BEYOND CARRIER PROTEINS

Bone defects in latent TGF- β binding protein (Ltbp)-3 null mice; a role for Ltbp in TGF- β presentation

B Dabovic¹, Y Chen¹, C Colarossi¹, L Zambuto¹, H Obata¹
and D B Rifkin^{1,2}

¹Department of Cell Biology, New York University School of Medicine, 550 First Avenue, New York, New York 10016, USA

²Department of Medicine, New York University School of Medicine, New York, New York 10016, USA

(Requests for offprints should be addressed to D B Rifkin; Email: rifkid01@med.nyu.edu))

(H Obata is now at the Department of Ophthalmology, Jichi Medical School, Tochigi, Japan)

Abstract

The latent transforming growth factor (TGF)- β binding proteins (LTBP)-1, -3 and -4 bind the latent form of the multipotent cytokine TGF- β . To examine the function of the LTBP, we made a null mutation of *Ltbp-3* by gene targeting. The homozygous mutant animals developed cranio-facial malformations by 12 days. By three months, there was a pronounced rounding of the cranial vault, extension of the mandible beyond the maxilla, and kyphosis. The mutant animals developed osteosclerosis of the long bones and vertebrae as well as osteoarthritis between 6 and 9 months of age. These latter phenotypic changes were similar to those described for mice that have impaired TGF- β signaling. Thus, we suggest that *Ltbp-3* plays an important role in regulating TGF- β bioavailability as the phenotype of the *Ltbp-3* null mouse appears to result from decreased TGF- β signaling.

Histological examination of the skulls from null animals revealed no effects on calvarial suture closure. However, the synchondroses in the skull base were obliterated within 2 weeks of birth. This is in contrast to the wild-type synchondroses, which remain unossified throughout the

life of the animal and enable growth of the skull base through endochondral ossification. Histological changes in mutant basooccipital-basosphonoid synchondrosis were observed 1.5 days after birth. Compared with wild-type or heterozygous littermates, the basooccipital-basosphonoid synchondrosis of *Ltbp-3* null mice contained increased numbers of hypertrophic chondrocytes. The expression of bone sialoprotein-1 (a marker for osteoblasts) was observed in cells surrounding the synchondrosis at postnatal day 1.5 indicating ectopic ossification. The expression of Indian hedgehog (*Ihh*) (a marker for chondrocytes committed to hypertrophic differentiation) was found through the basooccipital-basosphonoid synchondrosis, whereas the expression of parathyroid hormone related protein (PTHrP), which inhibits chondrocyte differentiation, appeared to be diminished in *Ltbp-3* null mice. This suggests that *Ltbp-3* may control chondrocyte differentiation by regulating TGF- β availability. TGF- β may regulate PTHrP expression either downstream of *Ihh* or independently of *Ihh* signaling.

Journal of Endocrinology (2002) **175**, 129–141

Introduction

The latent transforming growth factor (TGF)- β binding proteins (LTBP-1, -2, -3 and -4) comprise a family of matrix molecules whose function(s) is (are) poorly understood (Oklu & Hesketh 2000, Koli *et al.* 2001). LTBP are composed of multiple (14–20) epidermal growth factor (EGF)-like domains (Fig. 1), many of which are Ca²⁺-binding, and four domains containing eight cysteines (8-cys) that are specific for the LTBP and fibrillins (FBN)-1 and -2 (Pereira *et al.* 1993, Zhang *et al.* 1994). The modular structure of *Ltbp-3* is shown in Fig. 1.

LTBP range in size from 125–240 kDa. The variation in molecular mass within and between isoforms is a result not only of different amino acid sequences but also of multiple translation initiation sites, splice variants and different glycosylation patterns. The LTBP are structurally similar to FBN-1 and -2, which also contain multiple EGF-like repeats as well as 8-cys domains. However, the FBNs are approximately 350 kDa and contain 47 EGF-like domains and ten 8-cys domains.

The *in vivo* biological functions of the LTBP are unknown. As might be expected from their resemblance to the microfibrillar FBNs, LTBP-1, -2 and -4 are

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

localized in the extracellular matrix (ECM). Both *in vivo* and *in vitro* immunolocalization studies have described LTBP-1 as a matrix constituent that colocalizes with both FBN-1 and fibronectin (Gibson *et al.* 1995, Saharinen *et al.* 1996, Taipale *et al.* 1996, Dallas *et al.* 2000). Matrix deposition appears to be covalent as LTBP-1 can only be incorporated in the presence of cells or cell products and most of the matrix-associated LTBP-1 can only be released by proteases (Taipale *et al.* 1995, Unsold *et al.* 2001). LTBP-2 is found predominantly as a component of elastic fibrils and has a much more restricted cellular distribution than LTBP-1 (Gibson *et al.* 1995, Shipley *et al.* 2000). LTBP-3 expression has been observed in osteoblasts and periosteal cells, endothelial cells, and a number of other cell types in developing embryos (Yin *et al.* 1995), whereas LTBP-4 is secreted by fibroblasts (Saharinen *et al.* 1998).

The proposed biological functions of the LTBP-1 relate primarily to the interaction of LTBP-1 with TGF- β and the possible role of LTBP-1 as a modulator of TGF- β availability (Oklu & Hesketh 2000, Koli *et al.* 2001). Indeed, LTBP-1 was originally isolated as a component of the latent TGF- β complex released by platelets (Kanzaki *et al.* 1990). The TGF- β s are 25 kDa homodimeric cytokines derived by proteolytic processing of larger propeptides within the trans Golgi (Massague 1998). However, unlike most propeptides, once cleaved from the cytokine, the TGF- β propeptides remain non-covalently associated with the mature TGF- β even after secretion into the extracellular milieu (Koli *et al.* 2001, Lawrence 1991). This complex of TGF- β and its propeptide, called the small latent complex (SLC), is unable to bind to the high affinity TGF- β receptor. The propeptide is often referred to as the latency associated peptide (LAP), and the dissociation of TGF- β from LAP is a crucial regulatory step in TGF- β presentation to its receptor. In most cells and tissues, the small latent complex is found bound to an LTBP. This large latent complex is formed via the interactions of cysteines in the LAP dimer with a pair of cysteines in the third 8-cys domain of LTBP-1, -3 or -4

(Gleizes *et al.* 1996, Saharinen *et al.* 1996, Saharinen & Keski-Oja 2000). LTBP-2 does not bind TGF- β nor do the FBNs (Saharinen & Keski-Oja 2000). All three TGF- β isoforms are reported to bind to LTBP-1 (Olofsson *et al.* 1992, Saharinen & Keski-Oja 2000).

