chondrocytes and in lateral chondrocytes of the central region of the synchondrosis. The signal is absent in the resting chondrocytes in the center of synchondrosis. The intensity of the signal is decreased in *Ltbp-3*-null synchondrosis (P).

Therefore, premature closure of the synchondroses in *Ltbp-3*-null mice is responsible for the observed craniofacial malformations. The failure to extend the presphenoid, basosphenoid, and the basoccipital bones results in a shortened cranial base. To accommodate the growing brain volume, the membranous bones of the vault expand outward and upward creating a domed skull. The anterior displacement of the foramen magnum may cause the development of the kyphosis, as the spinal column must be realigned with the overall body axis. The shortening of the cranial base also causes the alteration in the apposition of the incisors.

The wider zones of hypertrophic chondrocytes, as well as more advanced bone fronts in the *Ltbp-3*-null mice, resembled certain changes observed in animals deficient for the expression of parathyroid-hormone related protein (PTHrP) or its receptor PTH/PTHrP-R (for review see Karsenty, 2001). Vortkamp et al. (1996) proposed that chondrocyte differentiation is regulated by an inhibitory feedback loop in which Indian hedgehog (Ihh), produced by the most differentiated prehypertrophic and hypertrophic chondrocytes, stimulates the production of PTHrP by the periarticular cartilage and perichondrium, and PTHrP inhibits hypertrophic differentiation by interaction with its receptor on prehypertrophic chondrocytes (Chung et al., 2001). Therefore, we examined the expression of *Ihh*, PTHrP, and PTH/PTHrP-R in wild-type and mutant mice. In wild-type mice, *Ihh* was expressed by prehypertrophic and differentiating hypertrophic chondrocytes, whereas in null animals, *Ihh* expression was broader, indicating that chondrocytes in the central region of the synchondrosis were committed to hypertrophic differentiation (Fig. 4, K and L). In wild-type animals, PTH/PTHrP-R expression was detected in prehypertrophic chondrocytes, whereas in *Ltbp-3*-null animals, PTH/PTHrP-R transcripts were detected in the central region of the synchondrosis (Fig. 4, M and N). The expression pattern appeared less organized than that observed in wild-type synchondrosis, but the level of expression was similar as judged by the intensity of the staining. In sections probed for PTHrP, there was broad expression throughout the presumptive zone of proliferating chondrocytes, and the level of expression appeared lower in *Ltbp-3*-null animals compared to wild-type animals (Fig. 4, O and P). This decreased expression of PTHrP was also observed in the synchondroses of younger animals (see online supplemental material, available at <http://www.jcb.org/cgi/content/full/200111080/DC1>). A decreased level of PTHrP in *Ltbp-3*-null mice would allow more extensive chondrocyte differentiation and account for the more rapid ossification of the synchondroses.

The changes in *Ltbp-3*-null mice are consistent with previous reports describing a role for TGF- β ; in regulating PTHrP expression, although we have been unable to detect differences with antibodies that recognize either active TGF- β or phosphorylated Smads. Paderer et al. (2001) and Serra et al. (1999) demonstrated in cell and organ culture that TGF- β stimulates PTHrP expression and inhibits hypertrophic differentiation. We infer that in *Ltbp-3*-null mice there is a deficit in TGF- β that results in decreased PTHrP expression and early differentiation of the synchondroses, ectopic ossification, and synostosis. The decrease in PTHrP

