PERGAMON

Archives of Oral Biology 45 (2000) 19–25

Archives of Oral Biology

www.elsevier.com/locate/archoralbio

Thrombospondin-2 gene expression and protein localization during embryonic mouse palate development

M. Melnick*, H. Chen, Y. Zhou, T. Jaskoll

Laboratory for Developmental Genetics, University of Southern California, 925 W. 34th Street, Los Angeles, CA 90089-0641, USA

Accepted 18 August 1999

Abstract

The mammalian palate develops from projections of the paired maxillary processes termed palatal shelves. Shelf growth is an essential in normal palatal morphogenesis. Mesenchymal proliferation in the palatal shelves is modulated by transforming growth factor-β (TGF-β), among other growth factors. Several pathways effect TGF-β activation, including one which utilizes thrombospondin (TSP). TSP-1 is a major activator of TGF-β in vivo and has been localized in head mesenchyme, including palates. TSP-2 appears to inhibit TSP-1 activation of latent TGF-β by competitively binding the latent TGF-β. Here the TSP-2 mRNA transcript and the immunolocalization of TSP-2 protein with progressive palatogenesis were quantified. There was a significant ($p < 0.05$) decline of TSP-2 transcript with palatal maturation; there was no evidence correlating the TSP-2 transcription with the amount of activated TGF-β. At the vertical shelf stage of palatogenesis, TSP-2 protein was found throughout the extracellular matrix of shelf mesenchyme. By the horizontal shelf stage, TSP-2 protein was principally localized to the ossification centres of the developing maxilla, both in extracellular matrix and bone; far less was seen in palatal shelves proper. These results suggest that TSP-2 is multifunctional during embryonic palate formation.

Keywords: Thrombospondin-2; Gene expression; Palate; Mouse

1. Introduction

The mammalian palate begins development from projections of the paired maxillary processes of the first branchial arches, termed palatal shelves (or lateral palatine processes). Initially, these shelves are in a vertical position on each side of the developing tongue, but, as the mandible grows, the tongue moves downward, and the shelves become horizontal and grow toward each other (see Figs. 2A and 3A). Subsequently, the shelves grow sufficiently large to approximate one another and begin the fusion process. Thus, shelf growth is an essential of normal palatal morphogenesis.

The TGF-β family of autocrine/paracrine growth factors participates in regulating cell proliferation and differentiation, as well as in the formation and degradation of extracellular matrix (Derynck, 1994; Lawrence, 1996). TGF-βs and their cognate receptors

Abbreviations: E, embryonic day; IGF, insulin-like growth factor; TGF-β, transforming growth factor-β.

* Corresponding author. Tel.: +1-213-740-1417; fax: +1-213-740-7560.

E-mail address: mmelnick@hsc.usc.edu (M. Melnick).

0003-9969/00/$ - see front matter
PII: S0003-9969(99)00113-2

are present in the developing palate (Gehris et al., 1991; Jaskoll et al., 1996; Linask et al., 1991; Melnick et al., 1998; Pelton et al., 1990): TGF-β_1 and -β_3 are expressed primarily in the epithelium, whereas TGF-β_2 is expressed primarily in the mesenchyme. The amount of TGF-β_2 decreases significantly with progressive palatal shelf development; this downregulation in TGF-β_2 expression is associated with increased mesenchymal cell proliferation and palatal shelf growth (Jaskoll et al., 1996).

TGF-β is somewhat idiosyncratic among known growth factors in that it is secreted and found primarily in a latent (non-active) form in vivo (Gleizes et al., 1997). The amount of active ligand available for receptor binding is not necessarily correlated with total TGF-β transcription and translation (Gleizes et al., 1997; Melnick et al., 1998). Several pathways effect TGF-β activation. Most prominent is the binding of latent TGF-β to cell-surface IGF-II receptor and cleavage by plasminogen activator-dependent plasmin (Dennis and Rifkin, 1991). IGF-II receptor, plasminogen, and plasminogen activators are all present in embryonic palatal shelves (Melnick et al., 1998). The latent TGF-β can also bind to integrin $\alpha V\beta_6$ and other integrins on the cell surface, which induces activation via a conformational change in the latent complex rather than cleavage (Munger et al., 1999).

