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ORIGINAL ARTICLE

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Gene and protein expression of *Transforming growth factor* β*2* gene during murine primary palatogenesis

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Abstract The molecular mechanisms by which the primordia of the midface grow and fuse to form the primary palate are not well characterized. This is in spite of the fact that failure of growth and/or fusion of these facial primordia leads to the common human craniofacial birth defects, clefts of the lip with or without clefts of the palate. Members of the *transforming growth factor* β (*Tgf*β) superfamily have been shown to play critical roles during craniofacial development. Specifically, the role of *Tgf*β*-3* in mediating the fusion of the embryonic secondary palatal shelves is well documented. In a screen for genes expressed during fusion of the murine midfacial processes, *Tgf*β*2* was identified as a gene differentially expressed during fusion of the lateral and medial nasal processes. The objective of our study was to analyze the spatial and temporal expression of *Tgf*β*2* during critical stages of midfacial morphogenesis at both the transcript and protein levels. We also compared the pattern of expression of *Tgf*β*2* with that of *Bmp4*, a gene shown previously to be involved in mediating the fusion process in the midface. Our results showed *Tgf*β*2* expression in a very restrictive area of the epithelial layer along the borders of the midfacial primordia, in a pattern very similar to that of *Bmp4*. The highly restrictive and spatial and temporal pattern of expression of *Tgf*β*2* implicates its role in mediating the fusion of the midfacial processes, possibly through interacting with *Bmp4* in the regulation of apoptosis and/or epithelial–mesenchymal transformation. A greater understanding of the role of this gene will clarify how the normal midface grows and the mechanisms behind cleft development.

Key words midface · primary palate fusion · clefts of lip and palate · *in situ* hybridization · craniofacial development

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Introduction

Clefts of the lip and palate is the most common craniofacial birth defect found in humans, with an incidence as high as 1:500 in some racial populations (Natsume et al., 1987). This birth defect occurs because of defects in growth of the midfacial primordia during early embryonic development.

Three midfacial prominences are visible during normal midfacial development: the medial and lateral nasal prominences (MNP and LNP, respectively) medial and lateral to the nasal pits and the maxillary primordium (M×P) of the first branchial arch. As these three facial primordia reach the appropriate size, fusion of the midfacial processes is initiated. The maxillary primordium, M×P, first contacts the MNP, followed by fusion of the LNP with the MNP (Trasler, 1968). The MNP and LNP first contact at the posterior end of the

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nasal pits, where fusion between these two prominences starts and proceeds anteriorly (Trasler, 1968). During fusion, the epithelial layers of the facial processes contact each other to form an epithelial seam, a bilayer epithelium that increases in size and eventually disintegrates to allow penetration of the mesenchyme (Trasler, 1968; Wang et al., 1995). Failure of the growth and/or fusion of the three facial primordia results in the formation of the clefts of the lip with or without clefts of the palate (Pelton et al., 1990).

The molecular mechanisms by which the primordia of the midface grow and fuse to form the primary palate are not well characterized. *Transforming growth factor* β*2* (*Tgf*β*2*) was one of the molecules identified in the laboratory during a microarray screen of tissues of the three midfacial processes taken before and after fusion. Because of the well-documented evidence from the literature of the important roles of different members of the *Tgf* family e.g. *TGF*β*3* is critical for fusion of the secondary palatal shelves (Proetzel et al., 1995; Kaartinen et al., 1997), we performed spatial and temporal analyses of the expression pattern of *Tgf*β*2* during critical stages of growth and fusion of the three midfacial processes. In addition, we also compared *Tgf*β*2* expression pattern to that of another member of the *Tgf*β superfamily, *Bmp4*, during midfacial fusion and found a highly overlapping pattern of expression to the putative site of fusion of the midfacial processes. We speculate that *Tgf*β*2* and *Bmp4* play important roles in mediating the fusion event of the primary palate.

Materials and methods

Mouse embryos

C57BL/6 male and female mice were obtained from Jackson Laboratory (Bar Harbor, ME) and mated as described in Gong et al. (2000). Embryos were removed at 10 and 11 days post-coitum (dpc) and further substaged by tail somite count. Stages that represent the different stages of fusion of the MNP and LNP were collected: tail somite (TS) 4–6 (representing pre-fusion stage), TS 8–12 (fusion), TS 13–16 (post-fusion), and primary palate enlargement (16–20).

