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The Tyrosine Phosphatase, OST-PTP, is Expressed in Mesenchymal Progenitor Cells Early During Skeletogenesis in the Mouse

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Abstract Osteotesticular protein tyrosine phosphatase (OST-PTP; OST), is a signaling molecule which catalyzes the removal of phosphates from tyrosine residues. It is known to be highly regulated in bone cells and has been shown to be important for the in vitro progression from a preosteoblast to a mature, mineralizing cell. However, the in vivo expression of this phosphatase during skeletogenesis has not been examined. Using Northern analysis and in situ hybridization (ISH), we have observed that this gene is strongly expressed early during the formation of the mouse skeleton. By 12.5 days post-coitum (dpc), expression of OST mRNA transcripts increases and is localized within the mesenchyme of craniofacial bones, ribs, limbs, and Meckel's cartilage. Following initiation of chondrogenesis, OST mRNA becomes restricted to the perichondrium of all endochondral elements. With ossification, this gene is also expressed by cells, presumably osteoblasts, at the chondro-osseous border and along cortical and trabecular bone surfaces. Unlike other bone markers examined such as Osterix and type II collagen, OST transcripts do not appear to be expressed by chondrocytes of epiphyseal cartilage or by non-hypertrophic or hypertrophic chondrocytes. Because the temporal expression patterns of OST and Runx2 were similar suggesting a potential interrelationship in bone regulation and function, OST expression was examined in transgenic mice lacking a functional Runx2/Cbfa1 protein (Runx2/Cbfa1 delta C (ΔC)) and possessing a cartilaginous skeleton. Interestingly, the OST gene was expressed with localization similar in wild-type, homozygous, and heterozygous embryos. These studies suggest that the expression of the OST gene may be important during skeletogenesis, potentially from commitment of mesenchymal cells to the ossification of new bones. Early in embryogenesis, regulation of OST expression may be independent of Runx2/Cbfa1. J. Cell. Biochem. 93: 761–773, 2004. © 2004 Wiley-Liss, Inc.

Key words: tyrosine phosphatase; embryogenesis; in situ hybridization; perichondrium; Runx2

The skeleton serves many functions in the vertebrate: it gives an organism the ability to move, provides support, maintains a storage site for minerals, and serves a critical role in hematopoiesis [Karaplis, 2002]. Accordingly, the specialized bone tissue which comprises the skeleton must offer rigidity, strength, and elasticity. During embryogenesis, the development of this complex tissue is accomplished through either endochondral or intramembraneous ossification and these two types of bone formation provide the classification for the hundreds of individual bones of the skeleton [Olsen et al., 2000]. The multistep process of endochondral ossification begins with the

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migration and condensation of mesenchymal stem cells to form the cartilage model of each bone, followed by the eventual deposition of a rigid, calcified matrix. In contrast, bones formed by intramembraneous ossification do not develop from a cartilage template, but form directly from condensing mesenchyme.

These bone forming processes are dependent on the continual interaction of specific cell populations which arise from mesenchymal and hematopoietic progenitors to become the differentiated, specialized types of chondrocytes, osteoblasts, and osteoclasts [Aubin, 1998; Takahashi et al., 2002]. As for all cells, their function is modulated by a myriad of hormonal and adhesion signals that ultimately result in changes in phosphorylation-dependent intracellular signaling cascades [Hunter, 2000]. Protein tyrosine phosphorylation is one mechanism of regulating such cascades which is dependent on the relative activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). PTP activity, the catalytic removal of phosphate from tyrosine residues, can serve to activate or inhibit signaling resulting in the modulation of processes such as cell growth, differentiation, and adhesion [Hunter, 1998]. For most of the members of this enzyme family, determining the specific cellular substrates of the PTPs and the signaling pathways they regulate has remained elusive. One particular member of this family, osteotesticular protein tyrosine phosphatase (OST-PTP; abbreviated as OST), is a distinctive molecule in its structure, expression, and regulation and, as such, may be an essential player in the development of the skeleton.

OST is a member of the 'classical' tyrosine phosphatase subfamily, originally cloned from primary osteoblasts and osteoblast-like cell lines and predominantly expressed in bone, testis, and ovary [Mauro et al., 1994; Lee et al., 1996]. It is a transmembrane receptor PTP containing two tandem catalytic domains, with the membrane proximal domain containing the enzymatically active signature motif [Mauro and Dixon, 1994; Petrone and Sap, 2000]. The unique extracellular region of this molecule contains ten fibronectin type III-like (FN-III) domains which shares sequence homology to several adhesion proteins including fibronectin, tenascin, and type VII collagen. Although there is no known ligand for this protein, this "adhesive-like" structure and our recent studies showing transcriptional regulation of this gene via cell–cell interactions [Mauro et al., 2001; Wheeler et al., 2002] may be indicative of a role for this molecule during adhesion events.

At present, this phosphatase is the only PTP known to be highly regulated in bone cells and essential for the progression from a preosteoblast to a mature, mineralizing cell. The expression of OST has been shown to be: (1) necessary for osteoblast differentiation and mineralization [Mauro et al., 1994; Chengalvala et al., 2001]; (2) modulated in response to known regulators of osteoblast function, including parathyroid hormone (PTH) and vitamin D_3 [Mauro et al., 1994, 1996]; (3) uniquely regulated during osteoblast differentiation in comparison to other PTPs [Wheeler et al., 2002]; and (4) transcriptionally activated during osteoblast differentiation [Wheeler et al., 2002]. This growing body of evidence supports the potential importance of this gene in bone formation yet all these studies have relied on in vitro cell models. In this report, we describe the first studies to establish the spatial and temporal expression of this PTP during skeletogenesis in the mouse. Using Northern blot analysis and in situ hybridization (ISH), we have shown that the in vivo expression of OST is expressed not only by mature osteoblasts, as suggested by previous cell studies, but by mesenchymal progenitor cells early during the development of all skeletal elements. In addition, early in skeletogenesis, the regulation of this expression may be independent of Runx2/Cbfa1, potentially expressed upstream of this transcription factor or via an independent signaling pathway.

EXPERIMENTAL PROCEDURES

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from whole mouse embryos (strain CD-1) at 11, 12.5, 14, 16 days post coitum (dpc) and from 19 dpc heads, forelimbs, and hindlimbs. In addition, RNA was also isolated from 1 and 3-week-old neonatal limbs, brain, and liver tissues. Extraction of RNA (Trizol; Invitrogen, Carlsbad, CA) and subsequent poly A+-enrichment of samples (PolyATract kit; Promega, Madison, WI) were conducted according to manufacturer's protocols as previously described [Mauro et al., 1994, 1996]. Standard molecular biology protocols were used for the Northern blot analysis with modifications as described [Mauro et al., 1996]. DNA probes encoding the murine OST and the

rat alkaline phosphatase (AP), osteocalcin (OC), and cyclophilin genes were radiolabeled with ^{32}P by random-priming (Prime-a-Gene Labeling System; Promega). The properties of these probes have been previously reported [Mauro et al., 1994, 1996].