A third TGF-β activation pathway utilizes thrombospondin-1, a large, homotrimeric glycoprotein secreted by many cell types into the extracellular matrix (Bornstein, 1992). Binding by thrombospondin-1 of latent TGF-β induces a conformational change that exposes mature TGF-β to cognate receptors while remaining bound to thrombospondin-1 (Murphy-Ullrich et al., 1992; Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1994, 1995). It has recently been shown that thrombospondin-1 is a major activator of TGF-β in vivo (Crawford et al., 1998). It has also been demonstrated in vitro that thrombospondin-2, in many ways homologous to thrombospondin-1 (Bornstein, 1992), inhibits activation of latent TGF-β by competitively binding it (Schultz-Cherry et al., 1995).

Existing reports on the spatiotemporal distribution of thrombospondin transcript and protein suggest that thrombospondin plays an important part in the proliferation of embryonic mesenchymal cells, including head mesenchyme (Iruela-Arispe et al., 1993; Laherty et al., 1992; O'Shea and Dixit, 1988; Tooney et al., 1998). Thrombospondin-1 transcript, in particular, has been detected in head mesenchyme beginning on E10, including palates (Iruela-Arispe et al., 1993), and thrombospondin-1 protein from E13 to birth (Tooney et al., 1998). We report here the quantitation of thrombospondin-2 mRNA transcript and the immunolocalization of its protein with progressive palatogenesis.

2. Material and methods

2.1. Animal mating and tissue collection

B10.A/SgSn (B10.A) and C57BL/10SgSn (B10) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our animal facilities in accord with NIH Guidelines. Animals were mated overnight and females were examined for vaginal plugs the next morning; this day was considered day 0 of gestation. Dams were anaesthetized with metafane and killed by cervical dislocation on the appropriate day of gestation. Embryos were staged according to Theiler (1989). Palatal shelves were dissected from staged embryos on days 13 (E13), 14 (E14), or 15 (E15) of gestation, pooled by litter and stored at -70°C. E13–E15 heads were fixed for immunohistochemistry as described below.

2.2. Analysis of thrombospondin-2 transcripts by ribonuclease protection assay

Steady-state amounts of thrombospondin-2 transcripts were measured by RNase protection assays essentially as described in Melnick et al. (1998). Each independent sample was a pool of five to eight palates from one litter; a minimum of three independent samples per day of gestation were assayed. Each sample was assayed two to three times. β-actin transcripts provided an internal standard for each sample. The control without palatal RNA contained 50 μg yeast tRNA. To determine possible differences in thrombospondin-2 between B10.A and B10 mouse strains, palatal shelves were obtained from three litters of B10.A and B10 at E14 and assayed; assays were done on E14 palates because strain-specific differences were previously detected only on E14 (Melnick et al., 1998). A 400-nt thrombospondin-2 protected fragment and a 250-nt β-actin protected fragment are detected. Protected RNAs corresponding to hybrids between thrombospondin-2 probe and its target mRNAs were quantitated by phosphor image analysis (Molecular Dynamics, Sunnyvale, CA) and normalized to the amount of protected β-actin probe present in each sample. Ratios were mathematically transformed to meet the analytical assumptions of normality and homoscedasticity, means were calculated, and differences between mean ratios were compared by *t*-test.

2.2.1. Probes

A plasmid containing a 500-bp *Thbs2* cDNA was generously provided by Dr Paul Bornstein (Shingu and Bornstein, 1993). A 430-bp full-length probe was linearized with *Bgl*II and a ^{32}P-labelled antisense probe was generated using T3 RNA polymerase and ^{32}P-UTP (ICN, Costa Mesa, CA). A gel-purified plasmid