Tissue preparation

Embryos were prepared for whole mount and section *in situ* hybridization experiments according to Gong and Guo (2003) and for immunohistochemistry according to Gong (2001). Embryos for section *in situ* hybridization were embedded in OCT such that cryosections of 10 μm thickness in the transverse orientation were obtained. For immunohistochemistry, paraffin-embedded embryos were sectioned at 14 μm thickness were obtained in the transverse and frontal orientation (refer to Fig. 2I for orientation of the embryo).

In situ hybridization

*Tgf*β*2* plasmids (5′-*Tgf*β*2* and 3′-*Tgf*β*2*) were generated by subcloning amplified fragments generated by polymerase chain reactions using the following primers from the 5′ and 3′ portions of the *Tgf*β*2* gene:

5′ F-$^{5'}$ TGGGGCAGGCGTCAGGGATGGAG$^{3'}$
R-$^{5'}$ CGGAGCAGGCGGCGAGGAT$^{3'}$

Fragment length = 412 bp

3′ F-$^{5'}$ GAAGGCGTTAGTCTGCATCTCACC$^{3'}$
R-$^{5'}$ ACCTCCTTCCCCCTGGCTTATTTG$^{3'}$

Fragment length = 430 bp

Sequences of the two fragments generated from amplification of the primers were specific to the *Tgf*β*2* gene and share no homology with other members of the *Tgf*β family as evidenced from DNA database blasting. The fragments were inserted into *EcoRI* linearized and end-filled pGEM®-T Easy vector (Promega Corp. Madison, WI) linear by blunt end ligation. The sequences of the inserts were verified by sequencing. Both the 5′-*Tgf*β*2* and 3′-*Tgf*β*2* plasmids were linearized with *ApaI* and *SacI* restriction endonucleases, and transcribed with Sp6 and T7 RNA polymerases in the presence of digoxigenin nucleotides to generate sense and antisense riboprobes, respectively.

The *Bmp4* cDNA plasmid was a kind gift of Genetics Institutes (Cambridge, MA).

Riboprobes generated from both the 5′-*Tgf*β*2* and 3′-*Tgf*β*2* plasmids were used in *in situ* hybridization on midfacial sections and yielded identical expression pattern. All subsequent *in situ* hybridization experiments were performed with the 5-*Tgf*β*2* riboprobe. A temperature of 60°C was used for hybridization with both *Tgf*β*2* and *Bmp4* riboprobes.

Immunohistochemistry

Immunohistochemistry was performed according to Gong (2001). Briefly, frontal and transverse sections of the midfacial region of the different staged embryos were reacted with two antibodies. The antibody against Tgfβ2 was a polyclonal antibody generated with synthetic peptide that corresponded to amino acids 50–75 of the mature human Tgfβ2 (Santa-Cruz Biotechnology, CA) (dilution = 1:500). The corresponding sequence in the mouse differed only by two amino acids whose structures were closely related to each other (Burt and Paton, 1992; Olaso et al., 1997). The antibody against Bmp4 was a polyclonal antibody generated with a synthetic peptide mapping at the amino terminus of the BMP-4 of human origin and differs from the corresponding mouse sequence by two amino acids (sc-6896, Santa-Cruz Biotechnology) (dilution—1:150).

The Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used with biotinylated goat anti-rabbit IgG as the second antibody. Negative controls were performed concurrently without the presence of the primary antibody.

