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Restricted Localization of Thrombospondin-2 Protein During Mouse Embryogenesis: A Comparison to Thrombospondin-1

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Abstract

Thrombospondin-1 and -2 (TSP1 and TSP2) are multifunctional, multimodular extracellular matrix proteins encoded by separate genes. We compared the distributions of TSP1 and TSP2 in mouse embryos (day 10 and later) by immunohistochemistry. TSP1 was detected on day 10 in the heart and intestinal epithelium, on day 11 in megakaryocytes, and on day 14 in the lung. TSP2 was not detected until day 14, with strongest staining in mesenchymal condensation that gives rise to cartilage and bone. The distribution of TSP2 was different from but overlapped with the distribution of TSP1. TSP1 was found in cartilage proper with diminished staining around chondrocytes undergoing differentiation and hypertrophy, whereas TSP2 was restricted to the matrix surrounding chondrocytes of the growth zone cartilage. TSP2 and TSP1 were both expressed in centers of intramembranous ossification that form the skull bones, in reticular dermis, on the apical surface of nasal epithelium, in skeletal muscle, and in the sheath surrounding vibrissae. Areas of exclusive staining for TSP2 included the perichondrium surrounding the cartilage of the nasal cavities, developing bone of the lower mandible, and adrenal gland. The distinct localizations of TSP1 and TSP2 indicate that the two proteins have specific functions during mouse embryogenesis.

Key words: bone formation, embryogenesis, Thrombospondin-1, Thrombospondin-2.

Introduction

The thrombospondins (TSPs) are a family of structurally related glycoproteins. Five different TSP genes are present as highly conserved orthologs in vertebrates (Bornstein, 1992). TSPs are divided into two groups; one contains trimeric proteins, thrombospondin-1 (TSP1) and -2 (TSP2); the other contains pentameric proteins, TSP3, TSP4 and TSP5 (also known as cartilage oligomeric matrix protein, COMP) (Bornstein and Sage, 1994).

TSP1 has been the focus of the majority of research on TSPs. TSP1 is a 450 kDa glycoprotein released from platelet α-granules during platelet activation and also synthesized and secreted by normal and transformed cells (reviewed in Mosher, 1990; Frazier, 1991). TSP1 binds Ca^{++} (Misenheimer and Mosher, 1995) and interacts with cells and various molecules in the extracellular

Abbreviations used: TSP, thrombospondin.

matrix and blood, influencing cell attachment, migration and growth (reviewed in Mosher, 1990; Bornstein, 1995). There are a number of potential TSP1 receptors, including heparan sulfate proteoglycans and sulfatides (Roberts, 1988; Kaesberg et al., 1989; Sun et al., 1989; Sun et al., 1992a); CD36 (GPIV) (Asch et al., 1992); low density lipoprotein receptor-related protein (Godyna et al., 1995); integrins $\alpha_v\beta_3$ (Lawler et al., 1988; Sun et al., 1992b), $\alpha_{IIb}\beta_3$ (Lawler and Hynes, 1989; Tuszynski et al., 1989), $\alpha_3\beta_1$ (DeFreitas et al., 1995), $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (Yabkowitz et al., 1993); and integrin-associated protein (Gao et al., 1996).

The subunits of TSP1 and TSP2 are composed of the same set of modules: a globular N-terminal heparin-binding module; an oligomerization domain; a central stalk composed of three types of well-known protein modules, a procollagen module, three properdin or type I repeats and three epidermal growth factor-like or type 2 repeats; and a C-terminal segment composed of a number of calcium binding or type 3 repeats followed by a long nonrepeating sequence (Lawler et al., 1985; Lawler and Hynes, 1986; O'Rouke et al., 1992). There is a gradient of increasing sequence identity from 38% in the N-terminal heparin-binding module to 82% in the C-terminal segment (Bornstein, 1992).

Similar to TSP1, TSP2 binds Ca^{++} (Chen et al., 1996b) and supports adhesion of endothelial cells, osteosarcoma cells and colon carcinoma cells mediated by heparan sulfate proteoglycans and the $\alpha_v\beta_3$ integrin (Chen et al., 1994). TSP1 binds and activates latent TGF-β, probably through the GGWSHW hexapeptide and the RFK tripeptide sequence found in the type I repeat region (Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1995). In TSP2, which binds but does not activate latent TGF-β (Schultz-Cherry et al., 1995), the RFK sequence is replaced with RIR. TSP2 inhibits TSP1-mediated latent TGF-β activation, suggesting a modulatory role for TSP2 in controlling this process. TSP1 and TSP2 are metabolized efficiently by a common internalization and degradation pathway involving heparan sulfate proteoglycan and low density lipoprotein receptor-related protein (Chen et al., 1996a). TSP1 (Rastinijad et al., 1989; Bagavandoss and Wilks, 1990; Good et al., 1990; Taraboletti et al., 1990) and TSP2 (Volpert et al., 1995; Panetti et al., 1997) have been shown to inhibit migration, mitogenesis and sprouting of endothelial cells *in vitro* and neovascularization *in vivo*. Hence, TSP1 and TSP2 may be important regulators of angiogenesis.

Investigation of protein expression and localization during embryogenesis provides clues to protein function. O'Shea and Dixit (1988) and Corless et al. (1992) have described the distribution of TSP1 in embryos. TSP1 was found in basement membranes of many tissues including the surface ectoderm, neuroepithelium, forming bronchi, salivary glands, kidney, colonic epithelium and the dermo-epidermal junction (O'Shea and Dixit, 1988). TSP1 was also present in the epicardium, forming myocardium, and endocardium; between neuroepithelial cells; in areas of peripheral nerve outgrowth such as the neural crest migratory pathway, ganglia and nerve rootlets emerging from the dorsal root ganglia; and on the surfaces of myoblasts and chondroblasts (O'Shea and Dixit, 1988). In general, TSP1 was associated with morphogenetic processes of cellular proliferation and migration.

mRNA transcripts for TSP1, TSP2 and TSP3 display temporally and spatially distinct distributions in the developing mouse (Iruela-Arispe et al., 1993). The promoter sequences of TSP1 (Donoviel et al., 1988; Laherty et al., 1989; Lawler et al., 1991) and TSP2 (Shingu and Bornstein, 1993; Adolph et al., 1997) are quite different. *In vitro* experiments have shown that TSP1 responds rapidly to serum and platelet-derived growth factor, whereas the expression of TSP2 is constitutive and does not respond to these growth stimulants (Bornstein et al., 1991; Laherty et al., 1992). Whether TSP1 and TSP2 are present in tissues is determined by translation of mRNAs and protein metabolism as well as by gene expression. Therefore, we have characterized the distribution of TSP2 protein during mouse embryogenesis and compared the distribution to that of TSP1. TSP1 was detected as early as the tenth day of development, whereas TSP2 was not expressed until day 14. There was colocalization of TSP2 and TSP1 in many instances. However, TSP2 displayed a distribution pattern that was more restricted and was predominantly found in cartilage and developing bone.