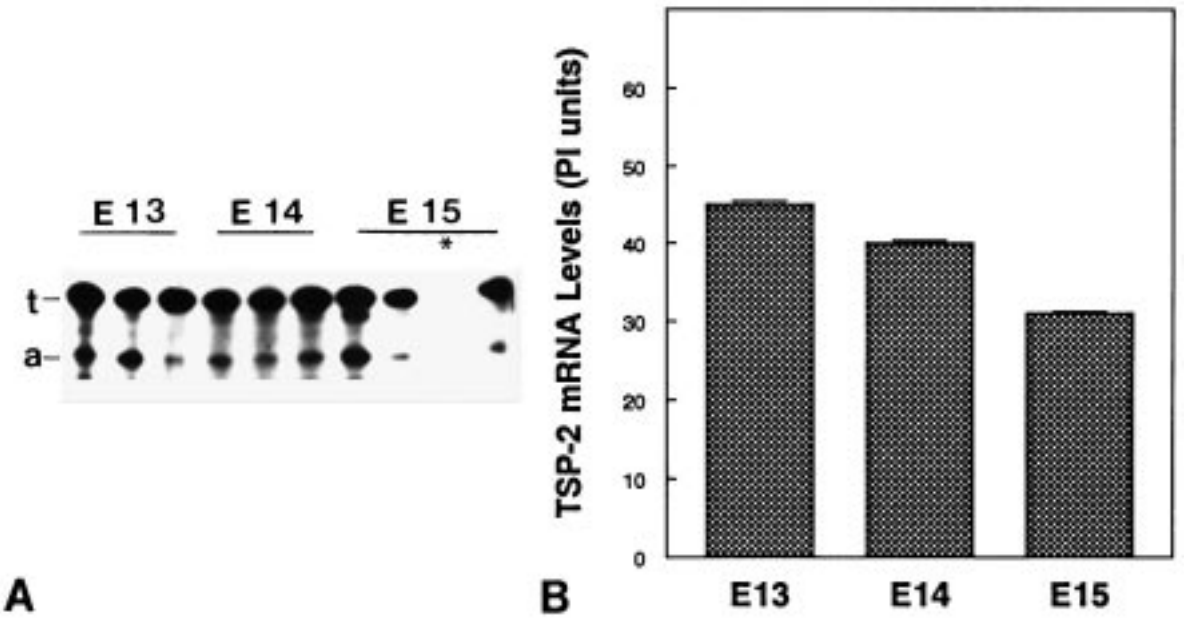

Fig. 1. Developmental expression of thrombospondin (TSP)-2 transcripts in embryonic B10.A mouse palates. (A) RNase protection assay comparing steady-state amounts of TSP-2 mRNA in E13–E15 B10.A palates. The autoradiograph demonstrates a 400-nt TSP-2 (t) protected fragment and a 250-nt β-actin (a) protected fragment in three independent E13, E14, and E15 samples. Note the absence of TSP-2 and β-actin protected fragments in a control lane (∗). (B) Bars represent mean phosphor-imaging (PI) units with their SEM for E13–E15 TSP-2 transcript.

containing a 250-bp mouse β-actin cDNA insert was purchased from Ambion (Austin, TX), linearized with *Eco*RI, and used to produce a ^{32}P-labelled antisense probe using T7 RNA polymerase.

2.3. Immunolocalization of thrombospondin-2 protein

E13–E15 heads were fixed in Carnoy's fixative, processed, and embedded in low-melting point Paraplast as described by Melnick et al. (1998). A minimum of three litters were collected for each day of gestation. The tissues were sectioned at 7 μm, placed on cleaned, gelatin-coated slides at 37°C for 3 h, and immediately immunostained for thrombospondin-2 protein expression according to the method of Kyriakides et al. (1998). In brief, the slides were preincubated in 3% hydrogen peroxide to eliminate endogenous peroxidase activity, blocked with 1% bovine serum albumin, and incubated overnight at 4°C with polyclonal rabbit anti-mouse thrombospondin-2 antibody (1:400; generously provided by Dr Paul Bornstein). Detection of thrombospondin-2 specific immunoreactivity was achieved using an Elite ABC Kit (Vector Labs, Inc, Burlington, CA). Following development of peroxidase activity, the sections were counterstained with haematoxylin, processed, and coverslipped as previously described (Melnick et al., 1998). A minimum of three palates were evaluated for each stage of development. Controls consisted of sections stained with secondary antibodies alone; controls were routinely negative.

3. Results

We quantitated thrombospondin-2 mRNA transcripts in B10.A mouse palates from E13 to E15, vertical to horizontal palatal shelves, respectively. There was a significant, 31% decline with palatal maturation ($t_4 = 2.80$; $p < 0.05$) (Fig. 1). As it has been reported that *Thbs2* gene expression in vitro is positively correlated with the amount of activated TGF-β (Negoescu et al., 1995), we also quantitated thrombospondin-2 transcripts in B10 embryonic mouse palates. B10 palates are known to have significantly less activated TGF-β than palates from B10.A embryos (Melnick et al., 1998). There were no significant differences in amounts of mRNA transcript between the strains ($t_7 = 1.70$; $p > 0.10$) and, thus, no in vivo evidence to support prior in vitro studies.