Tissue Preparation

Timed pregnant CD-1 mice (Charles River Laboratories, Wilmington, MA) were ordered at least 2 days prior to the desired time point of gestation. Pregnant females were anesthetized with Isoflurane (Halocarbon Laboratories) and humanely sacrificed at 12.5, 14, 16, and 19 dpc using protocols approved by the University of Minnesota's Institutional Animal Care and Use Committee. The embryos were dissected free of all extra-embryonic membranes in ice cold 1× phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS). Tissues were then transferred to freshly prepared, cold 4% paraformaldehyde/1 × PBS, fixed overnight at 4°C, and paraffin embedded. Neonatal mice were humanely euthanized as above and calvaria (at birth) and limbs at 2 weeks of age were collected and processed.

The Runx2/Cbfa1 delta C mutant embryos were produced as previously described where chimeric animals were obtained via microinjection of clonal embryonic stem cells containing the truncated form of Runx2 (delta C; ΔC) [Choi et al., 2001]. Heterozygous mice (Runx2 wild type/ΔC) were interbred to obtain wild-type, heterozygote, and homozygote mutant animals. Embryos were collected and processed at 12.5 and 14 dpc as described above. The genotype of these embryos was verified by PCR detection as previously published [Choi et al., 2001].

All tissues were cut into 4 μm sections, mounted on Superfrost Plus slides (Fisher Scientific, Fairlawn, NJ) and stored at room temperature until use.

In Situ Hybridization

To monitor expression of the OST gene and other bone markers, ISH analysis to detect specific mRNA transcripts was chosen. This was the optimal methodology considering the very low abundance of OST mRNA and the lack of an effective, high affinity antibody for the OST protein. Probes for ISH analysis were produced by designing a set of PCR primers to amplify a 250–350 bp primer product. For the OST antisense probe, primers were designed to span 286 bp from nt #4777–5035 of the mouse cDNA which encodes the amino terminal region of the divergent second catalytic domain (DII), an area with low homology to other PTPs. For the Runx2/Cbfa1 and the Type II collagen (Col II) probe, the cDNAs were kindly provided by G. Karsenty (Baylor College of Medicine) and B. de Crombrugghe (MD Anderson Cancer Ctr), respectively. To detect Osterix (Osx) transcripts, RNA harvested from 16 dpc embryos was reverse-transcribed using the RETROscript kit (Ambion, Austin, TX) and used to amplify a portion of the coding region of the Osterix gene. Sense and antisense riboprobes were single or double labeled using the Riboprobe In Vitro Transcription System (Sp6/T7; Promega) with ^{35}S-labeled UTP (Osx, Col II) or UTP/CTP (Runx2, OST).

Sections were treated according to published protocols [Pelton et al., 1990; Mauro et al., 1994; Alvarez et al., 2001]. Following hybridizations, slides were dipped into Ilford K-5 photoemulsion (1:1 solution in 2% glycerol; Polysciences) and dried overnight. Exposure was carried out from 3 days (Type II collagen) to up to 3 weeks (OST). Slides were developed and counterstained with hematoxylin and eosin (H&E) according to standard histological procedures. Brightfield and darkfield images were captured using a Olympus BH2 compound scope fitted with a Nikon DXM 1200 digital camera. Images were processed and pieced together with Adobe Photoshop 6.0 (Adobe Systems, Inc.) and Canvas 7 (Deneba) imaging and graphics software programs.

RESULTS

Temporal Expression of OST mRNA During Embryogenesis

To determine the temporal expression pattern of OST during skeletal development, the relative levels of the OST mRNA transcripts were examined by Northern blot analysis and compared to the transcripts of established markers of bone formation. Levels of OST transcripts increase dramatically in day 12.5 embryos and are maintained throughout gestation as shown in Figure 1A. Just prior to parturition at 19 dpc, expression of this gene is evident in the head and forelimbs, with the highest levels observed in the hindlimbs. Analysis of 1-week-old neonate limbs showed decreased transcript levels in comparison to day 19 limbs. Expression