Materials and Methods

Production of recombinant TSPs and anti-TSP polyclonal antibodies

Mouse TSP1 (mTSP1) and TSP2 (mTSP2) were expressed in Sf9 cells using recombinant baculovirus and purified from conditioned medium by heparin-agarose affinity chromatography (Chen et al., 1994, 1996b). Human TSP1 (Panetti et al., 1997) and TSP2 (Tooney et al., manuscript in preparation) were also expressed in Sf9 cells using recombinant baculovirus and purified from conditioned medium by heparin-agarose affinity chromatography.

Rabbit antibodies directed against mTSP2 were described previously (Chen et al., 1996a). Rabbit antibodies directed against mTSP1 were produced by the same method. Titers and specificity were assessed by enzyme-linked immunosorbent assay and Western blot analysis. The rabbit anti-mTSP1 antiserum had a titer of 1:4000 for mTSP1 and did not react with mTSP2. The rabbit anti-mTSP2 antiserum displayed some cross-reactivity with mTSP1 by Western blotting. This cross-reactivity was removed by two consecutive adsorptions with mTSP1 coupled to cyanogen bromide-activated agarose.

Immunohistochemistry

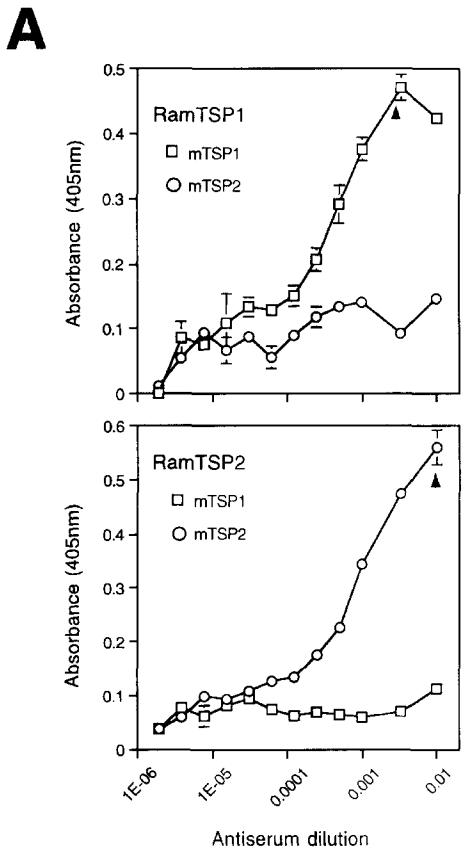
Embryos of C3H/HeJ females, newborn pups and femurs of 8-week-old Balb/c mice were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) (Sakai et al., 1994). After dehydration with water/ethanol solutions of increasing ethanol concentration, embryos and tissues were embedded in paraffin and serial sections, 4 μm thick, were cut. Immunohistochemistry was performed as described by Sakai et al. (1993a, 1993b) for tenascin-C with slight modifications. Sections were deparaffinized in xylene and rehydrated with water/ethanol solutions of increasing water concentrations. Sections were then treated with 0.4% (w/v) pepsin (Sigma, St. Louis, MO) in 0.01 M HCl for 2 h at 37 °C, followed by a rinse with water. The sections were immersed in methanol containing 0.3% (v/v) hydrogen peroxide for 30 min at room temperature to inactivate endogenous peroxidases. After washing with phosphate-buffered saline , the sections were incubated with phosphate-buffered saline containing 10% (v/v) normal donkey serum to block non-specific protein binding. Rabbit anti-TSP1 or -TSP2 or normal rabbit IgG was added to the section and incubated over night at 4 °C. Sections were then washed three times with phosphate-buffered saline before incubation with the secondary antibody, biotinylated donkey anti-rabbit IgG (Jackson Immuno-Res. Lab. Inc., West Grove, PA), for 1 h at room temperature. The sections were washed again and treated with peroxidase-conjugated streptavidin (DAKO, Denmark) for 40 min at room temperature. Bound enzyme-linked streptavidin was detected with 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Lab., Kumamoto, Japan) and 0.02% hydrogen peroxide in 50 mM Tris HCl (pH 7.6). Sections were counterstained with hematoxylin. The sections were examined on a Zeiss Axiovert 100 microscope using bright field optics. Images were captured with a CCD camera (DAGE DC330) and the built-in frame grabber of a Power Macintosh 8500 workstation loaded with the NIH image 1.6 software package. Composite images and figures were constructed using Adobe Photoshop 3.0 software.

Results

We characterized rabbit antibodies to recombinant mTSP1 and mTSP2 that had been expressed in Sf9 cells using recombinant baculoviruses. The antibodies displayed no cross-reactivity as assessed by enzyme-linked immunosorbent assay and Western blot analysis (Fig. 1) and recognized antigens with distinct localization patterns in pepsin-treated deparaffinized mouse embryonic tissues (Fig. 2). The antigen detected by our rabbit anti-mTSP1 antibodies had localizations similar to those described previously by O'Shea and Dixit (1988), using rabbit antibodies to human TSP1, and Corless et al. (1992), using a rat monoclonal anti-TSP1. Thus, results with our anti-mTSP1 antibodies serve as relevant positive and negative controls for the localizations found with the anti-mTSP2 antibodies. Comparisons of localizations of the two antigens with the expression patterns of TSP1 and TSP2 (Iruela-Arispe et al., 1993) are a further test of the validity of our findings.

TSP1 is present earlier during development than TSP2

TSP1 was present on day 10 and persisted throughout fetal development. Strong staining for TSP1 was found on day 10 in the myocardium (Fig. 3A). The staining intensity in the heart peaked on day 11 (Fig. 3C and 3E) and decreased thereafter. Strong staining for TSP1 was also found in the basement membrane of the intestinal epithelium and on the luminal surface of and between intestinal epithelial cells (Fig. 3A). In agreement with previous reports on protein and mRNA localization (O'Shea and Dixit, 1988; Iruela-Arispe et al., 1993), a strong positive reaction for TSP1 was detected on day 10 in the cytoplasm of large mononuclear cells sparsely scattered in connective tissue and small capillaries (not shown). Similar TSP1-positive cells, identified as megakaryocytes by their morphology, were present in the liver from day 11 to birth (Fig. 4). Megakaryocytes did not contain TSP2 (Fig. 4). TSP1 was detected in the choroid plexus from day 13 onward and in the basement membrane and columnar epithelium of bronchi from day 14 onward (not shown). Day 10–13 embryos lacked detectable TSP2 (Fig. 3).