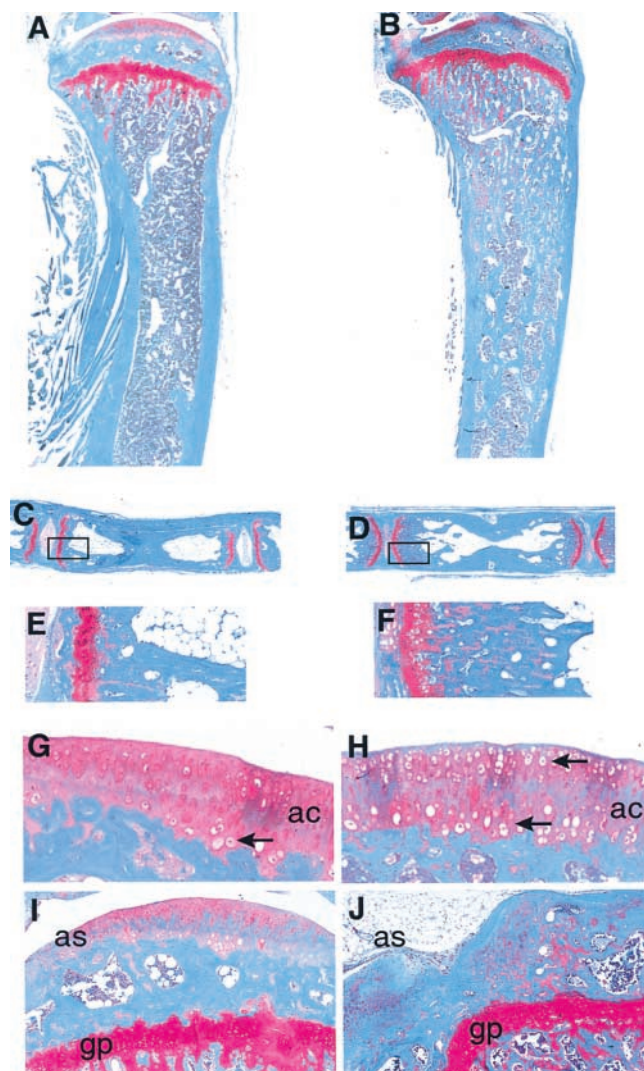


Figure 5. Changes in the structure of long bones in *Ltbp-3* mice. (A–F) Mid-sagittal sections of tibiae (A and B) and caudal vertebrae (C–F) of 9-mo-old wild-type (A, C, and E) and mutant (B, D, and F) animals stained with Weingart hematoxylin/Safranin O/Fast green. (E and F) Higher (5 \times) magnification of the regions boxed in C and D. (G and H) Mid-sagittal sections of the articular region of proximal tibiae in 6-mo-old wild-type (G) and mutant mice (H). (I and J). Mid-sagittal section of epiphyses of 9-mo-old wild-type and *Ltbp-3*-null mice. ac, articular cartilage; as, articular surface; gp, growth plate; arrowheads, hypertrophic chondrocytes.

coincident with *Ihh* expression in the central zone of the synchondroses suggests that TGF- β acts before the induction of PTHrP expression and after *Ihh* expression.

Defective bone remodeling

Because TGF- β has been shown to be important for long bone physiology, we examined the structure of the long bones of *Ltbp-3*-null mice to determine if other TGF- β -mediated functions were affected. By day 8 after birth, null animals showed growth retardation. The weight of adult *Ltbp-3*-null animals was 30–80% of sex-matched littermates, and the endochondral bones were shorter by ~10–25%. Histological analysis of the growth plates of tibiae, femora, and vertebrae from 1 d to 2-mo-old animals re-

vealed no obvious differences between *Ltbp-3*-null and wild-type littermates (unpublished data). Consistent with the report of Filvaroff et al. (1999), who expressed a type II TGF- β receptor with a truncated cytoplasmic domain (RIIDN) in osteoblasts and found age-dependent increases in trabecular bone mass, increased trabecular mass in long bones was observed at 3 mo of age in *Ltbp-3*-null mice with a body weight <60% of sex-matched wild-type littermates (unpublished data). This osteosclerosis was more pronounced in 6- and 9-mo-old mutant animals (Fig. 5, compare A and B). Staining for proteoglycans revealed unmineralized cores within the trabecular bones close to the growth plates, suggesting rapid extracellular matrix deposition and initiation of trabecular bone formation. The increased number of metaphyseal trabeculae also suggested slow turnover. Similar changes occurred in the vertebrae (5, C–F). Interestingly, mutations in LAP cause Camurati–Engelmann syndrome, which is characterized by sclerosis and hyperostosis (Janssens et al., 2000; Kinoshita et al., 2000), whereas transgenic animals overexpressing TGF- β 2 in osteoblasts develop osteoporosis (Erlebacher and Derynck, 1996). Hence, we conclude that an *Ltbp-3* defect mirrors long bone phenotypes caused by impaired TGF- β signaling.