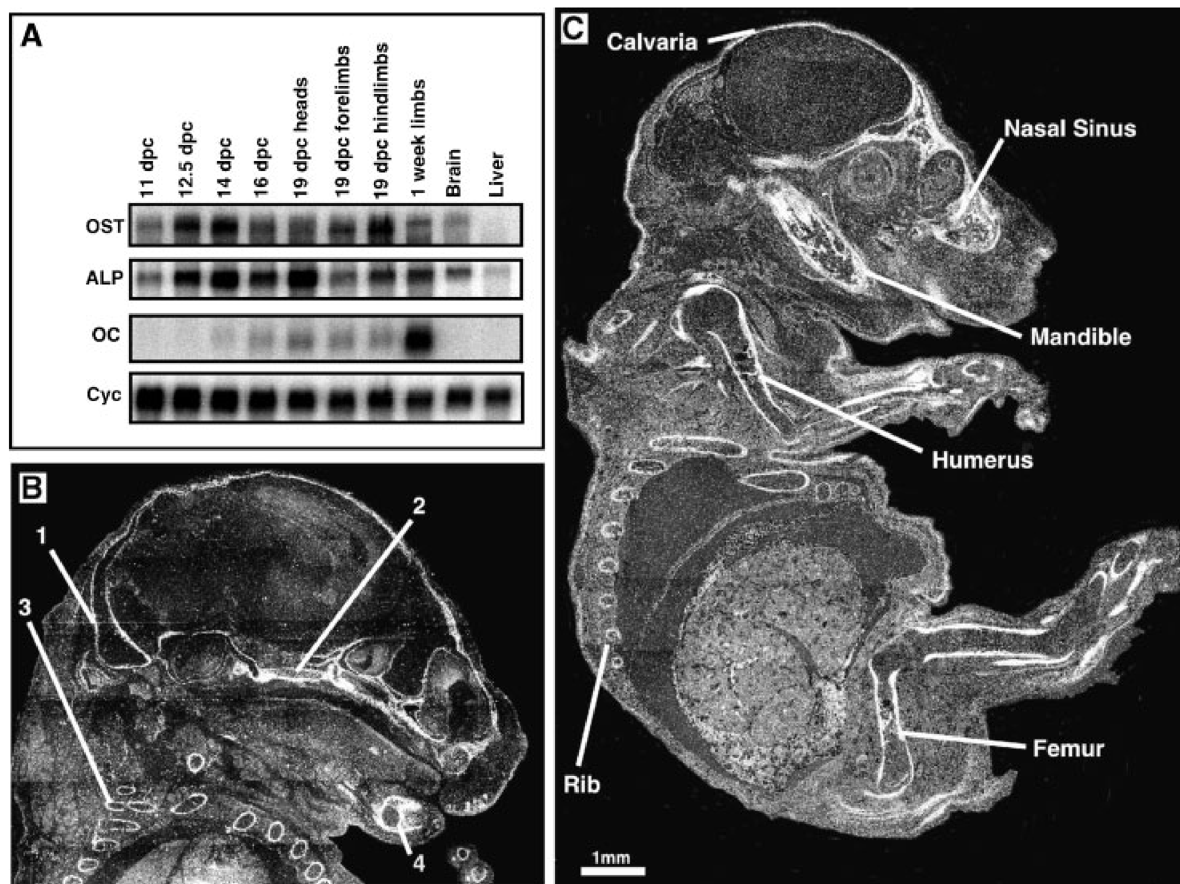

Fig. 1. OST expression is regulated both temporally and spatially during murine embryogenesis. Embryos were processed for RNA extraction (**A**) and in situ hybridization analysis (ISH; **B** & **C**). **A:** Tissues were collected, polyadenylated RNA isolated and Northern blot analysis (3–5 μg Poly (A^+) RNA/lane) performed using DNA probes for OST (osteotesticular protein tyrosine phosphatase), ALP (alkaline phosphatase), OC (osteocalcin), and Cyc (cyclophilin). Representative blot shown with brain and liver RNA and Cyc as a control for RNA integrity and concentration. **B:** Sagittal section of the head of a 16 dpc embryo. Tissue were hybridized with ^{35}S labeled anti-sense riboprobes. White granules indicate the localization of OST mRNA in: (1) calvaria, (2) basisphenoid bone, (3) vertebrae, and (4) Meckel's cartilage. **C:** Photomontage of a 16 dpc embryo. Scale bar = 1 mm.

of alkaline phosphatase (ALP), which is expressed primarily by differentiating osteoblasts and hypertrophic chondrocytes [Tuckermann et al., 2000], also increases at 12.5 dpc, with peak expression seen in heads from 19 dpc embryos. As expected, transcripts encoding the matrix protein OC, a gene expressed by mature, mineralizing osteoblasts, are evident later in development.

The spatial patterns of OST were determined by ISH of embryo sections. A dramatic, highly localized expression of this phosphatase is observed at 16 dpc, within most endochondral skeletal elements as well as within the mesenchyme of intramembraneous elements (Fig. 1B,C). Examination of the head of the day 16 embryo shows discrete distribution of OST transcripts in the developing calvaria and the vertebrae (Fig. 1B, #1,3). Expression is also evident in cartilaginous models of many craniofacial bones including the basisphenoid bone and Meckel's cartilage (Fig. 1B, #2,4). A photomontage darkfield image shows OST mRNA within the ribs, the mandible, and the nasal sinuses as well as the long bones and digits of the limbs (Fig. 1C).

OST Expression in Skeletal Elements Formed by Endochondral Ossification

The process of endochondral bone formation involves early chondrogenesis to form the cartilaginous template followed by osteogenesis to ossify the final bone [Karsenty and Wagner, 2002]. To determine the changes in the localization of OST throughout this process, chondrified skeletal elements at various developmental

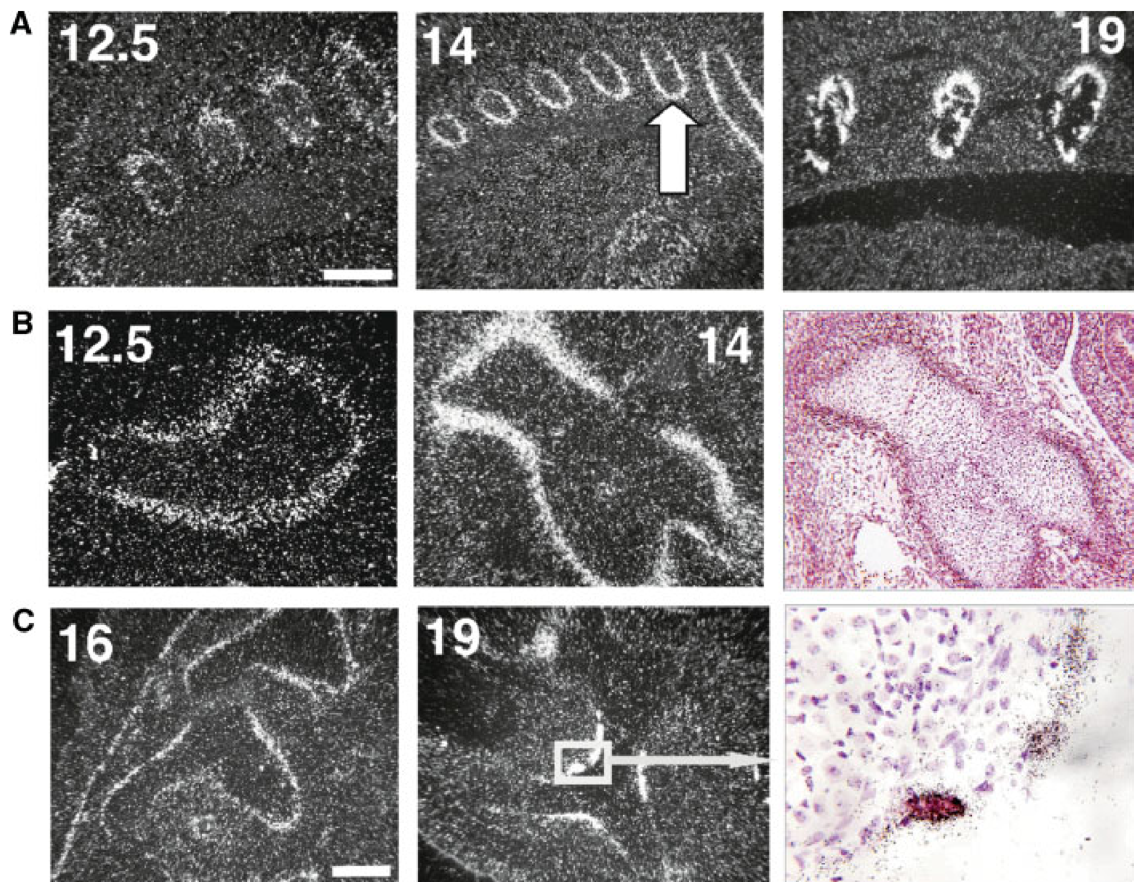

Fig. 2. During endochondral ossification, OST transcripts are detected in perichondrium surrounding cartilaginous elements. **A**: Ribs. ISH images where numbers indicated days post coitum (bar = 200 μm). White arrow points to the periochondrial layer. **B**: Skeletal elements in the shoulder region. Darkfield and corresponding H&E image at 12.5 and 14 dpc (bar = 200 μm). Vertebral elements at 16 and 19 dpc. Darkfield (bar = 100 μm) and H&E stained images (boxed right; bar = 20 μm).

ages were initially selected and OST expression patterns were compared. Examination of the sequential expression of this phosphatase mRNA within the ribs of CD-1 embryos shows that OST transcripts become localized to the perichondrial layers of the ribs as the embryo matures (Fig. 2A). By day 19, expression is observed in the periosteal/perichondrial layer as well as in the center of the rib within the ossified matrix. A similar spatial distribution is observed at 12 and 14 dpc in two other chondrified elements found in the shoulder region of the embryo (Fig. 2B). At 12.5, OST expression is diffuse and localized to the outer region of the mesenchymal condensation. In contrast, at 14 dpc, OST transcripts are highly localized within the perichondrium of the skeletal element and the signal is more intense suggesting a greater amount of mRNA. The vertebrae of 16 and 19 dpc embryos also express OST mRNA in the perichondrium (Fig. 2C). A heavy concentration of granules is seen over an area of ossified matrix in the 19 dpc vertebrae (Fig. 2C; boxed area magnified at right).