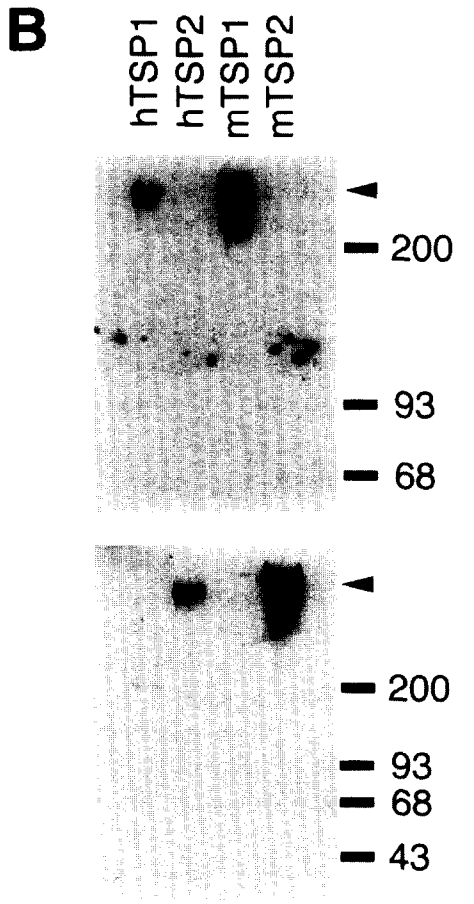


Figure 1. Specificity of the rabbit anti-mTSP1 and -mTSP2 antisera. (A) Wells were coated with 100 µl of mTSP1 (squares) or mTSP2 (circles) at 1 µg/ml, before addition of 100 µl of rabbit anti-mTSP1 (RamTSP1, top panel) or -mTSP2 (RamTSP2, bottom panel) antiserum diluted from 1/100 to $1/(5.12 \times 10^5)$. Bound antibody was detected by addition of alkaline phosphatase-conjugated goat anti-rabbit IgG (100 µl/well), followed by 100 µl of substrate solution and the absorbance was measured at 405 nm. Results are expressed as mean ± SE of triplicate wells from which the background binding to wells coated with chick egg ovalbumin (1 µg/ml) has been subtracted. Arrows point to the dilutions of antisera used for immunohistochemistry. (B) Purified recombinant hTSP1, hTSP2, mTSP1 and mTSP2 (1 µg/lane) were resolved under non-reducing conditions by SDS-PAGE on 5% acrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes and probed with rabbit anti-mTSP1 (top) or -mTSP2 antisera (bottom) diluted 1:2000 or 1:4000, respectively. Bound antibody was detected using horse radish peroxidase-conjugated goat anti-rabbit IgG and visualized using the enhanced chemiluminescence system. The positions of the molecular mass (kDa) markers are shown on the right; arrowheads indicate the positions of the TSPs.

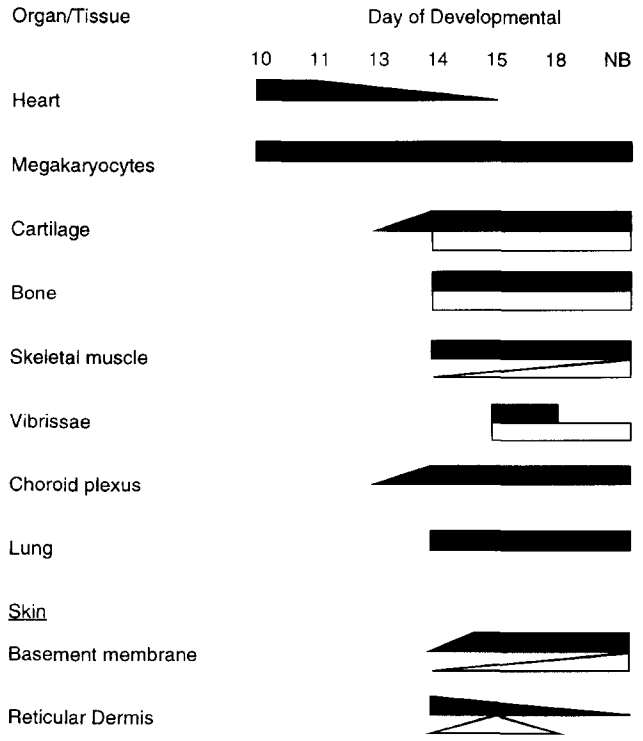


Figure 2. Localization of TSP1 and TSP2 during mouse embryogenesis. Closed bars represent TSP1, open bars TSP2. Sloped bars represent increase or decrease in staining intensity. NB, newborn.