Immunolocalization of thrombospondin-2 protein in B10.A mouse embryos at the vertical shelf stage of palatogenesis (c.E13) showed the protein throughout the extracellular matrix of palatal shelf mesenchyme (Fig. 2B,C). It was also localized in the basement

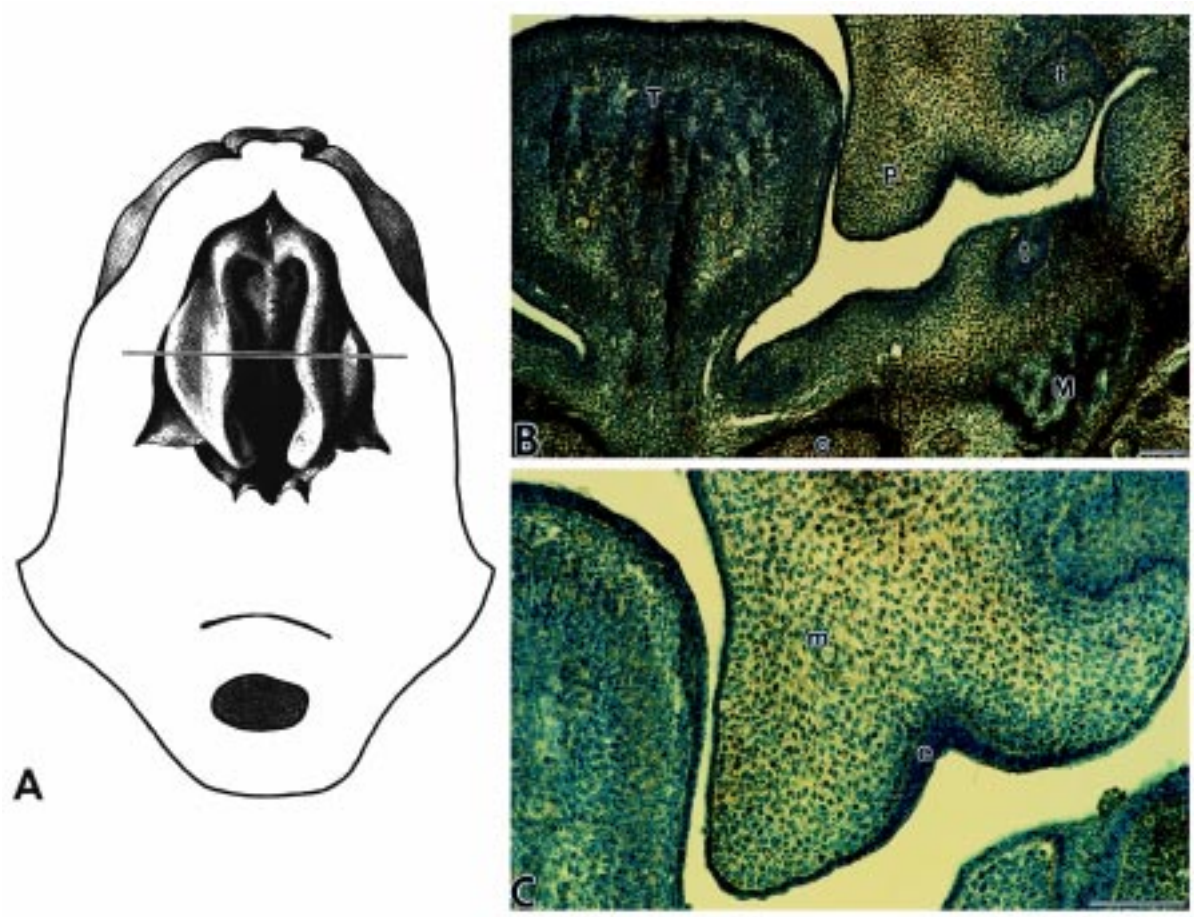

Fig. 2. Spatial distribution of thrombospondin (TSP)-2 protein in early embryonic palates. (A) View of the palate as seen with removal of the lower jaw. At this early stage of palatal development, both shelves are vertical. Line indicates the plane of section for (B). (B, C) Immunolocalization of TSP-2 in developing vertical palatal shelves. (B) Anti-TSP-2-stained section shows the relation between vertical palatal shelves (p) and the developing tongue (T). (C) Higher magnification of the palatal shelf shown in (B). TSP-2 protein is immunodetected throughout the extracellular matrix of palatal shelf mesenchyme (m) and is absent from the epithelium (e). TSP-2 is also immunolocalized in the basement membranes of the toothbuds (t), in Meckel's cartilage (c) and its perichondrium, and in ossification centres of the mandible (M). Bar = 100 μm.