Results

Expression of *Tgf*β*2* in the developing midface

*Tgf*β*2* was one of the genes identified in an original microarray screen to search for genes that may play a critical role in the growth and fusion of the midfacial region to become the primary palate (Gong et al., 2005). Confirmation of the differential expression of this gene at two stages (pre-fusion and fusion) of midfacial development by reverse-transcriptase quantitative polymerase chain reaction showed a small (less than twofold), but statistically significant, up-regulation of the gene as the midfacial

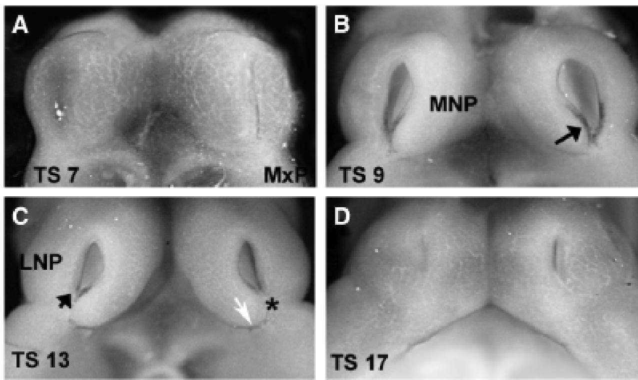

Fig. 1. *Transforming growth factor* β*-2* (*Tgf*β)*-2* mRNA expression. Frontal views of whole embryos at (**A**) pre-fusion (**B**) early fusion, (**C**) mid-fusion, and (**D**) late fusion stages of midfacial development and hybridized with *Tgf*β*2* riboprobe. *Tgf*β*2* expression was present at the leading edges of the lateral and medial nasal prominences (LNP and MNP) (black arrows in B and C) before fusion. Expression of the gene was also obvious at the fusion site of the MNP with the M×P. There was no visible expression of the gene before fusion (A) or after fusion had occurred (D).

processes fuse (S.-G. Gong, T.-W. Gong, and L. Shum, data not shown). We proceeded to map the spatial distribution of this gene during midfacial growth.

Before fusion of the midfacial processes, the expression of *Tgf*β*2* was not observed at the transcript level (Fig. 1A). Once the process of fusion is initiated, expression of the *Tgf*β*2* gene became apparent at the posterior ends of the MNP and LNP as they come into contact with each other to initiate the process of fusion (Fig. 1). *Tgf*β*2* transcripts were tightly restricted to the leading edges of the "zipper" formed by the posterior ends of both the LNP and MNP (black arrows in Fig. 1B, C). At the cellular level, the expression of the gene transcripts was clearly restricted to the ectodermal layer of the M×P and MNP (Fig. 2I, A–C) and the MNP and LNP (Fig. 2I–D). The gene was also expressed in the ectoderm of the LNP and MNP more anterior to the fusion site (Fig. 2I–E), corresponding to the expression observed in Fig. 1C. At the protein level, Tgfβ2 was observed in the epithelial seams of the contact of M×P/MNP (Fig. 2II–A, B) and LNP/MNP (Fig. 2II–C, D). However, in contrast to its mRNA expression, Tgfβ2 protein was present at very low levels or absent in the epithelial layers of the LNP and MNP that were not in contact (compare Fig. 2II–E with Fig. 2I–E). At a later developmental stage, when the epithelial seam had broken down and there was mesenchymal bridge formation, remnants of the Tgfβ2-expressing cells were only found in the epithelial triangles found on the nasal and oral part of the fusion site (arrows in Fig. 2II–F, H, J).

Expression of *Bmp4* and *Tgf*β*2* in the midface

The expression of *Tgf*β*2* in the developing midfacial region was reminiscent of that of *Bmp4* (Gong and Guo, 2003). We therefore conducted *Bmp4* and *Tgf*β*2* expression analyses at both the transcript and protein levels with commercially available antibodies. Consecutive sections through the developing midfacial region were utilized for the experiments for ease of comparison.

At the transcript levels, there appeared to be overlapping expression in the epithelial seam of the fusion site between the two genes. *Bmp4*, however, had a broader domain of expression, being present in ectodermal layer of the M×P (no expression of *Tgf*β*-2* in Fig. 3A compared with *Bmp4* in Fig. 3B) and strong expression in the medial aspect of the MNP (white arrow in Fig. 3F; Gong and Guo, 2003). By comparison, *Tgf*β*-2* mRNA expression was restricted to the layers of epithelial seam (white arrow in Fig. 3E) and decreased in amount anterior to the contact site (Fig. 3I). *Bmp4* transcripts persisted more anteriorly in the LNP and MNP i.e. more rostrally upwards the tips of the LNP and MNP (compare Fig. 3I and J). Both proteins were found to be expressed at the site of fusion of the LNP and MNP, specifically in and around the epithelial seam of the fusion site. One major difference, however, was the broader distribution of Bmp4 in the epithelial seam cells and some mesenchymal cells around the seam (Fig. 4A, arrow in a). Tgfβ2 protein, however, was restricted to cells in the epithelial seam (Fig. 4B, arrow in b) and never observed in any mesenchymal cells around the site of fusion.