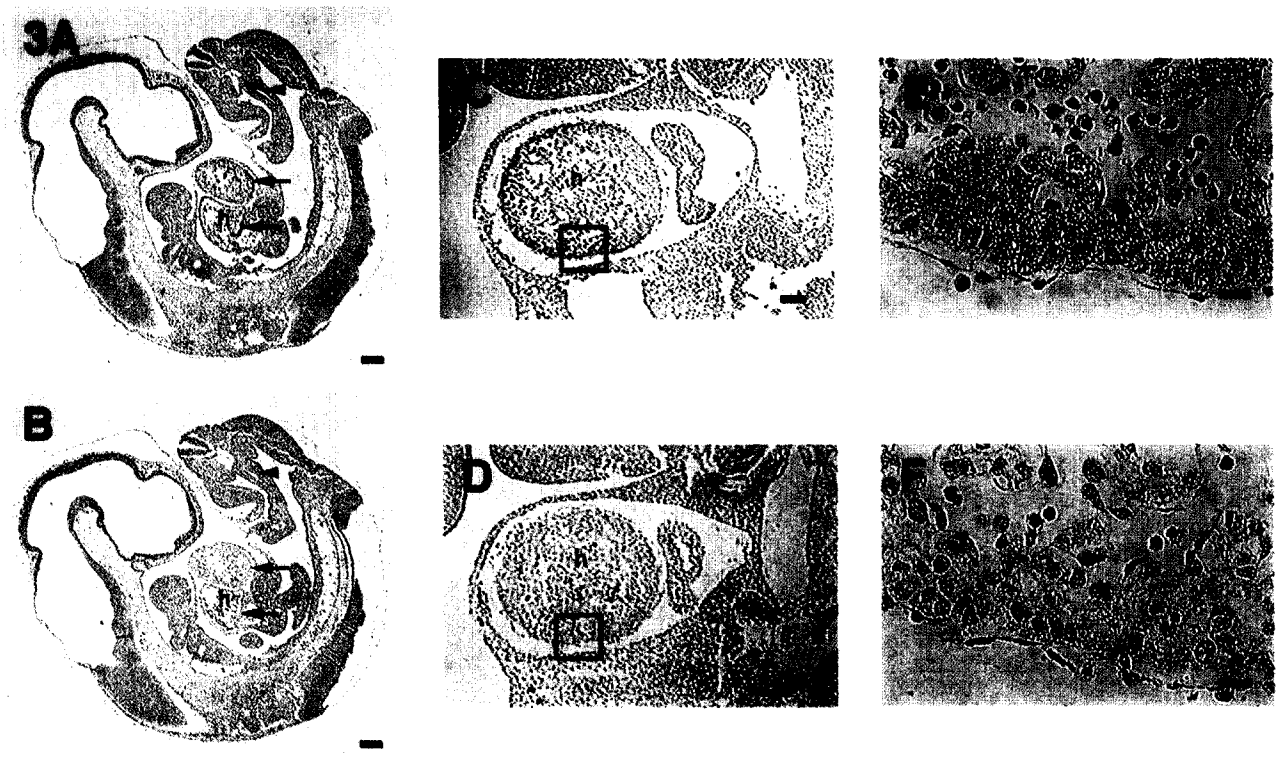


Figure 3. Localization of TSP1 and TSP2 in mouse embryos on the 10th and 11th day of gestation. Serial sagittal sections of day 10 and 11 mouse embryos were immunostained with rabbit anti-mTSP1 (A, C and E) or rabbit anti-mTSP2 (B, D and F). (A) and (B) Day 10 whole mouse embryo demonstrating TSP1 but not TSP2 staining in the heart (arrows) and intestinal epithelium (arrowhead). Bar, 200 μm. (C) and (D) Sagittal section through the thorax of the day 11 embryo demonstrating TSP1 localization in the developing heart. Bar, 100 μm. (E) and (F) Higher magnification of the day 11 heart representing the boxed areas in (C) and (D); TSP1 but not TSP2 is localized to the myocardium (arrowheads). h, heart. Bar, 20 μm.

Localization of TSP2 in chondrogenic and osteogenic tissues

TSP2 was detected from day 14 to birth predominantly in mesenchyme and cartilage anlagen that give rise to the skeletal elements. We describe these localizations in terms of intramembranous ossification, endochondral ossification, and the perichondrium rather than strictly temporally.

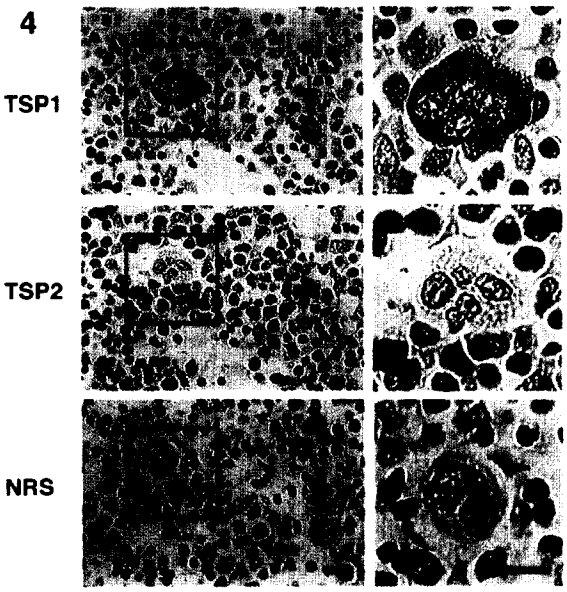


Figure 4. Megakaryocytes in the day 14 mouse embryo contain TSP1, but not TSP2. Serial sagittal sections through the liver of the day 14 mouse embryo immunostained with either rabbit anti-mTSP1 or anti-mTSP2 antisera or normal rabbit serum (NRS). TSP1 was detected in the cytoplasm of megakaryocytes (arrowhead and arrow), whereas TSP2 staining was not above the background seen with NRS (left panel). Bar, 20 μm. The right panel represents the boxed areas on the left at higher magnification. Bar, 10 μm.

Intramembranous ossification. The skull bones and mandibles develop by intramembranous ossification (direct conversion of mesenchymal tissue into bone) (Solursh, 1989). Both TSP2 and TSP1 were present in the head mesenchyme that gives rise to the skull bones from day 14 (Fig. 5A and 5B) to birth. TSP2 colocalized with TSP1 in ossification centers of the developing skull bones from day 14 onward (not shown) and in the mandibles on days 14 to 18 (Fig. 5C and 5D). In the newborn mouse, ossification centers in the developing mandibles stained strongly for TSP2 but not for TSP1 (Fig. 5E-5H).

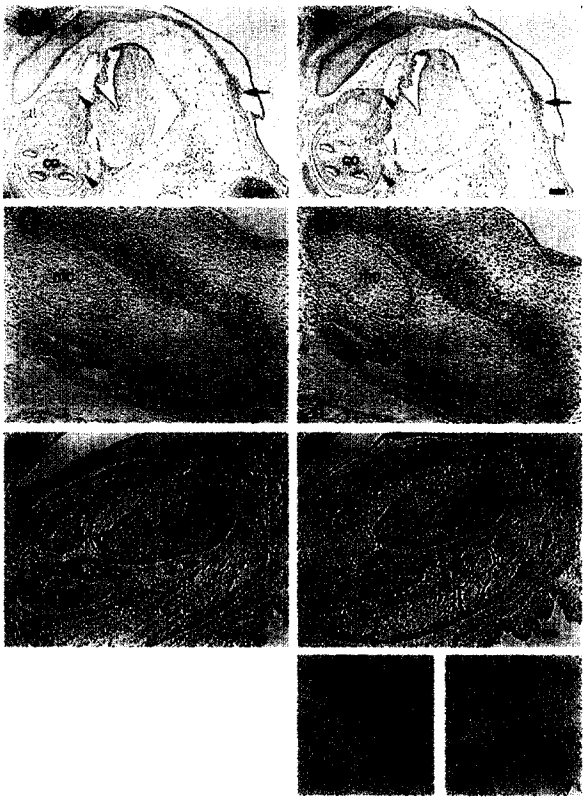


Figure 5. Sagittal sections through the head and mandibles of the day 14 embryo and the mandibles of the new born mouse. Serial sagittal sections were immunostained with rabbit anti-mTSP1 (A, C, E and G) or rabbit anti-mTSP2 (B, D, F and H). (A) and (B) TSP1 and TSP2 localize to the head mesenchyme (arrow) and to cartilage anlagen at the base of the skull (asterisk) and around the developing cochlea (co) in day 14 embryos (arrowheads). Bar, 100 μm. (C) and (D) Co-expression of TSP1 and TSP2 in ossification centers of the mandibles on day 14. Arrowhead indicates an area of colocalization. Faint staining for both TSP1 and TSP2 was also seen in Meckle's cartilage (mc). Bar, 50 μm. (E) and (F) Bone deposits around the tooth germ (tg) and mandibles of the newborn mouse contained strong staining for TSP2 but not TSP1. Bar, 100 μm. (G) and (H) Higher magnification of the boxed area in (E) and (F). Bar, 50 μm.