The best documented function for an LTBP is that described by Miyazono *et al.* (1991), who demonstrated that the binding of LTBP-1 to the small latent complex facilitated secretion of the growth factor. The secretion of the small latent complex not bound to LTBP-1 was much slower than that of the large latent complex and there was a corresponding increase in the number of TGF- β 1 molecules with abnormal disulfide bonds. This was suggested to result from disulfide bond shuffling in the absence of LTBP-1. Thus, LTBP-1 appears to facilitate the appropriate folding, and consequently the secretion of TGF- β 1 by binding reactive cysteines at position 33 in the LAP dimer. An additional function for LTBP-1 is its potential role in modulating TGF- β bioavailability. Sato *et al.* (1990) and Flaumenhaft *et al.* (1993) reported that an antibody to LTBP-1 blocked the activation of latent TGF- β in cocultures of endothelial and smooth muscle cells. Nakajima *et al.* (1997) demonstrated that an antibody to LTBP-1 prevented the endothelial-mesenchymal transition required for formation of the endocardial cushions in embryonic mouse heart cultures. In these experiments, addition of TGF- β 1 or - β 2 overcame the effect of the LTBP-1 antibody suggesting that LTBP-1 modulates TGF- β levels during formation of the endocardial cushions. Gualandris *et al.* (2000), also using antibodies to LTBP-1, suppressed the appearance of endothelial cells in cultures of differentiating embryonic stem (ES) cells. This effect on ES cells was mimicked by the addition of either antibodies to TGF- β or free LAP and was overcome by the application of free TGF- β 1. These results are most easily interpreted as indicating a role for LTBP-1 in modulating the bioavailability of TGF- β .

The *in vivo* functions of the LTBP-1 are poorly understood. An LTBP-2 null mouse has been described with a severe embryonic lethal phenotype at embryonic day 3.5–6.5 (Shipley *et al.* 2000). The reason for lethality is not clear but is not related to TGF- β availability, because LTBP-2 does not bind the growth factor. These *in vivo* results imply that LTBP-2 has a role in early development either through structural functions or, perhaps, by binding to an as yet unidentified effector molecule. Experiments addressing the *in vivo* biology of LTBP-1, -3 or -4 have not yet been described.

We have generated an *Ltbp-3* null mouse as an approach to understanding the biological functions of this protein (Dabovic *et al.* 2002). *Ltbp-3* null animals had an altered skull shape caused by the premature ossification of the synchondroses. This resulted in a shortened skull base, a disproportionately short upper jaw, occipital bossing, and displacement of the foramen magnum to a more anterior

position. In addition, there was enhanced accumulation of trabecular bone in the long bones and vertebrae and degeneration of the articular cartilage as the animals aged. We suggest that these phenotypic changes result from decreased TGF- β availability.

Materials and Methods

Construction of the Ltbp-3 targeting vector, ES cell gene targeting, and generation of Ltbp-3 null mice

The methods for producing *Ltbp-3* null mice are described in Dabovic *et al.* (2002). Briefly, an *Ltbp-3* cDNA fragment, bases 461–893, from the beginning of the open reading frame (ORF), was generated by RT-PCR amplification of RNA from 2T3 osteoblasts (L Bonewald, University of Texas Health Science Center, San Antonio, TX, USA). This cDNA was used to screen a 129 SvEv mouse Lambda FixII genomic library (A Joyner, New York University School of Medicine, New York, NY, USA). A genomic clone in the 5' region of the *Ltbp-3* locus was characterized by restriction mapping. Partial exon–intron organization was established by sequencing of the phage DNA fragments subcloned into pBluescriptSK. A replacement-type targeting vector was designed using the pKSloxPNT plasmid. A 1.7 kb KpnI–EcoRI fragment, 5' region of homology, was subcloned into the corresponding restriction sites between the neo resistance (*neo*^r) gene expression cassette and the TK gene cassette. The vector was linearized and 40 μ g purified DNA was electroporated into 5×10^6 W4 cells. Cells were selected using G418 (200 μ g/ml) and gancyclovir (2 mM). Cell clones with a disrupted *Ltbp-3* gene were identified by Southern blot analysis. Three ES clones (17, 22 and 25) with homologous recombination events were identified. There were no gross chromosomal rearrangements as shown by karyotype analysis. Chimeric mice were produced by aggregation with morulas of SW mice (line 25) or by injection into blastocysts of C57BL/6 mice (line 22).

X-ray radiography

X-ray radiography was performed on anaesthetized 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, tissue samples were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4 °C, decalcified in 10% EDTA/2.5% PFA in PBS for 7–14 days at 4 °C, dehydrated through an ethanol series, cleared in xylene and embedded in paraffin. Sections 5 μ m thick were cut, and stained with either Masson's Trichrome Stain (Sigma),

or Weingart's hematoxylin/Fast Green/Safranin O stain (Luna 1992) for bone and cartilage histological analysis. All immunostaining was performed at room temperature. Immunostaining for type II collagen was performed using mouse monoclonal 2B1.5 antibody (NeoMarkers) and an MOM kit (Vector). Collagen X was immunostained using rabbit antiserum pXNC1–89 (gift from G. Lunstrum, Shriners Hospital for Children, Portland, OR, USA) and a Vector Elite kit (Vector).

Northern blot analysis

The presence of *Ltbp-3* mRNA was determined on total RNA samples prepared from the lungs of genotyped wild-type and LTPB-3 null animals. RNA was extracted using Trizol Reagent (Gibco-BRL). Northern blot analysis was performed using the probe spanning bases 1369–2916 of the ORF, obtained by PCR amplification of *Ltbp-3* cDNA, using the primers AAGCGACTTCTC CCTCTTCC (L3–5F) and GAGGGCAGGAATGCAT AGTT (L3–3R). This PCR product covers the region 3' to the deletion introduced by the recombination with the targeting vector. For the analysis of *Ltbp-3* tissue distribution, a mouse multiple tissue Northern blot (Clontech) membrane was hybridized with a 432 bp PCR amplification product spanning 462–894 bp of ORF (L3–4FR).

In situ hybridization

RNA probes were prepared using the DIG RNA labeling kit (Roche). The probes used were bone sialoprotein-1 (*Bsp-1*, gift from I Thesleff, University of Helsinki, Helsinki, Finland), Indian hedgehog (*Ihh*, gift from A McMahon, Harvard University, Cambridge, MA, USA), PTHrP-R (gift from A Broadus, Yale University Medical School, New Haven, CT, USA) and parathyroid hormone related protein (PTHrP) (gift from H Kronenberg, Massachusetts General Hospital, Boston, MA, USA). *In situ* hybridization was performed as previously described (Wassarman *et al.* 1997). Briefly, deparaffinized sections were treated with proteinase K (20 μ g/ml) for 6 min at room temperature: hybridization was performed overnight at 55 °C in 50% formamide. The slides were washed with $2 \times$ SSC containing 50% formamide at 65 °C for 30 min, and treated with RNase A (20 μ g/ml at 37 °C for 30 min). Anti-DIG-alkaline phosphatase (AP)-coupled antibody and BM Purple AP substrate (Roche) were used to detect the signal.

Animals

All animals were maintained and treated according to NIH guidelines.

Figure 2 X-ray radiography of 2-month-old wild-type (WT) and *Ltbp-3* null (KO) mice. The skull of the mutant animal is domed, the foramen magnum is shifted anteriorly, the upper jaw is short, and the closure of upper and lower incisors is altered with the lower incisors extending outside the upper incisors. The null animal also demonstrates thoracic kyphosis.