membranes of tooth epithelium, Meckel's cartilage (particularly the perichondrium), and in the ossification centres of the developing mandible (Fig. 2B). By the horizontal shelf stage of palatogenesis (c.E14–15), thrombospondin-2 protein was principally localized to the ossification centres of the developing maxilla, both extracellular matrix and bone; far less was seen in the growing palatal shelves proper (Fig. 3). These results were concomitant with the decline in palatal thrombospondin-2 mRNA transcript from E13 to E15 (Fig. 1).

4. Discussion

Thrombospondins are multifunctional proteins secreted by a variety of cells, including fibroblasts (Bornstein, 1992). They have an affinity for cell surfaces and many macromolecules of the extracellular matrix such as heparan sulphate proteoglycans, fibronectin, laminin, several collagens, plasminogen, and histidine-rich glycoproteins (Bornstein, 1992).

In 1991, Bornstein and colleagues identified and partially characterized a second mouse thrombospondin gene (*Thbs2*), which was related to, but distinct from, previously characterized mouse and human thrombospondins (Bornstein et al., 1990, 1991). *Thbs2* gene expression was particularly evident in connective tissues such as bone and cartilage (Bornstein et al., 1991). Thrombospondin-1 and -2 are similar in structure in that both proteins contain NH_2-terminal, COOH-terminal, and procollagen homology domains, and type I (thrombospondin or properdin), type II (epidermal