Discussion

Our original microarray screen was initiated as an attempt to identify molecules that are involved in growth and fusion of the palate (Gong et al., 2005). The spatial expression of this gene, as presented in this paper, showed a pattern that suggested that *Tgf*β*2* may play an important role during midfacial morphogenesis, specifically in mediating fusion of the M×P with the MNP and the fusion of the MNP with the LNP.

The expression of *Tgf*β*2* in the primary palate is reminiscent of the expression pattern of *Tgf*β*3* in the developing secondary palate. Both *Tgf*β*2* and *Tgf*β*3* belong to the large *Tgf*β family, all of which regulate a broad spectrum of biological responses on a large variety of cell types (e.g. Rizzino, 1988; Pelton et al., 1991; McCartney-Francis and Wahl, 1994). *Tgf*β*3* is expressed at relatively high levels in the leading borders of the palatal shelves (known as the medial edge epithelium, MEE) (Ferguson, 1988) before fusion of the palatal shelves and disappears soon after fusion of the shelves, very similar to what we observed with *Tgf*β*2* expression in the primary palate. Following the first expression mapping of the *Tgf*β*3* gene to the MEE, numerous studies have shown a critical role for *Tgf*β*3* in the fusion event of the secondary palate. Not only does

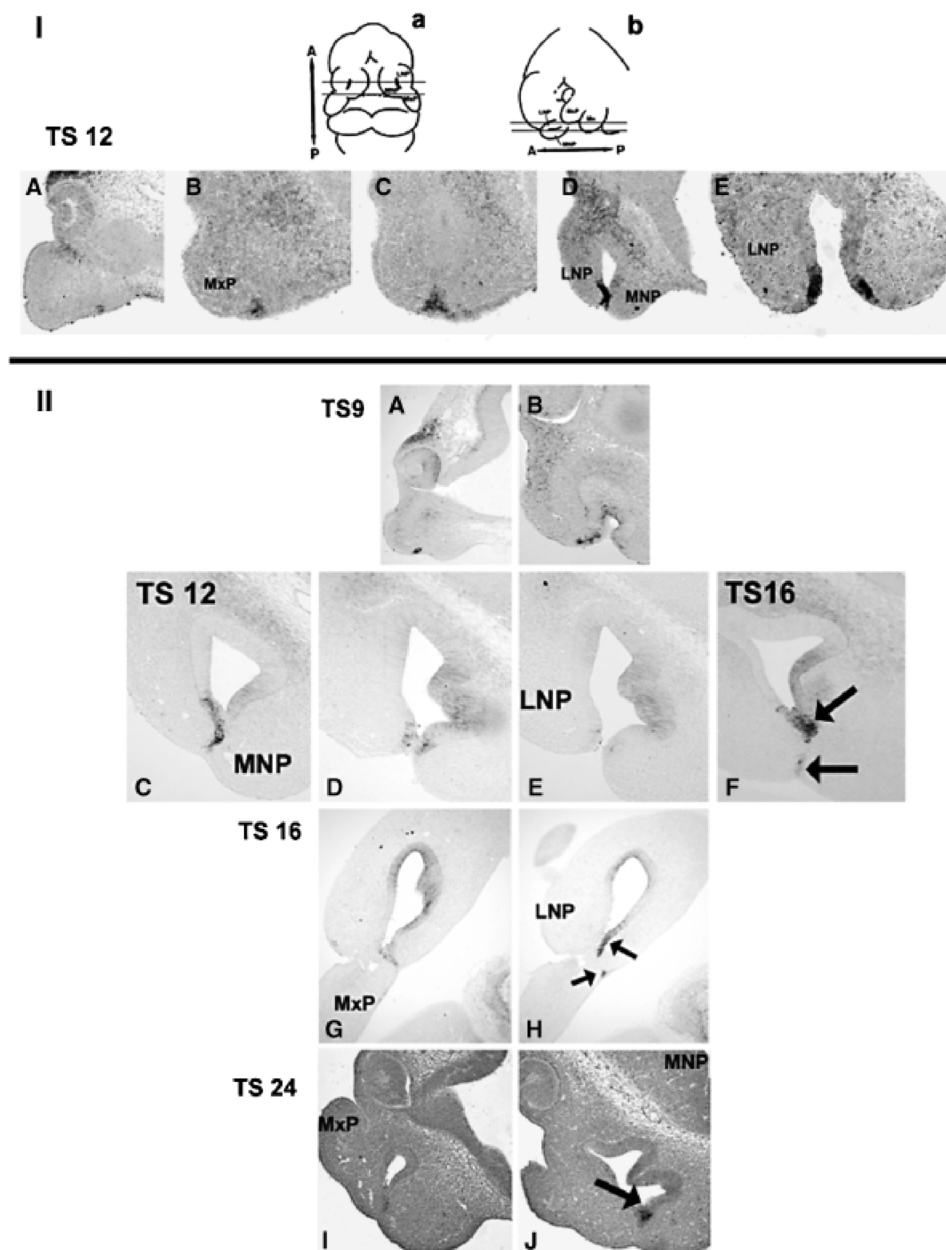

Fig. 2 Midfacial expression of *transforming growth factor* β-*2* (*Tgf*β-*2)* mRNA and protein. (**I**) Transverse sections through the midface of a tail somite (TS) 12 embryo hybridized with *Tgf*β*2* riboprobe. The sections were from a posterior to anterior direction of the same embryo during the fusion stage. TGFβ-2 was restricted to the epithelia of