By examining OST localization during the process of endochondral ossification, we determined if this gene was expressed in a variety of chondrogenic and osteogenic cell types through ISH analysis of developing long bones. First, a comparison of OST expression with that of established chondrocyte and osteoblast marker genes was conducted. Limbs from 16 dpc embryos were utilized because of the ease of acquiring high quality longitudinal sections through a single bone. These sections were hybridized with probes for OST, Runx2 (Cbfa1/AML3), Osterix (OSX), and type II collagen (Col II) and probe localization was analyzed (Fig. 3).

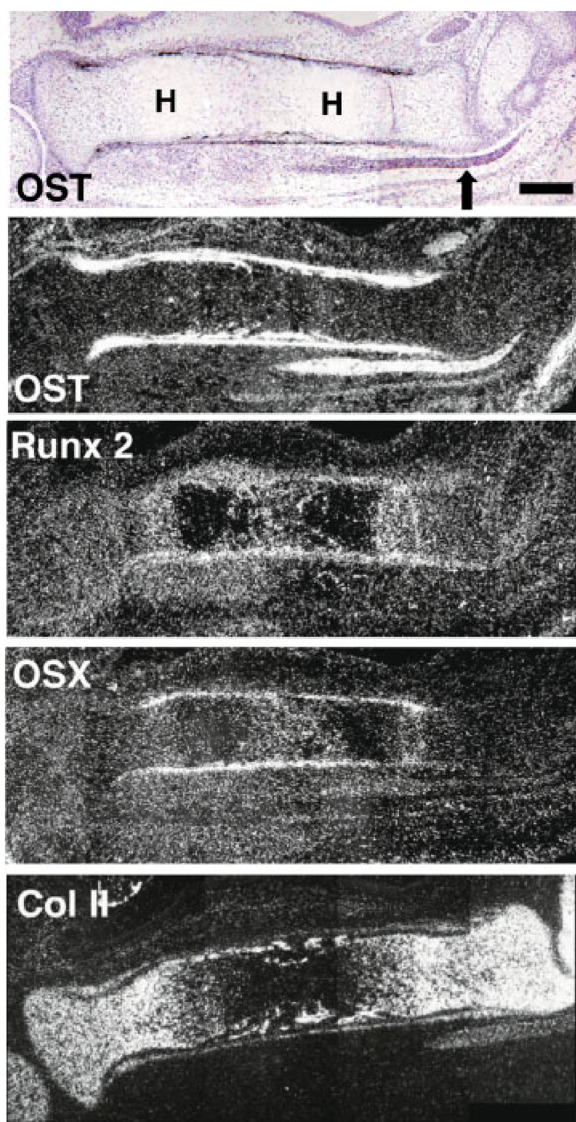

Fig. 3. OST expression patterns are unique when compared to other markers of bone formation. Serial sections of a long bone from 16 dpc mouse embryos were processed for ISH. OST localization is depicted in top two panels—H & E stained and darkfield montage of the same section (bar = 0.5 mm). Labeled structures are: arrow pointing to tendon; 'H' indicating the zones of hypertrophic cells. The subsequent panels are darkfield images indicating localization of other markers of bone formation including Runx2 (Cbfa1/AML3), Osterix (OSX), and type II collagen (Col II).

Similar localization patterns are observed for the transcription factors, Runx2 and Osterix. Both of these genes are necessary for osteoblast commitment and thought to be expressed by 'osteochondrocyte' progenitor cells as well as differentiated osteoblasts [Ducy et al., 1997; Komori et al., 1997; Nakashima et al., 2002]. Transcripts can be detected in the hypertrophic zone, in the primary ossification center, and along the perichondrium/periosteum of these embryonic long bones (Fig. 3; panel 3 and 4 from top). Type II collagen mRNA, an abundant extracellular matrix protein secreted mainly by non-hypertrophic chondrocytes [Scheven et al., 1988; Oshima et al., 1989; Karaplis, 2002], is strongly expressed in chondrocytes within the epiphyseal region of these bones (Fig. 3; bottom panel). Sense probes for these marker genes showed little non-specific signal similar to the OST sense probe (see Fig. 5).

In contrast to these marker genes, OST is expressed in a unique population of cells in the embryonic long bones (Fig. 3; top two panels). Granules are observed in the bony collar and along the shaft of the bone in both layers of the perichondrium/periosteum as seen previously. This localization is different from that of the other genes, particularly as compared to the type II collagen signal. Punctate distribution of granules is noted in the primary ossification center, expressed presumably by ossifying osteoblasts. In addition, a very strong OST signal localizes to the cells of a tendon connecting this bone to an adjacent muscle.

Developing bone tissue from 2.5-week-old neonates was also examined. As shown in Figure 4, OST expression is very intense with a high concentration of granules observed within the bony collar, the periochondrium and along the chondro-osseous junction (Fig. 4A). At this junction and within the primary spongiosa, OST mRNA is observed as punctate clumps of granules as well as long 'chains' along the trabecular surface of new bone. Due to this localization along the bony collar and trabecular bone, these OST-expressing cells are probably osteoblasts. Closer examination of the periosteum adjacent to the developing cortical bone indicates that OST is also expressed within the outer fibrous layer of the periosteum (Fig. 4B; *), the inner perichondrial layer (Fig. 4B; **) and the compact cortical bone (Fig. 4B; #). In contrast to these structures, white granules are strikingly absent from the epiphyseal cartilage at the end of the bone and the cells of the epiphyseal growth plate suggesting that this gene is not expressed by these types of chondrocytes (Fig. 4A; *). Analysis of a joint space shows intense expression within the perichondrium of both articulating surfaces and in the ligament connecting these developing bones (Fig. 4C,D). Again, no expression is observed within the epiphyseal regions.