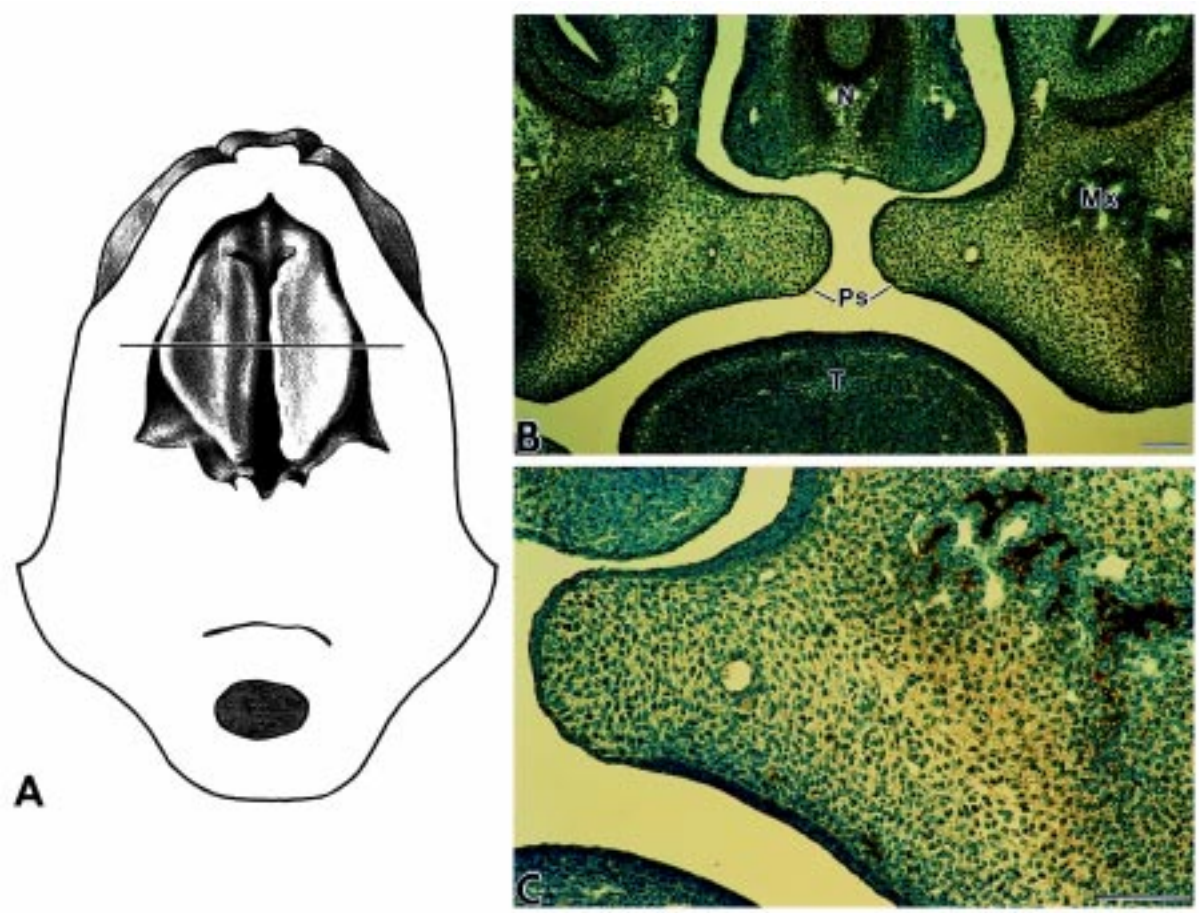

Fig. 3. Spatial distribution of thrombospondin (TSP)-2 protein in late embryonic palates. (A) View of palate with removal of lower jaw. At this later stage of development, both shelves are horizontal. Line indicates the plane of section for (B). (B, C) Immunolocalization of TSP-2 protein in developing horizontal palatal shelves. (B) Anti-TSP-2-stained section shows the relation between the horizontal palatal shelves (Ps), nasal septum (N), and tongue (T). (C) Higher magnification of palatal shelf shown in (B). TSP-2 is primarily seen in the extracellular matrix of mesenchymal cells more proximal to maxillary (Mx) bone formation, in maxillary ossification centers, and in developing cartilages and their perichondria. Far less TSP-2 is seen in the extracellular matrix of the medially growing palatal shelves (Ps) proper. Bar = 100 μm.

growth factor-like), and type III (Ca^{2+}-binding) repeats (Bornstein, 1992). The sequence identity, however, varies from 82% in the COOH-terminal domain to 32% in the NH_2-terminal domain, and there are striking differences in the promoter regions, which would account for their sometimes distinct expression and functional differences (Bornstein, 1992; Shingu and Bornstein, 1993).

Morphogenesis of the embryonic palate is a carefully orchestrated balance between the mitogenesis promoted by such factors as epidermal growth factor, TGF-α, and IGF-II and the mitostasis promoted by such factors as TGF-β (Dixon and Ferguson, 1992; Jaskoll et al., 1996; Melnick et al., 1998). As noted above, TGF-β_2 and thrombospondin-1 transcript and protein are both prominent in head mesenchyme, including palatal mesenchyme. Thrombospondin-1 serves, in part, to activate latent TGF-β in vivo (Crawford et al., 1998); thrombospondin-2 appears to be antagonistic to this function of thrombospondin-1 by competitively binding the latent TGF-β but not activating it (Schultz-Cherry et al., 1995). Thrombospondin-2, like thrombospondin-1, also appears to be antiangiogenic (Volpert et al., 1995). There is evidence that this function may be extant in both pre- and postnatal development (Kyriakides et al., 1998), as well as in tumorigenesis (Tokunaga et al., 1999). Nevertheless, how, when, and where thrombospondin-2 inhibits angiogenesis in embryos remains obscure. Although its precise role in embryogenesis is poorly understood and somewhat controversial (Kyriakides et al., 1998), our investigation of its tran-

script expression and protein localization provides some clues to its function during palatogenesis.