Results

Ltbp-3 null animals display skeletal defects

Ltbp-3 null mice were born in the expected Mendelian ratio of 1:2:1 (wild-type (wt), heterozygous and null). Null animals were distinguished from wild-type animals by a rounded skull and shortened snout by day 10. Two-month-old mutant mice had a domed skull as revealed by X-ray radiography (Fig. 2). These changes in skull shape also altered the normal apposition of the upper and lower incisors. In essentially all null animals the lower incisors extended outside of/abutted the upper incisors. X-ray radiography also demonstrated curvature of the cervical and thoracic vertebrae (thoracic kyphosis) and a distorted ribcage (Fig. 2).

Long bone phenotype

Whole mount skeletal preparations of *Ltbp-3* null embryos 18.5 days post-coitus revealed no defects in patterning and growth of the long bones (data not shown). By day 8 after

birth the null animals weighed 70–80% of wild-type and heterozygous sex-matched littermates. The decrease in size of mutant animals was even more pronounced by 3 weeks of age, and the weight of adult *Ltbp-3* null animals was 30–80% of wild-type and heterozygous littermates. The endochondral bones in null animals were approximately 10–25% shorter than those of wild-type littermates. The reason(s) for this decrease in growth of long bones and body weight is (are) not clear. Histological analysis of the growth plates of tibiae, femora and vertebrae from 1-day- to 2-month-old animals revealed no differences between *Ltbp-3* null and wild-type littermates (data not shown).

Osteosclerosis of Ltbp-3 null mutant mice

Differences consisting of an increase in trabecular mass in long bones of the mutant animals were observed at 3 months of age in animals with a severe phenotype (body weight less than 50% of sex-matched wild-type littermates; data not shown). The osteosclerosis was progressive and more pronounced in 6- and 9-month-old mutant

animals (Fig. 3A vs. B) and was most evident close to the growth plate, although an increased number of trabeculae were also observed in the metaphysial region. Safranin O staining for proteoglycans revealed unmineralized cores within the trabecular bones close to the growth plates, indicating rapid extracellular matrix deposition and new trabecular bone formation. A slow turnover of the mutant trabecular bone was suggested by the increased number of trabeculae in the metaphysial bone region. Similar changes were observed in the vertebrae of 9-month-old animals (Fig. 3C–F).

Osteoarthritis of Ltbp-3 null animals

Normal articular cartilage consists of mature chondrocytes arrested at a stage before hypertrophic differentiation. Pathological changes in the articular cartilage of 6-month-old *Ltbp-3* null animals were revealed by histological analysis of the knee joints of mutant and wild-type littermates. Proteoglycan staining was less intense in the articular cartilage of the tibiae from *Ltbp-3* null mice than in the articular cartilage of wild-type littermates (compare Fig. 3G and H). In addition, the superficial layers of the articular cartilage contained hypertrophic chondrocytes (Fig. 3H), whereas in wild-type mice the articular cartilage was composed almost exclusively of mature non-hypertrophic chondrocytes (Fig. 3G). In older animals (9 months old) the articular surface appeared fibrotic and/or ossified and staining for proteoglycans revealed the absence of articular cartilage (Fig. 3J). The maintenance of cartilage on the articular surface is necessary for proper joint function. These changes in the articular cartilage of the *Ltbp-3* null mice resemble the changes in human osteoarthritis.

Cranial synostosis in Ltbp-3 null animals

The enlargement of the skull requires the coordinated growth of multiple cranio-facial bones of two different types. The membranous bones, which comprise the cranial vault, are derived directly from mesenchymal condensations and, therefore, do not go through a cartilaginous stage. After development, growth occurs primarily at the bone ends in the regions of the sutures. The bones that constitute the base of the skull are formed by endochondral ossification. Two opposed growth plates, with a common zone of resting chondrocytes and separate zones of proliferating and hypertrophic chondrocytes, link the base of the occipital and the base of sphenoid bones (basooccipital–basosphenoid synchondrosis) and the base of the sphenoid and the presphenoid bones (basosphenoid–presphenoid synchondrosis) (Gakunga *et al.* 2000). After embryonic development, bone growth in the skull base is restricted to the zones of the synchondroses. The appropriate anatomical development of the skull requires that the growth of the membranous and endochondral bones continue post-

Figure 3 Increase in trabecular bone mass and degenerative changes in articular cartilage in *Ltbp-3* null mice. Mid-sagittal sections of the tibiae (A and B) and caudal vertebrae (C–F) of 9-month-old wild-type (A, C, E) and mutant (B, D, F) animals were stained with Weigert's hematoxylin, Safranin O and Fast green. Extensive osteosclerosis was observed in *Ltbp-3* deficient animals, with trabecular bone present in the diaphysis and metaphysis of tibiae and in the central part of the vertebrae. Unossified cores of the trabeculae were observed in the metaphysis of the tibiae and in vertebrae of the mutants as revealed by Safranin O staining. E and F show higher magnifications of the regions boxed in C and D, respectively. G and H, mid-sagittal sections of the articular region of the proximal tibiae in 6-month-old wild-type (G) and mutant mice (H). Safranin O staining for proteoglycans is less intense in the articular cartilage (ac) of *Ltbp-3* null mice than in the wild-type animals. The arrows point to the differentiated chondrocytes that are observed on the articular surface in the mutant, where only few differentiated chondrocytes are detected in the deep layers in the articular cartilage of the wild-type. I and J mid-sagittal show sections of the epiphyses of the 9-month-old wild-type and *Ltbp-3* null mice. as, articular surface; gp, growth plate. The cartilage chondrocytes are absent from the bone surface in the joint and the articular surface appears fibrotic and/or ossified in the mutant.

Figure 4 Histology of wild-type and *Ltbp-3* null mouse calvaria. Histology of posterior frontal sutures of 2-month-old wild-type (A) and *Ltbp-3* null (B) animals. Masson's trichrome staining stains bone dark blue. The arrow points to the connective tissue separating two frontal bones in a mutant animal.

nately to accommodate the increasing size of the brain. Therefore, the sutures between the bones of the cranial vault as well as synchondroses in the skull base remain non-ossified for an extensive period after birth. A common cause of abnormal skull shape is the premature fusion (craniosynostosis) of one or more of the calvarial sutures. Approximately ninety different craniosynostosis syndromes have been described in humans. Several of these synostosis syndromes (Pfeiffer, Crouzon and Apert syndromes and thanatophoric dysplasia Type II) have been replicated in mice by producing mutations in FGF receptors 1, 2 or 3 (Carlton *et al.* 1998, Iwata *et al.* 2000, Zhou *et al.* 2000, Hajihosseini *et al.* 2001). Mice with these mutations have a skull phenotype with occipital bossing similar to that which we observed in *Ltbp-3* null mice. Therefore, we initiated histological studies on the state of the sutures in wild-type and *Ltbp-3* null animals.