Endochondral ossification. Chondrogenesis begins with the proliferation and subsequent condensation of mesenchymal cells (Solursh, 1989; Gilbert, 1997). Once condensed, the cells differentiate into chondrocytes and secrete the chondrocyte-specific cartilaginous matrix (Solursh, 1989; Gilbert, 1997). The cartilage may remain throughout life (e.g., ear and nose cartilage). Alternatively, the cartilage of the growth zones serves as an intermediate for subsequent bone formation. This process, known as endochondral ossification, gives rise to most bones, including the ribs, vertebrae, clavicles and the temporal bone around the cochlea. Endochondral ossification starts with the hypertrophic differentiation of chondrocytes and ends with the resorption of the cartilage matrix and replacement with bone. On day 13, the condensed mesenchyme that forms the cartilage nodules of the developing ribs stained faintly for TSP1 but not for TSP2 (not shown). On day 14, TSP1 and TSP2 were found in chondrocytes and their surrounding extracellular matrix in the cartilage of the temporal bone around the developing cochlea (Fig. 5A and 5B) and ribs (Fig. 6A-6D). Strong staining for TSP1 and TSP2 was seen in bone deposits around hypertrophic chondrocytes in the ribs (Fig. 6A and 6B) and clavicle (Fig. 6E and 6F) from day 14 onward. TSP2 protein displayed a more restricted localization than TSP1 within cartilage from day 15 to birth. Cartilage proper and resting chondrocytes of the growth zone were largely negative for TSP2, whereas there was TSP2 staining in the extracellular matrix of proliferating and hypertrophic chondrocytes in the growth zone. This was clearly demonstrated in serial sections of developing vertebrae on day 18 (Fig. 6G and 6H). TSP2 was absent in chondrocytes and the surrounding extracellular matrix of the resting zone adjacent to the vertebral disc; it was present in the extracellular matrix around chondrocytes in the growth zone from the proliferative through to the hypertrophic zones and in depositing bone (Fig. 6H). TSP1 was localized to the surface and extracellular matrix surrounding chondrocytes in cartilage proper and to a lesser degree surrounding the resting and proliferating chondrocytes in the growth zone (Fig. 6G). As chondrocytes became hypertrophic, TSP1 staining diminished further. Staining for TSP1 in bone deposits, however, was equivalent to the intensity of staining for TSP2.

Perichondrium. A layer of mesenchyme (perichondrium) remains after the initial condensation of cartilage and is believed to proliferate to form chondrocytes (Gilbert, 1997). The perichondrium around cartilage in the ribs and nasal cavity (Fig. 6I-6N) and temporal