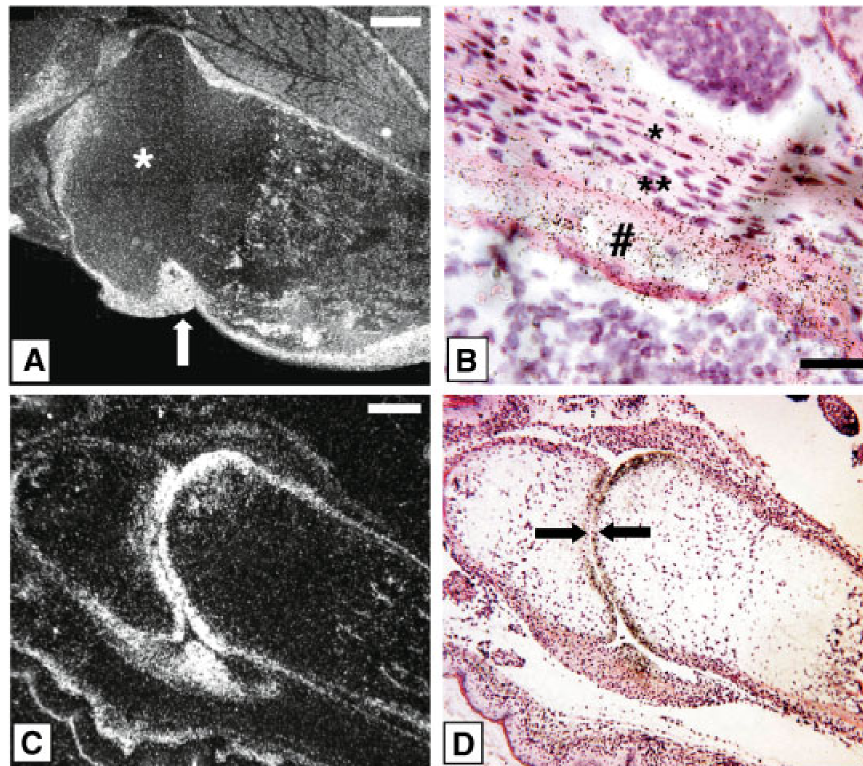

Fig. 4. Localization of OST during primary ossification of 2 week neonate long bones. **A**: Montage labeled structures are: (*) indicating epiphysis lacking OST expression; arrow pointing to bony collar (bar = 0.5 mm). **B**: H&E enlargement. Dark granules indicate localization of OST mRNA Labeled structures are: (*) the outer fibrous layer of the perichondrium, (**) the inner perichondrial layer and (#) the compact cortical bone (bar = 20 μm). **C**: Joint region. Developing bones of the distal hindlimb. Darkfield ISH (bar = 100 μm). **D**: H & E image. Facing black arrows indicate the articulating surfaces of adjoining bones.

OST Expression in Skeletal Elements Formed by Intramembraneous Ossification

The expression of this molecule was also followed during the development of the skull and the Meckel's cartilage, both arising without a cartilaginous template through the direct differentiation of mesenchymal stem cells to ossifying osteoblasts [Ishizeki et al., 1999; Opperman, 2000; Wilkie and Morriss-Kay, 2001]. Interestingly, the same pattern of increased specificity of expression and greater relative amount of signal, noted during endochondral ossification, is also observed during the intramembraneous ossification of these bones. Within the developing skull from 12.5 to 19 dpc, the expression of OST becomes tightly localized to the band of developing mesenchyme that is to become the ossified skull (Fig. 5A,B). Corresponding stained images of boxed area show dark granules over mesenchymal cells at all days post coitum (Fig. 5B). In addition, on 14 and 19 dpc, granules are observed within the osteoid of the developing skull. Upon examination of Meckel's cartilage and the surrounding condensing mesenchyme, the striking absence of OST mRNA within the cartilage is most notable (Fig. 5C,D). White granules on darkfield images outline the nodule of Meckel's cartilage and are also present within the mesenchyme that surrounds Meckel's cartilage. By day 16, OST mRNA expression was highly localized to the mesenchymal cell population, and a distinct boundary of expression could be drawn between the mesenchyme and the surrounding tissue. The darkfield and stained images (labeled SENSE) show a representative OST sense control indicating the low background present for these ISHs (Fig. 5C,D, last panel on right).

OST Expression in the Delta C Runx2 Functional Knockout

Knowing that Runx2/Cbfa1 is essential for osteoblast commitment and maturation and

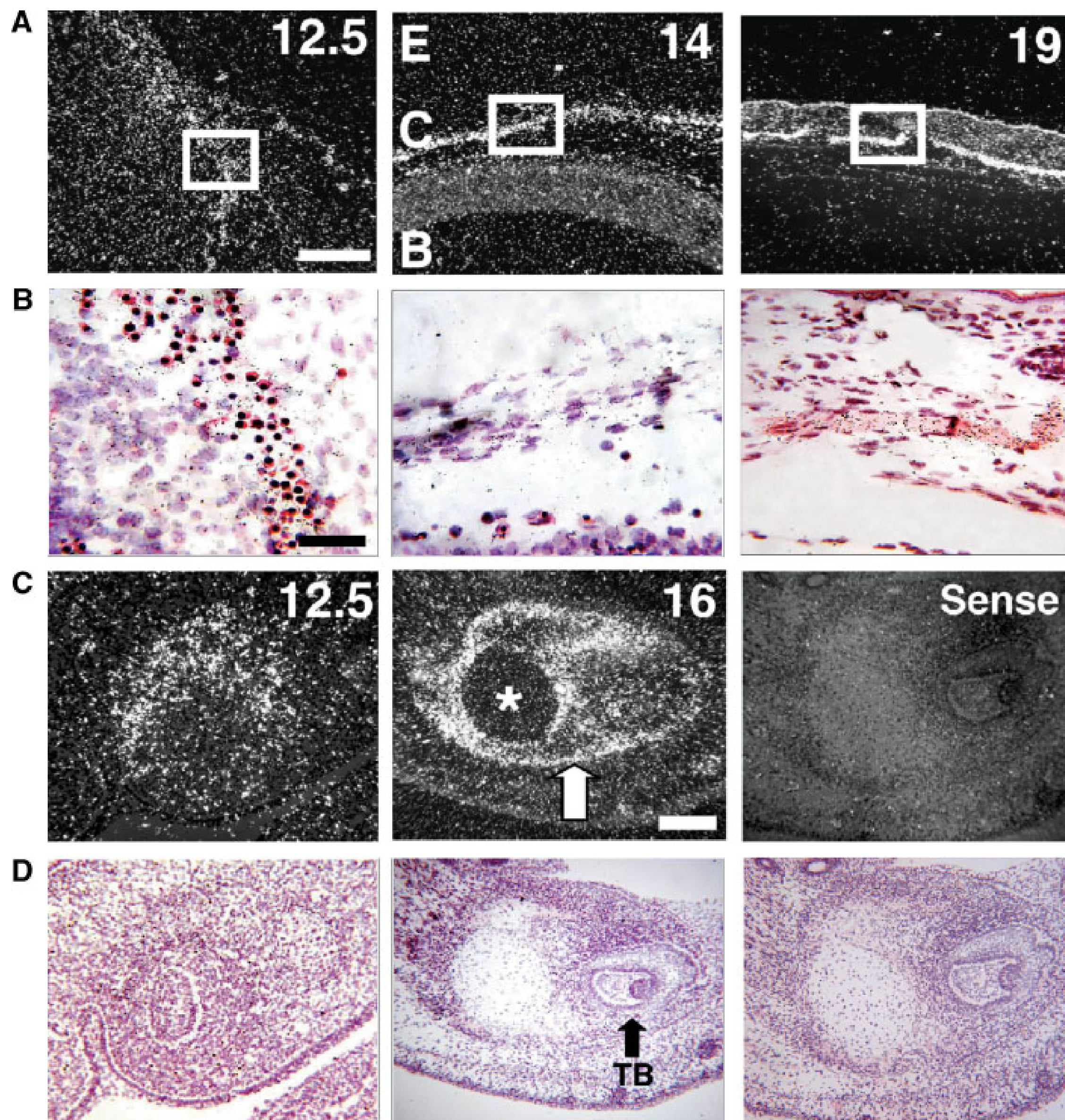

Fig. 5. OST expression in intramembraneous ossification centers. **A** & **B**: Calvaria. A: Darkfield images showing expression at 12.5, 14, and 19 dpc (bar = 200 μm). Boxed area delineates magnified image below. For orientation, E = exterior, C = calvaria, B = brain; and (B) H & E images (bar = 40 μm). **C** & **D**: Meckel's cartilage (C) Darkfield ISH. The sense probe for OST is shown on an adjacent 16 dpc section and is used as a control to show probe specificity. Labeled structures are: arrow = the intramembraneous region; (*) = Meckel's cartilage; and TB = tooth bud. (D) H & E images.