the site of fusion of the medial nasal prominences (MNP) with the M×P (A–C), and in the epithelia of the leading edges of the lateral nasal prominences (LNP) and MNP (D, E). (**II**) Immunohistochemistry with antibody against TGFβ2 on transverse (A–F) and frontal (G–H) sections through the midface of TS9 (A–B), TS12 (C–E), TS16 (F–H), TS 24 (I–J). (I) and (J) were counterstained with 1% hematoxylin. TGFβ2 protein was present in the bilayer epithelia of fusion of the MNP with M × P (A) and at the site of fusion of the LNP and MNP (C, D). The protein was absent where there was no contact between the epithelial layers. In older embryo, the protein was found in detectable levels in the olfactory epithelium of the medial wall of the nasal cavity (G). During the disintegration of the epithelial seam, remnants of the protein were present in the epithelial triangles on the nasal and oral sides (arrows in F, H, and J). Transverse and horizontal sections were prepared as in the horizontal lines in the diagram of an embryo at infero-frontal view.

Tgfb3-null mutation result in 100% penetrance of cleft secondary palate (Kaartinen et al., 1995; Proetzel et al., 1995), various studies have clearly shown that clefts of the secondary palate are because of failure of the fusion event. For example, blockade of *Tgf*β*3 in vitro* prevents palatal fusion and this blockade can be rescued by addition of Tgfβ3 to the culture medium (Brunet et al., 1995; Kaartinen et al., 1997; Taya et al., 1999).

In light of the similarity in the pattern of expression of *Tgf*β*3* and *Tgf*β*2* during secondary and primary palatogenesis, respectively, and the known role of *Tgf*β*3* in mediating fusion of the secondary palate, it is pos-