Synchondroses in Ltbp-3 null mice

Histological examination of 21-day-old animals failed to show abnormal fusion of the coronal, sagittal and lamboid sutures of the skull vault (data not shown). The only difference was observed in the posterior regions of the sutures between frontal bones. In wild-type mice, post-frontal (PF) suture fusion occurred during weeks 3–6 postnatally. Histological studies showed that at 2 months of age the PF suture in wild-type animals was fully closed with a continuous layer of bone linking the left and right frontal bones (Fig. 4A). However, at this time the PF suture was still patent in *Ltbp-3* null animals (Fig. 4B). Histological analysis of PF sutures of *Ltbp-3* null animals at 4 and 12 months showed that fusion of the frontal bones did not occur (data not shown). Although we observed histological changes in the mutant PF suture, the observed morphological difference, the failure to close, neither explained the early onset of the phenotype at 10–12 days of age nor did it explain the observed anatomical changes.

Staining for cartilage and bone in the skulls of 3-week-old animals gave the first clue concerning the histological change underlying the domed skull phenotype: the cartilaginous growth plates of the synchondroses between the bones of the skull base were absent in the null animals (Fig. 5B) but were still non-ossified in wild-type litter-

mates (Fig. 5A). The wild-type synchondroses are seen as the two dark blue lines representing cartilage in the basosphenoid–presphenoid and the basooccipital–basosphenoid synchondroses in Fig. 5A. Histological analysis of the cranial base in 21-day-old animals also illustrated that the wild-type synchondroses were still in the cartilaginous state (data not shown), whereas the mutant synchondroses had been replaced by bone (data not shown; Dabovic *et al.* 2002). Within the skull base, ossification occurred first in the occipital–basosphenoid synchondrosis and subsequently in the more anterior basosphenoid–presphenoid synchondrosis. In addition, within a synchondrosis, ectopic hypertrophic differentiation initiated along the midline and proceeded outward to the margins. At 3 days after birth, both cranial base synchondroses in wild-type mice remained open (Fig. 5C). However, all of the chondrocytes in the basooccipital–basosphenoid synchondrosis in *Ltbp-3* null animals appeared to be hypertrophic at this time, and the synchondrosis was obliterated by cortical bone (Fig. 5D). Between days 3–10, the first changes consisting of widening of the zone of hypertrophic chondrocytes were seen in the basosphenoid–presphenoid synchondrosis in the null animal (Fig. 5D). This premature ossification probably caused the shortening of the cranial base in mutant animals revealed by X-ray radiography (Fig. 2). The reduced growth of the bones of the skull base, in concert with relatively normal membranous bone development and growth in the cranial vault, may yield the abnormal skull shape observed in *Ltbp-3* null animals.

We next analyzed in detail the histology of the cranial base from birth to 10 days of age. The earliest changes in the skull base of *Ltbp-3* null animals were detected in the basooccipital–basosphenoid synchondrosis at 1–2 days after birth. At this time, the overall structure of the basooccipital–basosphenoid synchondrosis was altered. Columns of proliferating chondrocytes were not visible in the mutant synchondrosis (Fig. 6A vs. B). In addition, the zone of hypertrophic chondrocytes was wider in the null synchondrosis as verified by immunohistochemical staining using antibodies against collagen X, a protein specifically expressed by hypertrophic chondrocytes (Fig. 6C and D) (Elima *et al.* 1993). Collagen X expression was restricted to the ends of the synchondrosis in the wild-type

Figure 5 Histology of wild-type and *Ltbp-3* null mouse skull base. A and B, fusion of bones of the skull base in 3-week-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 the skull bases were dissected. The arrows point to the synchondroses between occipital (oc), sphenoid (sp) and presphenoid (ps) bones in wild-type mouse. These synchondroses are ossified in the mutant animal (B). C and D, mid-sagittal sections of 3-day-old wild-type (C) and mutant (D) skull bases. The synchondroses are stained in red by Safranin O. The anterior synchondrosis is on the right. The chondrocytes of the posterior (basooccipital–basosphenoid) synchondrosis in the mutant animal (D) are all in the hypertrophic stage. The organization of the anterior synchondrosis is also altered as the central region is narrower, and the zone of hypertrophic chondrocytes is wider compared with the wild-type synchondrosis.

animal (Fig. 6C), whereas the expression of collagen X was detected almost throughout the synchondrosis in *Ltbp-3* null animals (Fig. 6D); only at the very center was there a collagen X negative non-hypertrophic region. The ectopic ossification in mutant synchondroses was demonstrated by the expression of the bone sialoprotein 1 (*Bsp-1*) gene, an osteoblast specific marker (Bianco *et al.* 1991), in the cells surrounding basooccipital–basosphenoid synchondrosis (Fig. 6E and F). These results showed that pathological changes leading to ossification of the posterior skull base synchondroses in *Ltbp-3* null animals occurred as early as days 1–2 after birth.

The wider zone of hypertrophic chondrocytes as well as more advanced bone fronts in the *Ltbp-3* null mice resembled certain phenotypic changes observed in animals deficient for the expression of PTHrP or its receptor PTH/PTHrP-R (Amizuka *et al.* 1994, Karaplis *et al.* 1994, Lanske *et al.* 1996). Vortkamp *et al.* (1996), have

Figure 6 The histological changes in basooccipital–basosphenoid synchondroses in 1.5-day-old wild-type and *LTBP-3* null animals. A and B, Weigert's hematoxylin, Safranin O and Fast Green staining. The staining of wild-type (A) and mutant (B) basooccipital–basosphenoid synchondroses revealed a wider zone of hypertrophic chondrocytes in the mutant synchondrosis compared with the wild-type indicating more extensive differentiation. Cartilage is stained red. r, zone of resting chondrocytes; p, zone of proliferating chondrocytes; ph, zone of prehypertrophic chondrocytes; h, zone of hypertrophic chondrocytes. C and D, immunostaining for collagen X. Collagen X is a marker of the hypertrophic chondrocytes. Staining for collagen X showed the widening of the zone of hypertrophic chondrocytes in *Ltbp-3* null animals. C, wild-type; D, mutant. E and F, *in situ* hybridization for bone sialoprotein 1 (*Bsp-1*). *Bsp-1* is a marker of osteoblasts in different stages of differentiation. *In situ* hybridization with a *Bsp-1* probe revealed that the *in situ* hybridization signal is detected in the cells surrounding the synchondrosis in the mutant (F), but is missing in the wild-type synchondrosis (E). A weaker signal is also observed in the upper layer of hypertrophic chondrocytes both in wild-type and in the mutant tissues.

proposed that normal chondrocyte differentiation is regulated by an inhibitory feedback loop consisting of *Ihh* and PTHrP. *Ihh* is produced by the most differentiated prehypertrophic chondrocytes and hypertrophic chondrocytes (Bitgood & McMahon 1995). *Ihh* stimulates the production of PTHrP in cells in the periarticular cartilage and perichondrium. PTHrP, in turn, interacts with its receptor on prehypertrophic chondrocytes and inhibits hypertrophic differentiation.