considering the expression of both OST and Runx2 early in skeletogenesis, it seemed reasonable that there might be a dependence of OST expression on the presence of the transcription factor, Runx2. To address this possibility, we wished to determine if the OST gene would be expressed in embryos lacking functional Runx2. The transgenic mice utilized in our experiments were generated to contain a Runx2 gene lacking the C terminal region (Runx2/Cbfa1 delta C (ΔC)) and fail to form bone due to maturational arrest of osteoblasts [Choi et al., 2001]. The heterozygotes (+/ΔC) lack clavicles but otherwise exhibit normal bone development. The homozygous (ΔC/ΔC) mice are phenotypically indistinguishable from the mice with complete ablation of Runx2/Cbfa1 (Runx2 −/−; [Komori et al., 1997]) suggesting

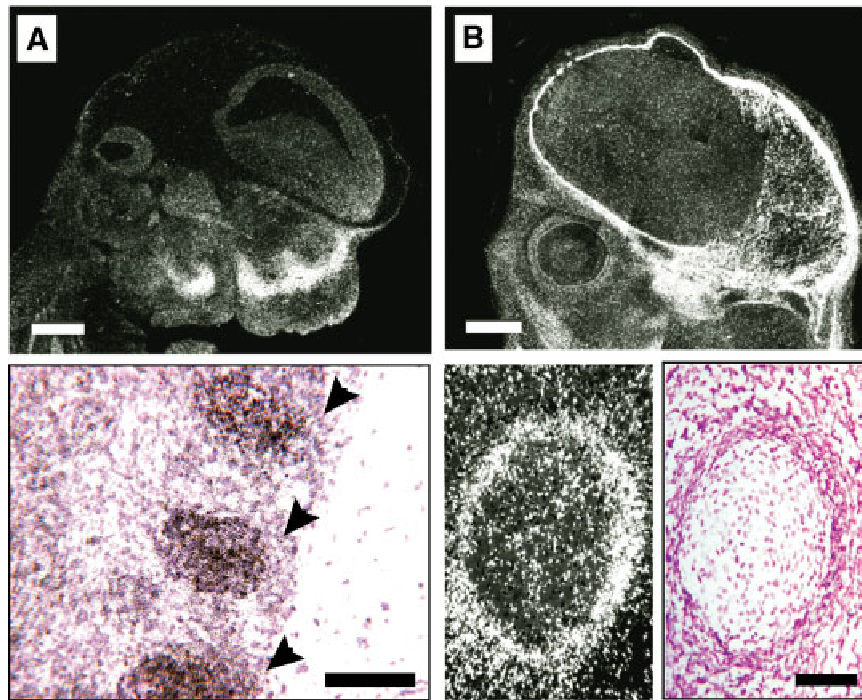

Fig. 6. OST is expressed in skeletal elements when functional Runx2 protein is absent. Delta C homozygote (Runx2 ΔC/ΔC) embryos at 12.5 and 14 dpc were collected and processed for ISH as described in Materials and Methods. **A**: 12.5 dpc Top = darkfield montage of head region (Bar = 0.5 mm); Bottom = H&E image of mesenchymal condensations of the ribs (Bar = 100 μm). Dark granules indicate localization of OST mRNA; **B**. 14 dpc. Top = Darkfield montage of head region (Bar = 0.5 mm); Bottom = darkfield and H&E image of a skeletal element in the upper forelimb (bar = 200 μm).

that this deleted region is critical, providing a valuable 'functional knockout' model. The homozygote (ΔC/ΔC), heterozygote (+/ΔC), and wild-type (Runx2 ΔC +/+) embryos at 12.5 and 14 dpc were used for these studies. Analysis of ISH results showed that OST transcripts are expressed at 12.5 dpc in the homozygous embryos (ΔC/ΔC; Fig. 6A). These transcripts are localized within the condensing mesenchyme of the facial region (Fig. 6A, top montage showing head of embryo) as well as within the thoracic condensations destined to formed the ribs (Fig. 6A, bottom montage). This expression pattern mimics what is observed in wild-type and heterozygous littermates (data not shown). No evidence of the initiation of chondrogenesis or a distinct perichondrial layer was detected at this stage. This observation of transcripts within mesenchymal condensations of all embryos at 12.5 dpc suggests that these tissues were collected at a slightly earlier time in development than the embryos of the outbred CD-1 strain illustrated in previous figures (e.g., see Fig. 2). At day 14, the transcripts are discretely localized to the perichondrial layer of endochondral elements, with little expression within the differentiated core cells (Fig. 6B). Again, this pattern is essentially the same as that observed in the CD-1 embryos illustrated in the earlier figures.

DISCUSSION

Previous studies suggest that OST may be a key regulator of signaling events during bone formation in vitro, particularly important in osteoblast differentiation and function. Lacking in this research is any evidence of the in vivo expression of this phosphatase in the tissues of developing bone. Here, we report the first studies showing that OST is strongly expressed in mesenchyme and osteoprogenitor cells with regulated expression continuing during endochondral and intramembraneous ossification in vivo.

Skeletal Patterning and OST mRNA Expression

The process of skeletogenesis involves the migration and condensation of stem cells of neural crest or mesodermal origin at specific

sites throughout the embryo [Karaplis, 2002]. Once in place, these stem cells undergo commitment and differentiation along chondrogenic or osteogenic lineages to ultimately give rise to all the bones of the skeleton. It appears from our results that OST is strongly expressed in the mesenchymal cells that form these condensations. OST transcripts are detected as early as 12.5 dpc throughout the mesenchyme of ribs and limbs of the Runx2 delta C transgenic and the wild-type CD-1 embryos. In intramembraneous elements, OST is also detected in the mesenchyme of the skull as well as the mesenchyme surrounding Meckel's cartilage. Although mesenchymal cells prior to or during condensation were not observed in our sections, previous embryological studies of mouse Esp (embryonic stem cell phosphatase; sequence identity to OST) expression from 1 cell stage to 7.5 dpc did observe Esp/OST transcripts in newly formed mesoderm [Lee et al., 1996]. Further studies are needed to determine if OST is expressed by such migrating stem cells or if expression is activated following mesenchymal condensation.