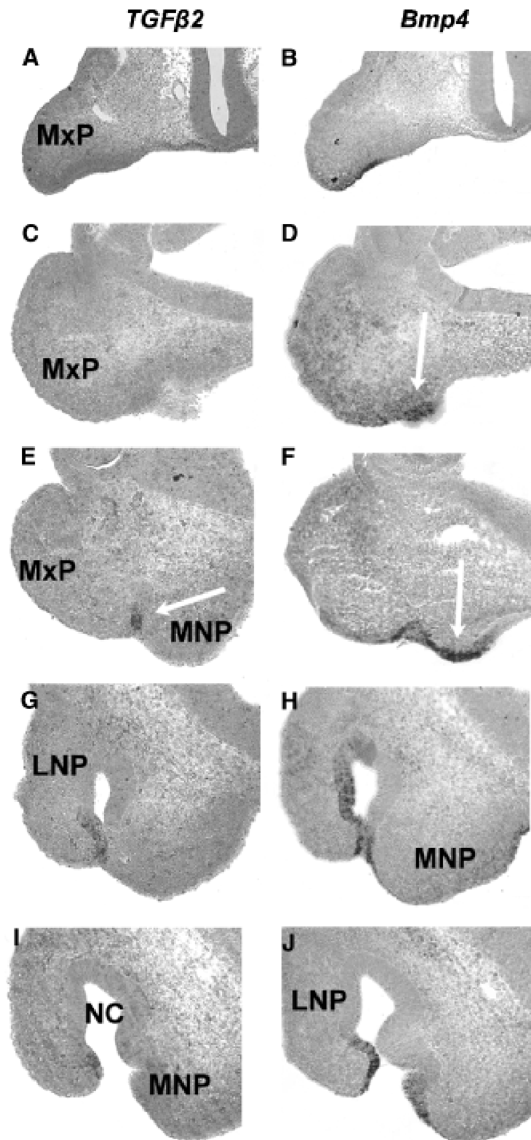

Fig. 3 Comparison of expression of midfacial expression of (*Tgf*β*-2*) and *Bmp4* transcripts. (**A**) Transverse sections (from posterior to anterior direction of embryo during fusion stage tail somite (TS) = 12. TGFβ-2 is restricted to the growing edges of the lateral and medial nasal prominences (LNP and MNP) in the epithelial layers. (**B**) Sections showing Bmp4 expression, in the same orientation and stage of development in that of the Tgfβ-2 sections. However, Bmp4 has a wider domain of expression, and appears earlier in a more posterior section, featured on the maxillary process in addition to the MNP and LNP.

sible that *Tgf*β*2* is involved in mediating fusion of the primary palate. At the structural level, *Tgf*-β *2* and *3* are very similar: each is synthesized as a prepro monomeric polypeptide cleaved at a multibasic peptide sequence to yield a 112–114 amino acid molecule (Miller et al., 1990). Mature TGF-β2 protein has approximately 80% identity to TGF-β3 (Derynck et al., 1988; ten Dijke et al., 1988). However, several differences are worth noting. Although *Tgf*β*2* and *Tgf*β*3* show high similarity at the structural level, the N-terminal regions of these two molecules show only 27% sequence identity to each other. In addition, although *Tgf*β*2*-null mice exhibit developmental defects in several systems, clefts of the lip have not been reported in these mice (Sanford et al., 1997). Interestingly, the *Tgf*β*2* loss-of-function mutation does result in cleft palate; the penetrance, however, is only 23% (Sanford et al., 1997) and could result from its expression in the cranial neural crest derived mesenchyme beneath the MEE. *Tgf*β*2* is restricted during secondary palatogenesis to the mesenchyme (Fitzpatrick et al., 1990; Pelton et al., 1990); clefts of the secondary palate in these null mutants are likely the result of an interference in the mesenchymal growth component of the developing secondary palatal shelves.