To test whether this inhibitory loop might be affected in the synchondroses of *Ltbp-3* null mice, 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 the null animals, there was an expanded region of *Ihh* expression indicating that

Figure 7 *Ihh* and PTHrP in *Ltbp-3* null synchondroses. A and B, *in situ* hybridization for *Ihh*. *Ihh* is preferentially expressed in prehypertrophic chondrocytes. *In situ* hybridization revealed a signal in the zone of hypertrophic chondrocytes of the wild-type animal (A). A strong signal was observed in the middle of the mutant mouse synchondrosis (B) suggesting that small chondrocytes in this region are already committed to hypertrophic differentiation. C and D, *in situ* hybridization for *PTHrP*. *PTHrP* is expressed in wild-type prehypertrophic chondrocytes. *In situ* hybridization indicated that the expression pattern is less defined in the mutant animal sample (D) compared with the wild-type (C), and the transcript is detected through the central region of the synchondrosis. The intensity of the signal is similar in the wild-type and in *Ltbp-3* null mouse. E and F, *in situ* hybridization for *PTHrP*. *In situ* hybridization with a probe for *PTHrP* in wild-type synchondroses (E) revealed expression signal in proliferating chondrocytes, in lateral chondrocytes of the central region of the synchondrosis and no signal in the resting chondrocytes in the center of synchondrosis. The intensity of the signal is decreased in *Ltbp-3* null synchondrosis (F).

some of the chondrocytes in the central region of the synchondrosis were already committed to hypertrophic differentiation (Fig. 7A and B). In the wild-type animals, there was a zone of PTH/PTHrP-R expression in the prehypertrophic chondrocytes over the zone of hypertrophic chondrocytes. In *Ltbp-3* null animals, PTH/PTHrP-R transcripts were detected in the central region of the synchondrosis (Fig. 7C and D). The expression pattern appeared less organized than that observed in wild-type synchondroses, but the degree of expression was similar as judged by the intensity of the staining. In the sections probed for PTHrP expression, there was broad expression throughout the presumptive zone of proliferating chondrocytes. However, the level of expression appeared lower in the *Ltbp-3* null animals compared with wild-type animals (Fig. 7E and F). A decreased level of PTHrP in *Ltbp-3* null mice would allow more extensive chondrocyte differentiation and could account for the more rapid ossification of the synchondroses.

Ltbp-3 expression pattern

The studies described above indicate that *Ltbp-3* has a function in the normal differentiation of the synchondroses in the skull base and in remodeling of the long bones. To evaluate the distribution of *Ltbp-3*, we performed RNA hybridization experiments on embryos as well as tissue sections. As can be seen in Fig. 8A *Ltbp-3* expression was widespread in day 13.5 and 16.5 embryos. Reactive regions included the bones of the base of the skull, nasal cartilage, tongue, digits, vertebrae, lung, heart and areas of the brain. In older animals transcripts for *Ltbp-3* were observed at a position corresponding to 4.6 kb by Northern blotting of RNA extracted from heart, brain, lung and kidney (Fig. 8B). Lower levels of transcript were present in testis and skeletal muscle, whereas there was essentially no expression in spleen and liver. In several tissues an additional band was observed at 7.0 kb. The identity of this band is not clear, but it is possible that it represents a long form of *Ltbp-3* similar to the longer transcript described for LTBP-1 (Koli *et al.* 2001). The expression pattern of *Ltbp-3* suggested that additional phenotypes, which are not obvious by the histological approaches taken thus far, may be present in the brain, lungs, heart and kidneys of the *Ltbp-3* null animals.

Discussion

By homologous recombination, we generated mice with a deletion in the *Ltbp-3* gene (Dabovic *et al.* 2002). Analysis of the exon structure of the *Ltbp-3* gene indicated that, this deletion should result in a frame shift in protein translation and lead to either production of a short truncated protein or a destabilization of the mRNA, essentially yielding a null mutation. Northern analysis revealed the absence of *Ltbp-3* mRNA (Dabovic *et al.* 2002), suggesting the latter postulate. Mice homozygous for the mutation displayed defects in skeletal development with domed skulls, shortened maxilla, anteriorized foramen magnum, kyphosis and smaller size. The cranio-facial phenotype developed in all animals and was observed as a rounded head by 10–12 days after birth. This skull abnormality occurred in mice from three different genetic backgrounds and with two independently targeted ES cells. The phenotype was not seen in heterozygous animals. Histological analysis revealed that the skull abnormalities result from premature closure of the synchondroses at the base of the skull. Changes in the synchondroses were observed as early as 1–2 days postnatally.

We believe that the premature closure of the synchondroses in *Ltbp-3* null mice was responsible for the observed cranio-facial malformations. The fusion of the two synchondroses prevented the endochondral bones at the base of the skull from extending along the anterior–posterior axis. The failure to extend the presphenoid, basosphenoid and the basooccipital bones resulted in a

Figure 8 Expression pattern of mouse *Ltbp-3*. A, *in situ* hybridization on para-sagittal sections of 13.5- and 16.5-day-old embryos. In E13.5 and E16.5 embryos *Ltbp-3* is preferentially expressed in cartilage primordia, in the forebrain, heart, lung and spinal cord. The arrow points to the cartilage primordia of the sphenoid bone. B, expression of *Ltbp-3* in adult mouse tissues. The highest level of expression is detected in heart, brain, lung and kidney. Control hybridization with a β -actin probe is shown in the lower panel. H, heart; B, brain; Sp, spleen; Lu, lung; L, liver; Sm, skeletal muscle; K, kidney; T, testis.

shortened cranial base. To accommodate the growing brain, the membranous bones of the vault expand outward and upward. This created the domed skull and may be responsible for failure of the post frontal (PF) suture to fuse. The anterior displacement of the foramen magnum was a consequence of the expanded volume of the cranial vault, together with the shortened cranial floor. We suggest that the altered position of the foramen magnum caused the development of the kyphosis, as the spinal column, which exits the skull more anteriorly, must be realigned with the overall body axis. The shortening of the cranial base also disrupted the normal closure of the upper and lower jaws which caused the alteration in the apposition of the upper and lower incisors.

The mechanism responsible for the synostosis in *Ltbp-3* null mice is not clear. We suggest two alternatives. First, *Ltbp-3* may have a structural function within the synchondroses, which in the absence of *Ltbp-3* ossify prematurely. Secondly, *Ltbp-3* may regulate the availability of TGF- β in the synchondrosis, and the alteration of TGF- β levels resulting from lack of *Ltbp-3* leads to premature obliteration of the synchondroses. Support for the first mechanism derives from the work of Dallas *et al.* (1995), who reported that LTBP-1 mediated the differentiation of cultured rat calvarial osteoblasts into mineralized bone nodules. Treatment of calvarial cell cultures with antibody or antisense oligonucleotides to LTBP-1 blocked mineralized nodule appearance. As exposure of control cultures to neutralizing antibodies to TGF- β 1 or TGF- β 2 did not block nodule formation, the authors suggested that LTBP-1 functioned directly to facilitate cell differentiation. Alternatively, as described in the Introduction, there are several published *in vitro* experiments indicating that LTBP-1 regulates TGF- β availability (Sato *et al.* 1990, Miyazono *et al.* 1991, Flaumenhaft *et al.* 1993, Nakajima *et al.* 1997, Gualandris *et al.* 2000). This may be accomplished both by enhancing secretion of the TGF- β small latent complex and by participating in the activation of latent TGF- β . Although there are no published experiments of this type for LTBP-3, LTBP-3 does bind the TGF- β 1, - β 2 and - β 3 small latent complexes (Y Chen and D B Rifkin, unpublished data) and therefore may have a similar function to LTBP-1.