For endochondral elements, the initiation of chondrogenesis leads to the differentiation of the core condensed cells to chondrocytes, with the outer undifferentiated mesenchymal cells forming the perichondrium [DeLise et al., 2000]. By 14 dpc, when a distinct perichondrial layer is evident in elements of the ribs, vertebrae, and long bones, OST transcripts are excluded from the differentiated core chondrocytes with expression restricted to these outer progenitor cells. This early pattern of OST localization is reminiscent of other genes important in the formation of the condensations and subsequent chondrogenesis. It is interesting to note that several cell adhesion molecules exhibit similar patterns of expression. Transcripts encoding *N*-cadherin [Oberlender and Tuan, 1994], neural cell adhesion molecule (N-CAM; [Tavella et al., 1994]), and isoforms of fibronectin [Gehris et al., 1996, 1997] are all localized throughout the condensed mesenchyme, then disappear from the chondrified core with expression solely in the perichondrium. These proteins mediate cell–cell and cell–matrix interactions that are important for the initiation of condensations and chondrogenic differentiation. Perturbation of these interactions can result in alterations of chondrogenesis and severe skeletal malformations [DeLise et al., 2000]. The OST molecule shares sequence homology with fibronectin and tenascin and is structurally similar to many CAMs due to its multiple fibronectin type III repeats [Mauro et al., 1994]. In addition, previous studies have shown that enhanced cell–cell interactions (e.g. cell density) can activate transcription and increase expression of endogenous OST transcripts [Mauro et al., 2001; Wheeler et al., 2002]. Considering the similarity in expression to such adhesion molecules and implications of these results and other studies, this phosphatase may be important in signaling earlier in skeletogenesis than previously appreciated. Preliminary reports suggest that other PTP molecules may also regulate cell signaling during this period of development. During chondrogenesis, PTP gamma (−) exhibits a similar expression pattern as OST and has been shown, in mandibular explant cultures, to modulate patterning and chondroblast proliferation [Augustine et al., 2000a,b]. The receptor PTP, RPTP beta/zeta, interacts with the matrix protein pleiotrophin and in this way may mediate adhesion and migration of osteoprogenitor cells [Rauvala et al., 2000; Qi et al., 2001; Yang et al., 2003]. The possibility that this regulated expression of OST we observed during early embryogenesis is necessary for proper mesenchymal patterning and chondrogenesis remains to be addressed by further functional studies.

Cell Commitment and OST Expression

The dramatic change in distribution of this phosphatase gene from mesenchymal core to perichondrial layer may indicate that the commitment to a chondrogenic lineage results in suppression of OST expression. Later in the development of long bones or Meckel's cartilage, exclusion of OST transcripts from non-hypertrophic and hypertrophic chondrocytes is also observed. This observation is further substantiated by the expression of markers of non-hypertrophic (e.g., Type II collagen) and hypertrophic (e.g., Runx2 and osterix) chondrocytes in what are presumed to be cells not expressing OST transcripts. The lack of specific OST signal in these chondrogenic cells is in contrast to the strong OST expression seen in the undifferentiated mesenchymal cells of the perichondrial layer. Once the bony collar develops and cortical bone begins to be deposited, OST transcripts are still highly localized to the cells of the periosteum/perichondrium as well as

the periarticular surfaces between bones. Cells lining the endosteal surface of the cortical bone or the surfaces of trabecular bone are presumably actively secreting and mineralizing matrix and are also observed to highly express this gene.

Our observations could be interpreted to indicate that OST is expressed in a mesenchymal progenitor cell and, following commitment and differentiation, is predominantly expressed in cells of an osteogenic lineage. Localization of this gene in preosteoblasts and mature, mineralizing osteoblasts was obviously expected considering previous in vitro studies showing the regulated, differentiation-dependent expression of OST in primary osteoblasts and osteblast-like cell lines [Mauro et al., 1994; Chengalvala et al., 2001; Wheeler et al., 2002]. The expression of this phosphatase has also been shown to be critical for proper progression of osteoblast differentiation in vitro [Chengalvala et al., 2001]. More surprising was its discrete expression early in mesenchyme and later in the perichondrium, which is reminiscent of the expression of the critical transcription factor, Runx2/Cbfa1. It has been shown that ablation of the mouse *Runx2/Cbfa1* gene or transgenic manipulations which interfere with the transcriptional activity of the endogenous protein (e.g., Runx2/Cbfa1 delta C) will lead to arrest of osteoblast differentiation and lack of bone formation [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Choi et al., 2001]. The conclusions from these and other genetic studies is that a biologically active Runx2/Cbfa1 protein is necessary to drive the commitment of an undifferentiated mesenchymal stem cell which expresses the Runx2 gene to a differentiated osteoblast which also is Runx2 positive. In general, commitment to a chondrocytic lineage results in a loss of Runx2/Cbfa1 expression. With the similarities of in situ expression patterns, the differentiation-dependent osteoblast expression [Mauro et al., 1994; Chengalvala et al., 2001; Wheeler et al., 2002], and the existence of Runx2/Cbfa1 binding sites in the mouse OST promoter [Morrison and Mauro, 2000], a dependence of OST expression on the presence of functional Runx2/Cbfa1 was anticipated. But, in the mice expressing a C-terminal truncated Runx2 protein, the OST gene is still robustly expressed and shows the same changes in localization from core to perichondrium. Therefore, during this discrete period of skeletal development, presumably prior to the commitment of OST-expressing progenitor cells to an osteogenic lineage, Runx2/Cbfa1 may not be necessary for the expression of this phosphatase gene. Of course, it must be noted that Runx2/Cbfa1 protein, albeit 'non-functional' in its ability to drive osteoblast commitment, is still present in these animals. We cannot rule out the possibility that this protein could conceivably serve a 'scaffolding' function with other transcription factors with redundant function comparable to Runx2. Regardless, it is possible that, even though both genes would be expressed in some of the same subpopulations of cells as indicated by our studies, the regulation of OST expression during commitment of progenitor cells is via an independent, parallel signaling pathway. It is also feasible that the OST gene is expressed upstream of Runx2/Cbfa1 in the proposed lineage progression from multipotential mesenchymal cell to the preosteoblast. This would be in contrast to the transcription factor, Osterix, which is not expressed in Runx2/Cbfa1 knockout mice and is thought to be essential downstream of Runx2 in the establishment of this osteogenic lineage [Nakashima et al., 2002].

In light of these localization studies, the hypothetical role of OST may be broader than just promoting osteoblast differentiation and will need to be addressed in future studies. It is conceivable that this molecule may function to transmit 'adhesive' signals that are important for the commitment of these cells to specific lineages such as osteoblasts or chondrocytes. Whether regulation of OST expression is dependent on Runx2/Cbfa1 during such commitment will also need to be clarified.

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REFERENCES

Alvarez J, Horton J, Sohn P, Serra R. 2001. The perichondrium plays an important role in mediating the effects of TGF-beta1 on endochondral bone formation. Dev Dyn 221:311–321.

Aubin J. 1998. Bone stem cells. J Cell Biochem 30–31:73–82.

Augustine KA, Rossi RM, Silbiger SM, Bucay N, Duryea D, Marshall WS, Medlock ES. 2000. Evidence that the protein tyrosine phosphatase (PC12,Br7,Sl) gamma (−) isoform modulates chondrogenic patterning and growth. Int J Dev Biol 44:361–371.