Initially, thrombospondin-2 mRNA is highly expressed and the protein is localized throughout the extracellular matrix of palatal mesenchyme (Figs. 1 and 2), as is TGF-β2 and thrombospondin-1 (Iruela-Arispe et al., 1993; Jaskoll et al., 1996). With horizontalization of the palatal shelves, *Thbs2* gene transcription significantly declines (Fig. 1) and thrombospondin-2 protein is far less evident in palatal shelves proper (Fig. 3). There is also a significant decline of TGF-β_2 (Jaskoll et al., 1996) and thrombospondin-1 (Tooney et al., 1998), permitting mesenchymal cell proliferation and palatal shelf growth. Taken together, these data suggest that thrombospondin-2 is one factor, among others, modulating growth by modulating thrombospondin-1-dependent activation of TGF-β_2. From E15 onward, thrombospondin-2 is localized to the osteogenic tissues of maxillary skeletal elements, including the palate, and this is consistent with its previously purported role in the genesis of other craniofacial skelatal elements (Tooney et al., 1998). Finally, while thrombospondin-2 most certainly participates in embryonic palatal development, the absence of cleft palate or other craniofacial anomalies in *Thbs2* null mice would indicate that it is not a necessary participant (Kyriakides et al., 1998). In this regard, it is instructive to note that the *Thbs2* locus on mouse chromosome 17 is proximate to *Igf2r* and *Plg*, other modulators of TGF-β_2 activation in palates (Melnick et al., 1998). This is probably not fortuitous. It is likely that these genes adjust their expression as needed in *Thbs2* null mutants. It might also be so for genes elsewhere that regulate angiogenesis, such as *Thbs1*.

Acknowledgements

We gratefully acknowledge the generous gift from Dr Paul Bornstein of thrombospondin-2 cDNA probes and antibody and the most helpful advice of Dr Themis Kyriakides. This study was supported by NIDCR/NIH grant DE12111.

References

Bornstein, P., 1992. Thrombospondins: structure and regulation of expression. FASEB J. 6, 3290–3299.

Bornstein, P., Alfi, D., Devarayalu, S., Framson, P., Li, P., 1990. Characterization of the mouse thrombospondin gene and evaluation of the role of the first intron in human gene expression. J. Biol. Chem. 265, 16691–16698.

Bornstein, P., O'Rourke, K., Wikstrom, K., Wolf, F.W., Katz, R., Li, P., Dixit, V.M., 1991. A second, expressed thrombospondin gene (*Thbs2*) exists in the mouse genome. J. Biol. Chem. 266, 12821–12824.

Crawford, S.E., Stellmach, V., Murphy-Ullrich, J.E., Ribeiro, S.M.F., Lawler, J., Hynes, R.O., Boivin, G.P., Bouck, N., 1998. Thrombospondin-1 is a major activator of TGF-β1 in vivo. Cell 93, 1159–1170.

Dennis, P.A., Rifkin, D.B., 1991. Cellular activation of latent transforming growth factor β requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor. Proc. Natl. Acad. Sci. USA 88, 580–584.

Derynck, R., 1994. TGF-β-receptor mediated signaling. Trends Biochem. Sci. 19, 548–553.

Dixon, M.J., Ferguson, M.W.J., 1992. The effects of epidermal growth factor, transforming growth factors alpha and beta and platelet derived growth factor on murine palatal shelves in organ culture. Arch. Oral Biol. 37, 395–410.

Gehris, A.L., D'Angelo, M., Greene, R.M., 1991. Immunodetection of the transforming growth factors β_1 and β_2 in the developing murine palate. Int. J. Dev. Biol. 35, 17–24.

Gleizes, P.-E., Munger, J.S., Nunes, I., Harpel, J.G., Mazzieri, R., Noguera, I., Rifkin, D.B., 1997. TGF-β latency: biological significance and mechanisms of activation. Stem Cells 15, 190–197.

Iruela-Arispe, M.L., Liska, D.J., Sage, E.H., Bornstein, P., 1993. Differential expression of thrombospondin 1, 2, and 3 during murine development. Dev. Dyn. 197, 40–56.

Jaskoll, T., Choy, H.A., Chen, H., Melnick, M., 1996. Developmental expression and CORT-regulation of TGF-β and EGF receptor mRNA during mouse palatal morphogenesis: correlation between CORT-induced cleft palate and TGF-β_2 mRNA expression. Teratology 54, 34–44.

Kyriakides, T.R., Zhu, Y.-H., Smith, L.T., Bain, S.D., Yang, Z., Lin, M.T., Danielson, K.G., Iozzo, R.V., LaMarca, M., McKinney, C.E., Ginns, E.I., Bornstein, P., 1998. Mice that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. J. Cell Biol. 140, 419–430.