The literature regarding the role of TGF- β in the differentiation of the membranous bones of the skull is somewhat contradictory (Roth *et al.* 1996, Opperman *et al.* 1999). Although there are results from organ culture experiments indicating that closure of the PF suture is regulated in a positive manner by the addition of TGF- β 1, this effect is believed to be dependent upon interactions of the calvaria and the underlying dura (Opperman *et al.* 1995, Roth *et al.* 1996). A more refined study by Opperman *et al.* (1999) examined the roles of the different TGF- β isoforms in coronal suture closure by monitoring the effects of neutralizing antibodies to individual TGF- β isoforms. This approach indicated isoform specific

activities as the different TGF- β s had either promoting or inhibitory effects. However, experiments of this type have not been reported for skull base synchondroses, and therefore there is no information concerning TGF- β function in these structures.

There are a number of reports analyzing the role of TGF- β in chondrocyte differentiation. Paderer *et al.* (2001) showed that treatment with TGF- β 1 stimulated the expression of PTHrP in chick sternal chondrocyte primary cell culture and inhibited hypertrophic differentiation. Serra *et al.* (1999) demonstrated that treatment of cultured mouse metatarsal bone rudiments with TGF- β 1 inhibited chondrocyte proliferation and hypertrophic differentiation and stimulated the expression of *Pthrp*. Chung *et al.* (2001) demonstrated that *Ihh* signals the position of prehypertrophic and hypertrophic chondrocytes and thus regulates the amount of PTHrP synthesized in the peri-articular region *in vivo*. PTHrP then inhibits chondrocyte transition from the proliferative to the hypertrophic state and in this way controls the site and extent of hypertrophy. *Ihh* also couples cartilage differentiation and bone collar formation by inducing ossification in the perichondrium adjacent to hypertrophic chondrocytes. However, other regulatory molecules through which *Ihh* and PTHrP regulate the rate of chondrocyte differentiation and long bone collar formation may exist. Our data, the results of Serra *et al.* (1999), as well as the results from a recent paper by Alvarez *et al.* (2002) indicating that the ability of sonic hedgehog to regulate *Pthrp* expression in mouse embryonic metatarsal organ cultures is dependent upon TGF- β 2, indicate that TGF- β may be one such molecule.

Ltbp-3 null animals developed age-dependent osteosclerosis with increased trabecular bone mass in both the long bones and the axial skeleton. The mechanism underlying this defect in bone remodeling is not clear. Support for our hypothesis that the phenotype of the *Ltbp-3* null animals is the consequence of decreased active TGF- β comes from the work of Filvaroff *et al.* (1999), who reported that the inhibition of TGF- β signaling in osteoblasts caused age-dependent increase in trabecular bone mass. In this work transgenic mice were generated which expressed a type II TGF- β receptor with a truncated cytoplasmic domain (RIIDN) under the control of the osteocalcin promotor. As the RIIDN mice showed normal rates of bone deposition, the suggested mechanism underlying the observed osteosclerosis was decreased osteoclastic bone resorption, indicating that TGF- β signaling in osteoblasts is required for normal coupling of bone formation and resorption. These changes were similar to those we observed in the long bones of *Ltbp-3* null mutants. In addition, it has been reported that mutations in TGF- β 1:LAP cause Camarati-Engelman syndrome, which is characterized by hyperostosis and sclerosis of the diaphyses of the long bones (Janssens *et al.* 2000, Kinoshita *et al.* 2000). The osteosclerotic phenotype would be consistent with decreased TGF- β availability, perhaps,

resulting from improper SLC secretion. Other support for an important role for TGF- β in bone remodeling derives from the data of Erlebacher & Derynck (1996) who demonstrated that transgenic animals over-expressing TGF- β 2 in osteoblasts develop osteoporosis.

Serra *et al.* (1997) generated mice that express a TGF- β RIIDN in articular cartilage, synovium and periosteum/perichondrium, and showed that TGF- β plays an essential role in maintenance of periarticular cartilage. In the absence of TGF- β signaling, periarticular cartilage undergoes terminal differentiation and ossification – the pathological changes observed in osteoarthritis in humans. Similar degenerative changes were observed in the articular cartilage of *Ltbp-3* null animals, although the phenotype is less severe than in RIIDN animals. An essential role for TGF- β in maintaining articular cartilage is also supported by the work of Yang *et al.* (2001) who studied mice deficient for Smad-3. Smad-2 and Smad-3 are intracellular mediators of TGF- β signaling. Smad-2 and Smad-3 are phosphorylated in response to TGF- β binding to its receptors, and the phosphorylated Smad-2 and -3 bind to Smad-4, migrate into the nucleus, and modify transcription (Massague *et al.* 2000). Smad-3 deficient animals develop degenerative joint disease similar to RIIDN mice (Yang *et al.* 2001). The degenerative changes observed in the articular cartilage in *Ltbp-3* null animals correspond to both the pathological changes in human osteoarthritis and the degenerative changes described in the joints of both RIIDN transgenic and Smad-3 null animals. However, the degenerative changes of *Ltbp-3* null joints are observed later (6–9 months) than those in RIIDN transgenic and Smad-3 deficient animals. This may be explained by more moderate changes in TGF- β signaling caused by altered levels of active TGF- β in cartilage and bone as a consequence of the *Ltbp-3* deficiency, rather than the more complete inhibition of TGF- β signaling in dominant negative receptor II (DNRII) and Smad-3 null animals.

Our hypothesis predicts that there should be decreased active TGF- β in the synchondroses of *Ltbp-3* null animals. To date, we have been unsuccessful in attempts to detect such a difference with antibodies reported to recognize specifically active TGF- β . However, the detection of differences in the levels of active TGF- β in tissues is hindered by the short half-life of the free cytokine and its rapid clearance in the extracellular milieu. It is also possible that the absolute amount of extracellular TGF- β isoforms is equal in wild-type and *Ltbp-3* null mice, but in the mutant mice, a significant fraction of the TGF- β is misfolded (Miyazono *et al.* 1991). A third explanation is that LTBP-3 has a direct effect on the induction of *Pthrp* expression. These possibilities can be tested by *in vitro* experiments with synchondrosis organ cultures.