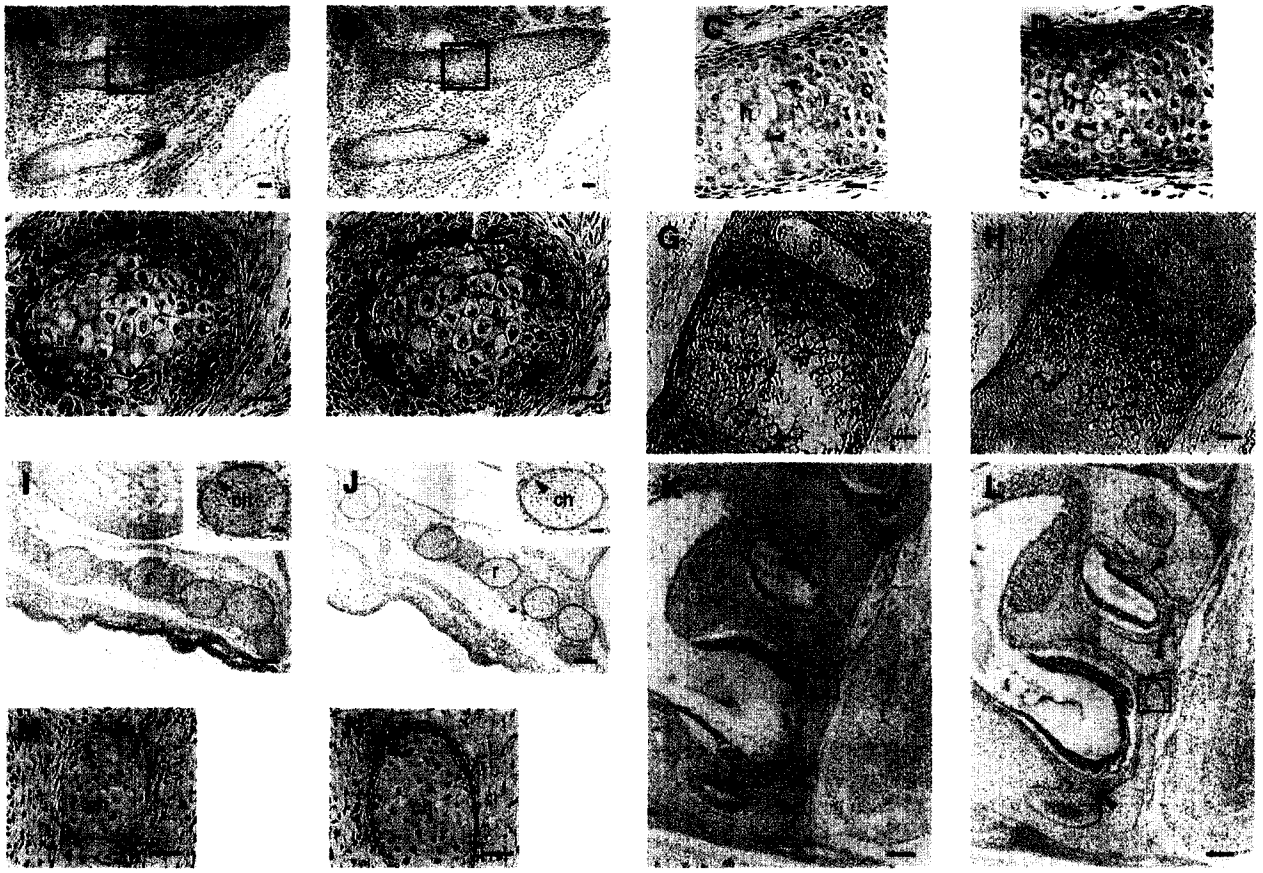


Figure 6. Localization of TSP1 and TSP2 in cartilage, bone and the perichondrium in the day 14, 15 and 18 embryos and newborn mouse. Serial sagittal sections were immunostained with rabbit anti-mTSP1 (A, C, E, G, I, K and M) or rabbit anti-mTSP2 (B, D, F, H, J, L and N). (A) and (B) TSP1 and TSP2 staining in the cartilage of the first and second ribs of the day 14 embryo. Bone deposits in between the differentiating chondrocytes and the perichondrium of the second rib stained for TSP1 and TSP2 (arrowhead). Bar, 50 μm. (C) and (D) High magnification image representing the boxed area in (A) and (B), demonstrating the localization of TSP1 and TSP2 around chondrocytes undergoing hypertrophy (arrowheads). Bar, 20 μm. (E) and (F) TSP1 and TSP2 are co-expressed in bone deposits (arrow) and around hypertrophic chondrocytes (arrowhead) in the clavicle of the day 14 embryo. Bar, 20 μm. (G) and (H) In developing vertebrae on day 18, TSP1 was seen in the resting and proliferating zones, with less in the hypertrophic zone. TSP2 staining intensity increased from the late proliferating zone through to the hypertrophic zone. Some bone trabeculae were also positive for TSP1 and TSP2 (arrowhead). Bar, 50 μm. (I) and (J) Day 15 embryo with strong TSP2 and weaker TSP1 staining in the perichondrium (inset, arrowhead) of the ribs (r). Bar, 100 μm. Inset, higher magnification image of the fifth rib from (I) and (J) showing TSP1 and not TSP2 staining of the cartilage matrix. Bar, 30 μm. (K) and (L) TSP2 staining in the perichondrium of the nasal cavities in serial sagittal sections from the newborn mouse (arrowheads). TSP1 staining was seen in the cartilage matrix but not in the perichondrium. Bar, 500 μm. (M) and (N) Higher magnification of the boxed area in (K) and (L), demonstrating strong TSP2 and not TSP1 staining in the perichondrium (arrowhead) surrounding the nasal cartilage. Bar, 20 μm. c, condensed chondrocytes; h, hypertrophic chondrocytes; p, proliferating chondrocytes; d, vertebral disc; ch, chondrocytes.

bones surrounding the cochlea (not shown) stained intensely for TSP2 from day 14 to birth, but it contained only low or undetectable levels of TSP1. In a femur from an 8-week-old mouse, the periosteum was also positive for TSP2, as was the matrix around hypertrophic chondrocytes in the not yet closed growth plate between primary and secondary ossification centers (not shown).

Localization of TSP2 in other tissues

Areas other than cartilage and developing bone also stained for TSP2. Fibroblasts in the reticular dermis (Fig. 7B) and surrounding part of the spinal cord stained faintly for TSP2 on day 14, with reduced staining seen on day 15 and no staining on day 18 or in the newborn mouse (not shown). Stronger anti-TSP1 staining was

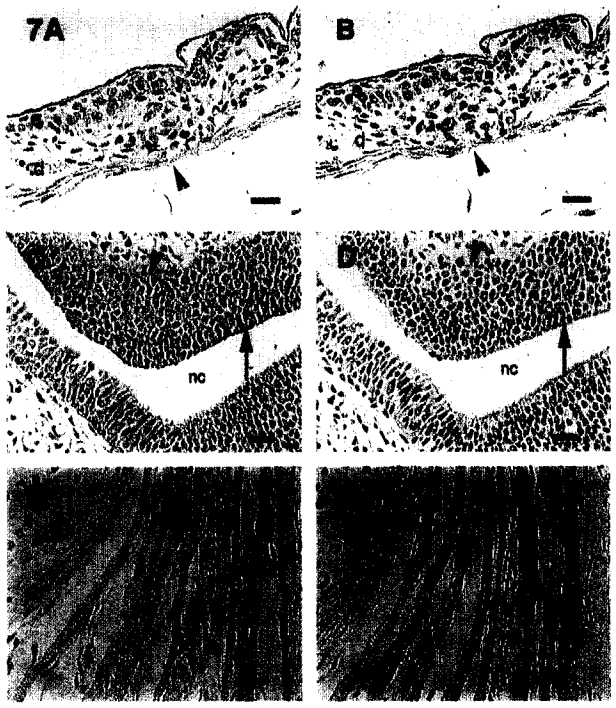


Figure 7. The localization of TSP1 and TSP2 in several tissues from the day 14 embryo and the newborn mouse. Serial sagittal sections were immunostained with rabbit anti-mTSP1 (A, C and E) or rabbit anti-mTSP2 (B, D and F). (A) and (B) TSP1 and TSP2 staining in the reticular dermis (arrowhead) of the skin on day 14. (C) and (D) TSP1 and TSP2 localized to the apical surface of nasal epithelium on day 14 (arrow). TSP1 was also seen between cells and along the basement membrane underneath the nasal epithelium (arrowhead). nc, nasal cavity. (E) and (F) Skeletal muscle (sm) around the developing vertebrae of the newborn mouse stained for both TSP1 (arrow) and TSP2 (arrowhead). ch, chondrocytes; pc, perichondrium. Bar, 20 μm.