The inhibition of TGF- β signaling has been shown to lead to periarticular cartilage terminal differentiation and ossification. Mice either expressing a TGF- β RIIDN in articular cartilage, synovium and periosteum/perichondrium (Serra et al., 1997), or deficient in *Smad-3* (Yang et al., 2001) develop degenerative joint disease. *Ltbp-3*-null animals also develop progressive degeneration of articular cartilage resembling osteoarthritis. Histological analysis of the knee joints of mutant and wild-type littermates revealed pathological changes in the articular cartilage of 6-mo-old *Ltbp-3*-null animals: proteoglycan staining was decreased (compare Fig. 5, G and H) and hypertrophic chondrocytes were detected in the superficial layers of the articular cartilage (Fig. 5 H). In wild-type mice, the articular cartilage consisted almost exclusively of mature nonhypertrophic chondrocytes (Fig. 5 G). In mutant mice, at 9 mo, articular cartilage was absent, the articular surface appeared ossified and fibrotic (Fig. 5 J), and osteophytes were present (unpublished data). Similar changes were observed in the vertebral joints (Fig. 5, C–F). The degenerative changes in *Ltbp-3*-null joints present later (6–9 mo) than those in RIIDN transgenic and *Smad-3*-deficient animals. We believe that *Ltbp-3* deficiency causes a more moderate decrease in TGF- β levels compared to the inhibition of TGF- β signaling in DNIIR and *Smad-3*-null animals.

Summary

This report describes the phenotype of the *Ltbp-3*-null mouse: premature obliteration of synchondroses, osteosclerosis, and osteoarthritis. These manifestations are consistent with published evidence suggesting TGF- β involvement in bone remodeling and homeostasis. Therefore, we propose that *Ltbp-3* regulates TGF- β bioavailability either by enhancing secretion of the TGF- β small latent complex or by participating in the activation of the latent TGF- β , as suggested from results obtained for LTBP-1 in cell culture

(Miyazono et al., 1991; Flaumenhaft et al., 1993). Irrespective of the mechanism, the phenotypic changes in *Ltbp-3*-null mice are consistent with previous results describing a role for TGF- β in regulating PTHrP expression, as well as with the effects of impaired TGF- β signaling on bone physiology in vivo. Hence, this represents the first report providing genetic evidence in support of the role for LTBP in regulating TGF- β bioavailability. Experiments to determine the precise mechanism underlying these phenotypes are underway.

Materials and methods

Ltbp-3 gene targeting

Ltbp-3 gene targeting strategy, as well as the production and characterization of mice with a disrupted *Ltbp-3* gene, are described in the online supplemental material (available at <http://www.jcb.org/cgi/content/full/200111080/DC1>). X-ray radiography was performed on anesthetized animals using a Micro 50 (Microfocus Imaging) at 30 kV for 10 s.

Histology and immunohistochemistry

Whole-skull staining with Alizarin red S and Alcian blue was performed as described (Lufkin et al., 1992). For histological analysis, samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C, decalcified in 10% EDTA/2.5% paraformaldehyde in PBS for 7–14 d at 4°C, dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. 5- μ m sections were stained with either Masson's Trichrome Stain or Weingart's hematoxylin/Fast Green/Safranin O (Luna, 1992) for bone and cartilage. Collagen II was detected using mouse monoclonal 2B1.5 antibody (NeoMarkers) and M.O.M. Kit (Vector) and collagen X using rabbit antiserum pXNC1-8, a gift from G. Lunstrum (Shriners Hospital for Children, Portland, OR) and the Vector Elite Kit.

In situ hybridization

RNA probes were prepared using DIG RNA labeling kit (Roche). The probes used were *Bsp-1*, a gift from I. Thesleff (University of Helsinki, Helsinki, Finland), *Ihh*, a gift from A. McMahon (Harvard University, Cambridge, MA), PTHrP-R, gift of A. Broadus (Yale University Medical School, New Haven, CT), and PTHrP, a gift of H. Kronenberg (Massachusetts General Hospital, Boston, MA). In situ hybridization was performed as described (Wassarman et al., 1997).

Online supplemental material

Included in the online supplemental materials (available at <http://www.jcb.org/cgi/content/full/200111080/DC1>) are details of the targeting strategy for production of *Ltbp-3* disruption and characterization of the genotypes of the mutant mice (Fig. S1), a picture of in situ hybridization of embryos probed for *Ltbp-3* expression (Fig. S2), and an illustration of PTHrP expression in the synchondroses of newborn animals (Fig. S3).

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