We suggest that lower concentrations of TGF- β lead to the decreased synthesis of PTHrP, misexpression of *Ihh*, widening of the zone of hypertrophic chondrocytes in the synchondroses and, consequently, to the observed ectopic

ossification and synostosis. Our data indicating a lower level of expression of *Pthrp* in the synchondroses of *Ltbp-3* null animals is consistent with this model. Exactly how TGF- β participates in the *Ihh*-PTHrP control loop is unclear. However, the observed decrease in *Pthrp* 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.

Acknowledgements

Figures 2, 3, 5A and B, 6, 7 and 8A are reproduced from the Journal of Cell Biology (2002) 156, 227–233 by copyright permission of The Rockefeller University Press. The authors would like to thank R Peralta, J Annes, B Arias, E Arteaga-Solis, A Auerbach, A Joyner, S Ortega, and M Vassallo for assistance. HO was supported by a Bausch and Lomb Overseas Research Fellowship. This work was supported by grants from the NIH CA 34282, CA 78422, and DE 13742, and from the National Marfan Foundation to DBR. BD was supported by DOD grant number DAMD 17–99–1–9319.

References

- Alvarez J, Sohn P, Zeng X, Doetschman T, Robbins DJ & Serra R 2002 TGF β 2 mediates the effects of Hedgehog on hypertrophic differentiation and PTHrP expression. *Development* **129** 1913–1924.
- Amizuka N, Warshawsky H, Henderson JE, Goltzman D & Karaplis AC 1994 Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *Journal of Cell Biology* **126** 1611–1623.
- Bianco P, Fisher LW, Young MF, Termine JD & Robey PG 1991 Expression of bone sialoprotein (BSP) in developing human tissues. *Calcified Tissue International* **49** 421–426.
- Bitgood MJ & McMahon AP 1995 Hedgehog and Bmp genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. *Developmental Biology* **172** 126–138.
- Carlton MB, Colledge WH & Evans MJ 1998 Crouzon-like craniofacial dysmorphism in the mouse is caused by an insertional mutation at the *Fgf3/Fgf4* locus. *Developmental Dynamics* **212** 242–249.
- Chung U-I, Schipani E, McMahon AP & Kronenberg HM 2001 Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *Journal of Clinical Investigation* **107** 295–304.
- Dabovic B, Chen Y, Colarossi C, Obata H, Zambuto L, Perle MA & Rifkin DB 2002 Bone abnormalities in latent TGF- β binding protein (*Ltbp*)-3 null mice indicate a role for *Ltbp*-3 in modulating TGF- β bioavailability. *Journal of Cell Biology* **156** 227–232.
- Dallas SL, Keene DR, Bruder SP, Saharinen J, Sakai LY, Mundy GR & Bonewald LF 2000 Role of the latent transforming growth factor beta binding protein 1 in fibrillin-containing microfibrils in bone cells *in vitro* and *in vivo*. *Journal of Bone and Mineral Research* **15** 68–81.
- Dallas SL, Miyazono K, Skerry TM, Mundy GR & Bonewald LF 1995 Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein. *Journal of Cell Biology* **131** 539–549.