Laherty, C.D., O'Rourke, K., Wolf, F.W., Katz, R., Seldin, M.F., Dixit, V.M., 1992. Characterization of mouse thrombospondin 2 sequence and expression during cell growth and development. J. Biol. Chem. 267, 3274–3281.

Lawrence, D.A., 1996. Transforming growth factor-β: A general review. Eur. Cytokine Netw. 7, 363–374.

Linask, K.K., D'Angelo, M., Gehris, A.L., Greene, R.M., 1991. Transforming growth factor-β receptor profiles of human and murine embryonic palate mesenchymal cells. Exp. Cell Res. 192, 1–9.

Melnick, M., Chen, H., Buckley, S., Warburton, D., Jaskoll, T., 1998. Insulin-like growth factor II receptor, transforming growth factor β and Cdk 4 expression and the developmental epigenetics of mouse palate morphogenesis and dysmorphogenesis. Dev. Dyn. 211, 11–25.

Munger, J.S., Huang, X., Kawakatsu, H., Griffiths, M.J.D., Dalton, S.L., Wu, J., Pittet, J.-F., Kaminski, N., Garat, C., Matthay, M.A., Rifkin, D.B., Sheppard, D., 1999. The integrin αVβ6 binds and activates latent TGFβ$_1$: a mech-

anism for regulating pulmonary inflammation and fibrosis. Cell 96, 319–328.

Murphy-Ullrich, J.E., Schultz-Cherry, S., Höök, M., 1992. Transforming growth factor-β complexes with thrombospondin. Mol. Biol. Cell 3, 181–188.

Negoescu, A., Lafeuillade, B., Pellerin, S., Chambaz, E.M., Feige, J.J., 1995. Transforming growth factors β stimulate both thrombospondin-1 and CISP/thrombospondin-2 synthesis by bovine adrenocortical cells. Exp. Cell Res. 217, 404–409.

O'Shea, K.S., Dixit, V.M., 1988. Unique distribution of the extracellular matrix component thrombospondin in the developing mouse embryo. J. Cell Biol. 107, 2737–2748.

Pelton, R.W., Hogan, B.L., Millet, D.A., Moses, H.L., 1990. Differential expression of genes encoding TGFs β_1, β_2, β_3 during murine palate formation. Dev. Biol. 141, 456–460.

Schultz-Cherry, S., Murphy-Ullrich, J.E., 1993. Thrombospondin causes activation of latent transforming growth factor-β secreted by endothelial cells by a novel mechanism. J. Cell Biol. 122, 923–932.

Schultz-Cherry, S., Lawler, J., Murphy-Ullrich, J.E., 1994. The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-β. J. Biol. Chem. 269, 26783–26788.

Schultz-Cherry, S., Chen, H., Mosher, D.F., Misenheimer, T.M., Krutzsch, H.C., Roberts, D.D., Murphy-Ullrich, J.E., 1995. Regulation of transforming growth factor-β activation by discrete sequences of thrombospondin 1. J. Biol. Chem. 270, 7304–7310.

Shingu, T., Bornstein, P., 1993. Characterization of the mouse thrombospondin 2 gene. Genomics 16, 78–84.

Theiler, K., 1989. The House Mouse: Atlas of Embryonic Development. Springer-Verlag, New York.

Tokunaga, T., Nakamura, M., Oshika, Y., Abe, Y., Ozeki, Y., Fukushima, Y., Hatanaka, H., Sadahiro, S., Kijima, H., Tsuchida, T., Yamazaki, H., Tamaoki, N., Ueyama, Y., 1999. Thrombospondin 2 expression is correlated with inhibition of angiogenesis and metastasis of colon cancer. Br. J. Cancer 79, 354–359.

Tooney, P.A., Sakai, T., Sakai, K., Aeschlimann, D., Mosher, D.F., 1998. Restricted localization of thrombospondin-2 protein during mouse embryogenesis: a comparison to thrombospondin-1. Matrix Biol. 17, 131–143.

Volpert, O.V., Tolsma, S.S., Pellerin, S., Feige, J.J., Chen, H., Mosher, D.F., Bouck, N., 1995. Inhibition of angiogenesis by thrombospondin-2. Biochem. Biophys. Res. Comm. 217, 326–332.