Augustine KA, Silbiger SM, Bucay N, Ulias L, Boynton A, Trebasky LD, Medlock ES. 2000. Protein tyrosine phosphatase (PC12, Br7,S1) family: Expression characterization in the adult human and mouse. Anat Rec 258:221–234.

Chengalvala MV, Bapat AR, Hurlburt WW, Kostek B, Gonder DS, Mastroeni RA, Frail DE. 2001. Biochemical characterization of osteo-testicular protein tyrosine phosphatase and its functional significance in rat primary osteoblasts. Biochemistry 40:814–821.

Choi JY, Pratap J, Javed A, Zaidi SK, Xing L, Balint E, Dalamangas S, Boyce B, van Wijnen AJ, Lian JB, Stein JL, Jones SN, Stein GS. 2001. Subnuclear targeting of Runx/Cbfa/AML factors is essential for tissue-specific differentiation during embryonic development. Proc Natl Acad Sci USA 98:8650–8655.

DeLise AM, Fischer L, Tuan RS. 2000. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage 8:309–434.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Gehris AL, Oberlender SA, Shepley KJ, Tuan RS, Bennett VD. 1996. Fibronectin mRNA alternative splicing is temporally and spatially regulated during chondrogenesis in vivo and in vitro. Dev Dyn 206:219–230.

Gehris AL, Stringa E, Spina J, Desmond ME, Tuan RS, Bennett VD. 1997. The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. Dev Biol 190:191–205.

Hunter T. 1998. The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: Its role in cell growth and disease. Philos Trans R Soc Lond B Biol Sci 353:583–605.

Hunter T. 2000. Signaling—2000 and beyond. Cell 100: 113–127.

Ishizeki K, Saito H, Shinagawa T, Fujiwara N, Nawa T. 1999. Histochemical and immunohistochemical analysis of the mechanism of calcification of Meckel's cartilage during mandible development in rodents. J Anat 194: 265–277.

Karaplis AC. 2002. Embryonic development of bone and the molecular regulation of intramembraneous and endochondral bone formation. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of bone biology. 2nd edn. Vol. 1: San Diego: Academic Press. pp 33–58.

Karsenty G, Wagner EF. 2002. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2:389–406.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Lee K, Nichols J, Smith A. 1996. Identification of a developmentally regulated protein tyrosine phosphatase in embryonic stem cells that is a marker of pluripotential epiblast and early mesoderm. Mech Dev 59:153–164.

Mauro LJ, Dixon JE. 1994. 'Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular 'address'. Trends Biochem Sci 19:151–155.

Mauro LJ, Olmsted EA, Skrobacz BM, Mourey RJ, Davis AR, Dixon JE. 1994. Identification of a hormonally regulated protein tyrosine phosphatase associated with bone and testicular differentiation. J Biol Chem 269: 30659–30667.

Mauro LJ, Olmsted EA, Davis AR, Dixon JE. 1996. Parathyroid hormone regulates the expression of the receptor protein tyrosine phosphatase, OST-PTP, in rat osteoblast-like cells. Endocrinology 137:925–933.

Mauro LJ, Townsend MK, Wheeler M, Kishel E. 2001. Cell–cell/cell–matrix interactions and hormonal bone modulators regulate the mOST-PTP gene in MC3T3 osteoblasts. J Bone Miner Res 16:S489.

Morrison DF, Mauro LJ. 2000. Structural characterization and chromosomal localization of the mouse cDNA and gene encoding the bone tyrosine phosphatase, mOST-PTP. Gene 257:195–208.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Oberlender SA, Tuan RS. 1994. Spatiotemporal profile of *N*-cadherin expression in the developing limb mesenchyme. Cell Adhes Commun 2:521–537.

Olsen BR, Reginato AM, Wang W. 2000. Bone development. Annu Rev Cell Dev Biol 16:191–220.

Opperman LA. 2000. Cranial sutures as intramembranous bone growth sites. Dev Dyn 219:472–485.

Oshima O, Leboy PS, McDonald SA, Tuan RS, Shapiro IM. 1989. Developmental expression of genes in chick growth cartilage detected by in situ hybridization. Calcif Tissue Int 45:182–192.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765–771.

Pelton RW, Dickinson ME, Moses HL, Hogan BL. 1990. In situ hybridization analysis of TGF beta 3 RNA expression during mouse development: Comparative studies with TGF beta 1 and beta 2. Development 110:609–620.

Petrone A, Sap J. 2000. Emerging issues in receptor protein tyrosine phosphatase function: Lifting fog or simply shifting? J Cell Sci 113:2345–2354.

Qi M, Ikematsu S, Maeda N, Ichihara-Tanaka K, Sakuma S, Noda M, Muramatsu T, Kadomatsu K. 2001. Haptotactic migration induced by midkine. Involvement of protein-tyrosine phosphatase zeta. Mitogen-activated protein kinase, and phosphatidylinositol 3-kinase. J Biol Chem 276:15868–15875.

Rauvala H, Huttunen HJ, Fages C, Kaksonen M, Kinnunen T, Imai S, Raulo E, Kilpelainen I. 2000. Heparin-binding proteins HB-GAM (pleiotrophin) and amphoterin in the regulation of cell motility. Matrix Biol 19:377–387.

Scheven BA, Hamilton NJ, Farquharson C, Rucklidge GJ, Robins SP. 1988. Immunohisto-chemical localization of native and denatured collagen types I and II in fetal and adult rat long bones. Bone 9:407–414.

Takahashi N, Udagawa N, Takami M, Suda T. 2002. Cells of bone: Osteoclast generation. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of bone biology. vol. 1: San Diego: Academic Press. pp 109–126.

Tavella S, Raffo P, Tacchetti C, Cancedda R, Castagnola P. 1994. *N*-CAM and *N*-cadherin expression during in vitro chondrogenesis. Exp Cell Res 215:354–362.

Tuckermann JP, Pittois K, Partridge NC, Merregaert J, Angel P. 2000. Collagenase-3 (MMP-13) and integral membrane protein 2a (Itm2a) are marker genes of chondrogenic/osteoblastic cells in bone formation: Sequential temporal, and spatial expression of Itm2a, alkaline phosphatase, MMP-13, and osteocalcin in the mouse. J Bone Miner Res 15:1257–1265.

Wheeler MA, Townsend MK, Yunker LA, Mauro LJ. 2002. Transcriptional activation of the tyrosine phosphatase gene, OST-PTP, during osteoblast differentiation. J Cell Biochem 87:363–376.

Wilkie AO, Morriss-Kay GM. 2001. Genetics of craniofacial development and malformation. Nat Rev Genet 2:458–468.

Yang X, Tare RS, Partridge KA, Roach HI, Clarke NM, Howdle SM, Shakesheff KM, Oreffo RO. 2003. Induction of human osteoprogenitor chemotaxis, proliferation, differentiation, and bone formation by osteoblast stimulating factor-1/pleiotrophin: Osteoconductive biomimetic scaffolds for tissue engineering. J Bone Miner Res 18:47–57.

Yunker LA, Carlson CS, Mauro LJ. 2002. Spatial and temporal expression of the tyrosine phosphatase, OST-PTP, during osteogenesis. J Bone Miner Res 17:S246.