- Elima K, Eerola I, Rosati R, Metsaranta M, Garofalo S, Perala M, De Crombrughe B & Vuorio E 1993 The mouse collagen X gene: complete nucleotide sequence, exon structure and expression pattern. *Biochemical Journal* **289** 247–253.
- Erlebacher A & Derynck R 1996 Increased expression of TGF- β 2 in osteoblasts results in an osteoporosis-like phenotype. *Journal of Cell Biology* **132** 195–210.
- Filvaroff E, Erlebacher A, Ye J-Q, Gitelman SE, Lotz J, Heilman M & Derynck R 1999 Inhibition of TGF- β receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. *Development* **126** 4267–4279.
- Flaumenhaft R, Abe M, Sato Y, Miyazono K, Harpel J, Heldin CH & Rifkin DB 1993 Role of the latent TGF- β binding protein in the activation of latent TGF- β by co-cultures of endothelial and smooth muscle cells. *Journal of Cell Biology* **120** 995–1002.
- Gakunga PT, Kuboki Y & Opperman LA 2000 Hyaluronan is essential for the expansion of the cranial base growth plates. *Journal of Craniofacial Genetics and Developmental Biology* **20** 53–63.
- Gibson MA, Hatzinikolas G, Davis EC, Baker E, Sutherland GR & Mecham RP 1995 Bovine latent transforming growth factor beta 1-binding protein 2: molecular cloning, identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils. *Molecular and Cellular Biology* **15** 6932–6942.
- Gleizes PE, Beavis RC, Mazzieri R, Shen B & Rifkin DB 1996 Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1. *Journal of Biological Chemistry* **271** 29891–29896.
- Gualandris A, Annes JP, Arese M, Noguera I, Jurukovski V & Rifkin DB 2000 The latent transforming growth factor-beta-binding protein-1 promotes *in vitro* differentiation of embryonic stem cells into endothelium. *Molecular Biology of the Cell* **11** 4295–4308.
- Hajihosseini MK, Wilson S, De Moerloose L & Dickson C 2001 A splicing switch and gain-of-function mutation in *FgfR2-IIIc* hemizygotes causes Apert/Pfeiffer-syndrome-like phenotypes. *PNAS* **98** 3855–3860.
- Iwata T, Chen L, Li C, Ovchinnikov DA, Behringer RR, Francomano CA & Deng CX 2000 A neonatal lethal mutation in *FGFR3* uncouples proliferation and differentiation of growth plate chondrocytes in embryos. *Human Molecular Genetics* **9** 1603–1613.
- Janssens G, Gershoni-Baruch R, Gnañabens N, Migone N, Ralston S, Bonduelle M, Lissens W, Maldergem LV, Vanhoenck F, Verbruggen L & Hul WV 2000 Mutations in the gene encoding the latency-associated peptide of TGF- β 1 cause Camurati-Engelmann disease. *Nature Genetics* **26** 273–275.
- Kanzaki T, Olofsson A, Moren A, Wernstedt C, Hellman U, Miyazono K, Claesson-Welsh L & Heldin CH 1990 TGF- β 1 binding protein: a component of the large latent complex of TGF- β 1 with multiple repeat sequences. *Cell* **61** 1051–1061.
- Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM & Mulligan RC 1994 Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes and Development* **8** 277–289.
- Kinoshita A, Saito T, Tomita H, Makita Y, Yoshida K, Ghadami M, Yamada K, Kondo S, Ikegawa S, Nishimura G, Fukushima Y, Nakagomi T, Saito H, Sugimoto T, Kamegaya M, Hisa K, Murray JC, Taniguchi N, Niikawa N & Yoshiura K 2000 Domain-specific mutations in *TGFB-1* result in Camurati-Engelmann disease. *Nature Genetics* **26** 19–20.
- Koli K, Saharinen J, Hyytiäinen M, Penttinen C & Keski-Oja J 2001 Latency, activation, and binding proteins of TGF- β . *Microscopy Research and Technique* **52** 354–362.
- Kosher RA, Kulyk WM & Gay SW 1986 Collagen gene expression during limb cartilage differentiation. *Journal of Cell Biology* **102** 1151–1156.
- Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LHK, Ho C, Mulligan RC, Abou-Samra A-B, Jüppner H, Segre GV & Kronenberg HM 1996 PTH/PTHrP receptor in early development and indian hedgehog-regulated bone growth. *Science* **273** 663–666.
- Lawrence DA 1991 Identification and activation of latent transforming growth factor beta. *Methods in Enzymology* **198** 327–336.
- Lufkin T, Mark M, Hart CP, Dolle P, LeMeur M & Chambon P 1992 Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. *Nature* **359** 835–841.
- Luna L 1992 Histopathologic methods and color atlas of special stains and tissue artifacts. Gaithersburg, MD, USA: American Histolabs Inc.
- Massague J 1998 TGF- β signal transduction. *Annual Review of Biochemistry* **67** 753–791.
- Massague J, Blain SW & Lo RS 2000 TGF β signaling in growth control, cancer, and heritable disorders. *Cell* **103** 295–309.
- Miyazono K, Olofsson A, Colosetti P & Heldin CH 1991 A role of the latent TGF- β 1-binding protein in the assembly and secretion of TGF- β 1. *EMBO Journal* **10** 1091–1100.
- Nakajima Y, Miyazono K, Kato M, Takase M, Yamagishi T & Nakamura H 1997 Extracellular fibrillar structure of latent TGF beta binding protein-1: role in TGF beta-dependent endothelial-mesenchymal transformation during endocardial cushion tissue formation in mouse embryonic heart. *Journal of Cell Biology* **136** 193–204.
- Oklu R & Hesketh R 2000 The latent transforming growth factor beta binding protein (LTBP) family. *Biochemical Journal* **352** 601–610.
- Olofsson A, Miyazono K, Kanzaki T, Colosetti P, Engstrom U & Heldin CH 1992 Transforming growth factor-beta 1, -beta 2, and -beta 3 secreted by a human glioblastoma cell line. Identification of small and different forms of large latent complexes. *Journal of Biological Chemistry* **267** 19482–19488.
- Opperman LA, Chhabra A, Cho RW & Ogle RC 1999 Cranial suture obliteration is induced by removal of transforming growth factor (TGF)-beta 3 activity and prevented by removal of TGF-beta 2 activity from fetal rat calvaria *in vitro*. *Journal of Craniofacial Genetics and Developmental Biology* **19** 164–173.
- Opperman LA, Passarelli RW, Morgan EP, Reintjes M & Ogle RC 1995 Cranial sutures require tissue interactions with dura mater to resist osseous obliteration *in vitro*. *Journal of Bone and Mineral Research* **10** 1978–1987.
- Patader DB, Ferguson CM, Ionescu AM, Schwarz EM, Rosier RN, Puzas JE & O'Keefe RJ 2001 PTHrP expression in chick sternal chondrocytes is regulated by TGF-beta through Smad-mediated signaling. *Journal of Cellular Physiology* **188** 343–351.
- Pereira L, D'Alessio M, Ramirez F, Lynch JR, Sykes B, Pangilinan T & Bonadio J 1993 Genomic organization of the sequence coding for fibrillin, the defective gene product in Marfan syndrome. *Human Molecular Genetics* **2** 961–968.
- Roth DA, Bradley JP, Levine JP, McMullen HF, McCarthy JG & Longaker MT 1996. Studies in cranial suture biology: part II Role of the dura in cranial suture fusion. *Plastic and Reconstructive Surgery* **97** 693–699.
- Saharinen J & Keski-Oja J 2000 Specific sequence motif of 8-Cys repeats of TGF- β binding proteins, LTBP, creates a hydrophobic interaction surface for binding of small latent TGF- β . *Molecular Biology of the Cell* **11** 2691–2704.
- Saharinen J, Taipale J & Keski-Oja J 1996 Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. *EMBO Journal* **15** 245–253.
- Saharinen J, Taipale J, Monni O & Keski-Oja J 1998 Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4. *Journal of Biological Chemistry* **273** 18459–18469.
- Sato Y, Tsuboi R, Lyons R, Moses H & Rifkin DB 1990 Characterization of the activation of latent TGF- β by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *Journal of Cell Biology* **111** 757–763.

- Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R & Moses HL 1997 Expression of a truncated, kinase-defective TGF- β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *Journal of Cell Biology* **139** 541–552.
- Serra R, Karaplis A & Sohn P 1999 Parathyroid hormone-related peptide (PTHrP)-dependent and -independent effects of transforming growth factor β (TGF- β) on endochondral bone formation. *Journal of Cell Biology* **145** 783–794.
- Shipley JM, Mecham RP, Maus E, Bonadio J, Rosenbloom J, McCarthy RT, Baumann ML, Frankfater C, Segade F & Shapiro SD 2000 Developmental expression of latent transforming growth factor beta binding protein 2 and its requirement early in mouse development. *Molecular and Cellular Biology* **20** 4879–4887.
- Swalla BJ, Upholt WB & Solursh M 1988 Analysis of type II collagen RNA localization in chick wing buds by *in situ* hybridization. *Developmental Biology* **125** 51–58.
- Taipale J, Lohi J, Saarinen J, Kovanen PT & Keski-Oja J 1995 Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *Journal of Biological Chemistry* **270** 4689–4696.
- Taipale J, Saharinen J, Hedman K & Keski-Oja J 1996 Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils. *Journal of Histochemistry and Cytochemistry* **44** 875–889.
- Unsold C, Hyytiäinen M, Bruckner-Tuderman L & Keski-Oja J 2001 Latent TGF-beta binding protein LTBP-1 contains three potential extracellular matrix interacting domains. *Journal of Cell Science* **114** 187–197.
- Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM & Tabin CJ 1996 Regulation of rate of cartilage differentiation by indian hedgehog and PTH-related protein. *Science* **273** 613–622.
- Wassarman KM, Lewandoski M, Campbell K, Joyner AL, Rubenstein JL, Martinez S & Martin GR 1997 Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. *Development* **124** 2923–2934.
- Yang X, Chen L, Xu X, Li C, Huang C & Deng C-X 2001 TGF- β /Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. *Journal of Cell Biology* **153** 35–46.
- Yin W, Smiley E, Germiller J, Mecham RP, Florer JB, Wenstrup RJ & Bonadio J 1995 Isolation of a novel latent transforming growth factor- β binding protein gene (LTBP-3). *Journal of Biological Chemistry* **270** 10147–10160.
- Zhang H, Apfelroth SD, Hu W, Davis EC, Sanguineti C, Bonadio J, Mecham RP & Ramirez F 1994 Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *Journal of Cell Biology* **124** 855–863.
- Zhou Y-X, Xu X, Chen L, Li C, Brodie SG & Deng C-X 2000 A pro250 Arg substitution in mouse Fgfr1 causes increased expression of Cbfa1 and premature fusion of calvarial sutures. *Human Molecular Genetics* **9** 2001–2008.

Received 13 February 2002

Accepted 11 June 2002