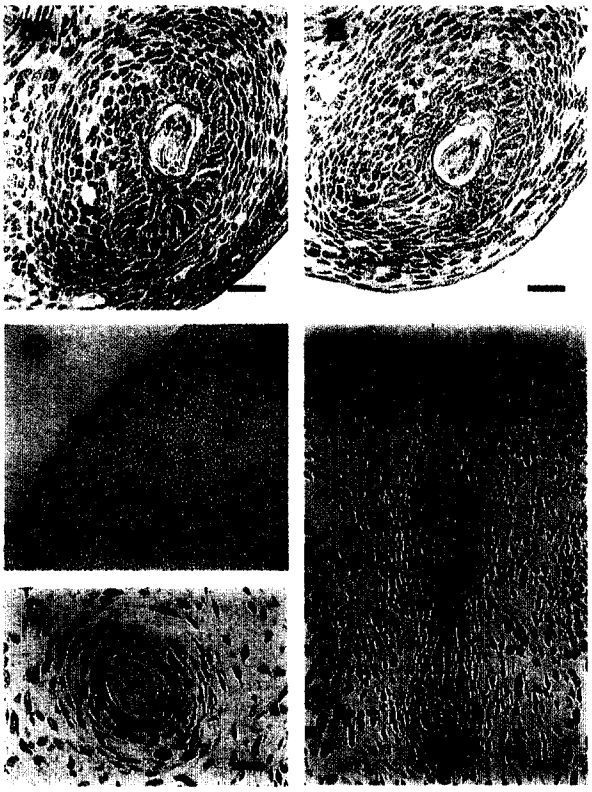


Figure 8. Sagittal sections of the vibrissae from the day 15 and 18 embryos. Sections were immunostained with rabbit anti-mTSP1 (A) or rabbit anti-mTSP2 (B, C, D and E). (A) and (B) A faint band (arrowhead) of TSP1 and TSP2 staining was detected on the inner side of the connective tissue sheath surrounding vibrissae on day 15. TSP1 was also detected in the epidermis of the developing skin. Bar, 20 μm. (C) Strong staining for TSP2 in the keratinized hair shaft of the vibrissae on day 18. Bar, 50 μm. (D) and (E) High magnification images of vibrissae from (C), indicated by the arrowhead and arrow, respectively. The apparent staining of the epidermis in C and E was not reproducibly above control and is not considered specific. Bar, 20 μm.

detected in the reticular dermis on day 14 (Fig. 7A) and 15, with lower staining on day 18 and no specific staining in the newborn mouse (not shown). Patchy staining for TSP1 was seen in the basement membrane at the dermo-epidermal junction of the skin from day 14 to newborn (not shown). The basement membrane of the dermo-epidermal junction stained faintly for TSP2 on day 14, with intensity increasing to the time of birth (not shown). The apical surface of the nasal epithelium was stained for TSP2 and TSP1 on day 14. TSP1 was also found in between nasal epithelial cells and along the basement membrane (Fig. 7C and 7D). TSP2 was not detected on the apical surface of the nasal epithelium after day 14, whereas the anti-TSP1 staining persisted on days 15 and 18 but was absent in the newborn mouse (not shown). Weak staining for TSP2 was seen in skeletal muscle cells on days 14, 15 and 18, with maximal intensity observed in the newborn mouse (Fig. 7F). In agreement with O'Shea and Dixit (1988), TSP1 stained strongly in these cells from day 14 onward (Fig. 7E). TSP1 was detected in the meninges of the forebrain on day 18, where only faint anti-TSP2 staining was observed (not shown). Staining for TSP2 was seen in the cortex of the adrenal gland on day 15 (not shown).

On day 15, faint TSP1 and TSP2 staining was detected in a ring on the inside of the connective tissue sheath surrounding vibrissae (Fig. 8A and 8B), correlating well with the mRNA data of Iruela-Arispe et al. (1993). On

day 18, the keratinized hair shaft of developing vibrissae stained strongly for TSP2 (Fig. 8C–8E), whereas there was no specific staining for TSP1.

Discussion

Previous reports have studied the immunolocalization of TSP protein in various tissues (Wright et al., 1985; O'Shea and Dixit, 1988; O'Shea et al., 1990a; O'Shea et al., 1990b; Corless et al., 1992). These studies used polyclonal or monoclonal antibodies directed against human TSP from platelets (i.e., TSP1). We developed specific antibodies to mTSP1 and mTSP2, studied the localization of TSP2 protein during mouse embryonic development, and compared the localizations of TSP2 and TSP1.

Localization of TSP1

The localization of TSP1 closely resembled that reported by O'Shea and Dixit (1988) and Corless et al. (1992) and is summarized in Figure 2. The presence of TSP1 and absence of TSP2 in the lung is noteworthy, since TSP1 deficient-mice develop acute and chronic organizing pneumonia in response to otherwise nonpathogenic bacteria (Lawler et al., 1998). Both O'Shea and Dixit (1988) and Corless et al. (1992) observed strong staining for TSP1 in the neuroepithelium of the brain and spinal cord. However, we could detect only low levels in these areas. This difference may be explained by the differences in staining methods. We pretreated sections with pepsin before addition of the antibody, because preliminary experiments showed that pretreatment with pepsin was necessary to obtain positive staining for TSP2 in areas such as the perichondrium where TSP2 transcripts were detected by *in situ* hybridization (Iruela-Arispe et al., 1993). O'Shea and Dixit (1988) reported that after pretreatment of their sections with testicular hyaluronidase, they could not detect TSP1 between neuroepithelial cells. Therefore, it is possible that our enzymatic pretreatment destroyed the TSP1 epitopes in the neuroepithelium.

Localization of TSP2

The expression of messages for TSP1 and TSP2 during development is tightly regulated (Iruela-Arispe et al., 1993). Message for TSP1 was found on day 10, whereas low levels of TSP2 mRNA expression were not detected until days 11 and 12, with levels increasing thereafter. In general, our immunolocalization studies correlated with the *in situ* hybridization studies. TSP2 protein displayed a restricted localization when compared to TSP1 (Fig. 2). TSP2 was predominantly confined to tissues derived from mesenchyme such as cartilage, bone and connective tissue. In cartilage, particularly striking staining was present in the late proliferative zone through to the hypertrophic zone of growth plates. TSP2 was also prominent in the perichondrium, whereas weak or no staining was seen for TSP1. TSP2 and TSP1 also colocalized within the mesenchyme that undergoes intramembranous ossification to form the bones of the skull. Ossification centers of the developing skull and mandibles contained both TSP1 and TSP2. One exception was observed in the newborn mouse, where exclusive staining for TSP2 was demonstrated in ossification centers of the lower mandibles. The localization of TSP2 in the adrenal gland is in accord with the finding that TSP2 is secreted prominently by adrenocortical cells upon stimulation with adrenocorticotropin (Pellerin et al., 1993a, 1993b). The low intensity of staining may reflect the levels of adrenocorticotropin present during embryogenesis.

Some differences were observed between *in situ* hybridization and immunolocalization. Both Iruela-Arispe et al. (1993) and Tucker (1993) reported the expression of TSP2 transcripts in endothelial cells of capillaries and large vessels, in the mesenchyme surrounding bronchi and bronchioles, in the smooth muscle layer of some segments of the intestine and in the kidney. We could not detect significant levels of TSP2 protein in these cells and tissues. This is probably not due to destruction of the antibody epitopes in these tissues during enzyme treatment of the sections, because no staining for TSP2 was seen without this treatment (not shown). Alternatively, if the turnover rate of TSP2 protein is high in these tissues, then rapid degradation could result in undetectable levels of protein being present.

Potential functional activities for TSP2

The distinct, yet overlapping, distribution of TSP1 and TSP2 in the skeletal elements suggests different functions for these proteins in chondrogenesis and osteogenesis. This is supported by the different skeletal abnormalities exhibited by mice lacking the genes for TSP1 (Lawler et al., 1998) and TSP2 (Kyriakides et al., 1998). The TSP1-deficient mice exhibit a lordotic curvature of the spine which is evident from birth, the cause of which is unknown (Lawler et al., 1998). The TSP2-deficient mice display increased cortical bone thickness and density, a mild kyphosis, and remarkable flexibility in the tail (Kyriakides et al., 1998). Previous reports have shown

that TSP1 is produced by osteocytes (Clezardin et al., 1989) and is involved in bone resorption (Carron et al., 1995). When sections of the femur from an 8-week-old mouse were studied, we found TSP2 in the periosteum and the matrix around hypertrophic chondrocytes in the growth plate between primary and secondary ossification centers. The osteocytes within the bone, and the bone itself, did not contain TSP2. Hence, TSP2 appears to be localized to areas where bone formation is occurring, suggesting that the bone defect seen in the TSP2-deficient mice may be due to an alteration in bone formation rather than bone resorption.

TSP2 may be involved in the differentiation of mesenchymal cells during intramembranous ossification, the differentiation of mesenchymal cells from the inner layer of the perichondrium into chondrocytes and, at later stages, the differentiation of chondrocytes to a hypertrophic state. Mackie et al. (1987) reported that another extracellular matrix protein, tenascin-C, is expressed in the perichondrium and condensing mesenchyme during intramembranous ossification and demonstrated that tenascin-C promotes chondroblast differentiation in vitro. Whether TSP2 performs similar functions as tenascin-C during chondrogenesis and osteogenesis remains to be determined. In contrast, TSP1 is present in the cartilage proper and may play a role in the proliferation of chondrocytes, as suggested by O'Shea and Dixit (1988), and maintenance of the chondrocyte phenotype.

The members of the other TSP subfamily (i.e., TSP3, TSP4 and TSP5) also display distinct localization patterns within chondrogenic and osteogenic tissue (reviewed in Adams et al., 1995). TSP3 message is seen in chondrocytes of the early proliferative zone (Iruela-Arispe et al., 1993). TSP3 protein is strongly expressed in bone spicules and weakly in mature and differentiating chondrocytes (Qabar et al., 1994). TSP4 message is associated with the onset of osteogenesis in the perichondrium, the periosteal collar and, similar to TSP2, the mesenchyme that forms the skull bones (Tucker et al., 1995). TSP5 is an integral component of cartilage (Franzen et al., 1987; Hedbom et al., 1992) and is found in articular cartilage and sites of chondronecrosis and to a lesser extent in proliferative and hypertrophic chondrocytes (Ekman and Heinegård 1992). Missense mutations of TSP5 are associated with pseudochondroplasia and multiple epiphyseal dysplasia (Briggs et al., 1995; Hecht et al., 1995). These findings suggest that the five TSPs may have distinct yet overlapping functions during development of the skeletal elements.

TSP2-deficient mice display abnormalities in connective tissue structure within both skin and tendon. Specifically, the collagen fibrils are disordered and abnormally large (Kyriakides et al., 1998), suggesting a role for TSP2 in their assembly. TSP2 was found in various extracellular matrices and may play a role in determining or maintaining the structure of these protein assemblies. TSP1 binds a number of plasma and extracellular matrix components, including several types of collagen, fibronectin, laminin, heparan sulfate, fibrinogen, plasminogen and osteonectin (reviewed in Mosher, 1990; Frazier, 1991; Bornstein, 1995). The similar modular structure and considerable sequence identity between TSP1 and TSP2 (Bornstein, 1992) suggest that TSP2 may bind some or all of these molecules.

Alternatively, TSP2 may have a role in cell adhesion to the extracellular matrix. When coated onto plastic surfaces, TSP2 supports the adhesion of bovine aortic endothelial cells, HT-29 human colon adenocarcinoma cells, and MG-63 human osteosarcoma cells (Chen et al., 1994) and bovine adrenocortical cells (Pellerin et al., 1993a). Skin fibroblasts from the TSP2-deficient mice display defective attachment to the substratum (Kyriakides et al., 1998). When secreted into the extracellular space, TSP2 may become incorporated into the extracellular matrix and function as an adhesive substrate during development.

TSP1 inhibits endothelial cell migration, proliferation and sprouting *in vitro* and neovascularization *in vivo* (Rastinijad et al., 1989; Bagavandoss and Wilks, 1990; Good et al., 1990; Taraboletti et al., 1990). TSP2 also exhibits anti-angiogenic properties in these assays but has less specific activity than TSP1 (Volpert et al., 1995; Panetti et al., 1997). The TSP1-deficient mice display prolonged angiogenesis after skin wounding (Polverini et al., 1995). TSP2-deficient mice display increased blood vessel density within a number of embryonic and adult tissues and an abnormally long bleeding time (Kyriakides et al., in press). Cartilage is with a few exceptions an avascular tissue. One exception is where capillaries invade the mineralized cartilage of the hypertrophic zone in the growth plate, which is then removed and replaced by bone (Schenk et al., 1967, 1968). TSP1 produced by chondrocytes in cartilage proper may inhibit angiogenesis completely, whereas TSP2 functions as an angiogenic regulator during endochondral bone formation to ensure that excessive blood vessel formation does not occur.

In what ways are the functions of TSP1 and TSP2 related?

Both TSP1 and TSP2 have a number of potential activities, as mentioned above. Our localization studies indi-

cate that these activities would be manifest in a restricted number of embryonic tissues and at distinct stages of morphogenesis. TSP2 colocalized with TSP1 in the connective tissue of the reticular dermis, cartilage and bone matrix, skeletal muscle, and the apical surface of nasal epithelium. This overlap is potentially important because the two TSPs may form heterotrimers (O'Rouke et al., 1992), may compensate for one another functionally or, as homotrimers, may modulate the activities of one another (Schultz-Cherry et al., 1995; Chen et al., 1996a). The distinct phenotypes of the TSP1-deficient and TSP2-deficient mice could be due to differences in expression of the two proteins or to different functional activities. The possibility that TSP1 and TSP2 share the same *in vivo* functions should become clearer when the phenotype of mice lacking both TSP1 and TSP2 is known.

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