*Tgf*β*3* is believed to play a role in enhancing the transformation of MEE cells into the palatal mesenchyme and inducing apoptosis in the MEE (Sun et al., 1998; Martinez-Alvarez et al., 2000; Ito et al., 2001, 2003; Cuervo et al., 2002; Cuervo and Covarrubias, 2004). The primary palate is also believed to undergo these two biological events during fusion (Sun et al., 2000). In the primary palate, however, we speculate that during fusion of the midfacial processes, *Tgf*β*2* may work together with *Bmp4*, another member of the *Tgf*β superfamily. The similarity of expression pattern in the epithelial layers of the site of fusion lends strong support to the possibility that both *Bmp4* and *Tgf*β*2* have roles in mediating apoptosis and/or EMT at the fusion site of the midface. Although there is overlapping expression pattern with *Tgf*β*2* in the leading edges of the fusion site of the M×P/MNP and MNP/LNP, *Bmp4* expression covers a more extensive area, being present in the M×P and further rostrally in the leading edges of the epithelium of fusion. Another distinct difference is that as fusion occurs, *Tgf*β*2* expression was lost and restricted to the oral and nasal triangles of the epithelial fusion site. In fact, Tgfβ2 protein was never observed in epithelial cells of the midfacial processes that were not in contact with each other and appeared to be more restricted to the superficial layer of the epithelia of facial processes. *Bmp4* expression, on the other hand, was observed in the epithelial seam as it breaks down to allow mesenchymal confluence, followed by a shift to the mesenchyme as its epithelial expression in the fusion site disappears completely (Gong and Guo, 2003). In addition, Bmp4 protein was observed not only in the epithelial seam but also in the immediate surrounding mesenchymal cells, suggesting a paracrine mode of action of the protein in the developing midfacial processes. The complementary, yet distinct, pattern of *Tgf*β*2* and *Bmp4* expression suggests a possible interactive role of these two molecules in mediating the fusion event in the primary palate. It is possible that one molecule, possibly *Tgf*β*2,* could be involved in mediating apoptosis, while the other, *Bmp4* possibly, in mediating EMT of the epithelial cells during fusion. Another possibility is that these two molecules, and perhaps others, work

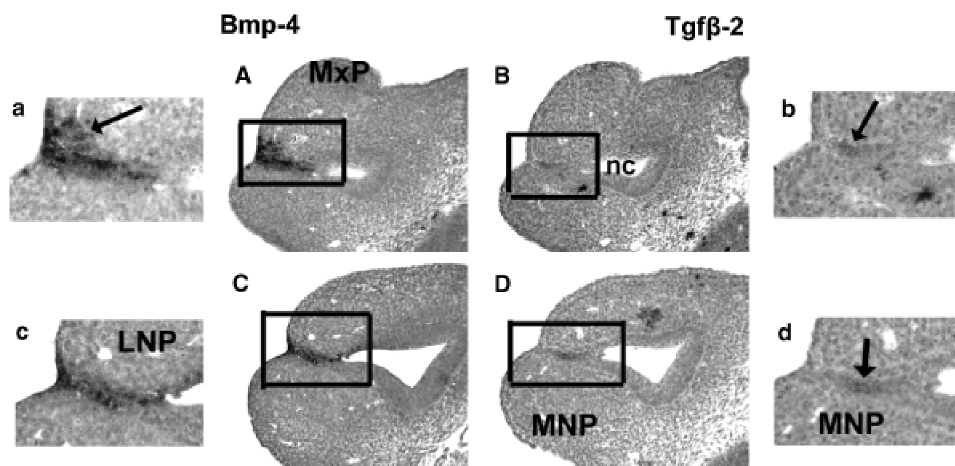

Fig. 4 Immunohistochemical localization (of Bmp-4 (**A, C, a, c**) and Tgf-β2 (**B, D, b, d**), counterstained with hematoxylin & eosin, in adjacent transverse sections through the midfacial processes of 10.5 days post-coitum embryo at tail somite (TS) 14. The boxes in (A)–(D) are magnified in the diagrams labeled in corresponding lower case letters. Tgf-β2 protein was expressed only in the epithelial cells of the seam (arrows in b, d) whereas Bmp4 was present in both the epithelial and some mesenchymal cells surrounding the seam (arrow in a).

together to "share" the job of mediating fusion at different stages of development.

One potential mechanism for "sharing" functions during fusion of the primary palate could be the interplay and the convergence of the signaling pathways used by these two members of the *Tgf*β superfamily. All members of the *Tgf*-β superfamily mediate their biological responses through a receptor signaling complex, consisting of a ligand binding Tgf-β receptor type II (TβR-II), and a Tgf-β receptor type I (activin receptor-like kinase, Alks) (Heldin et al., 1997; Shi and Massague, 2003; Waite and Eng, 2003). Upon ligand binding, type II receptors associate with and phosphorylate type I receptors which then bind and phosphorylate the intracellular signaling mediators, Smads (Heldin et al., 1997; Shi and Massague, 2003; Waite and Eng, 2003). Smad1, Smad5, and Smad8 mediate the Bmps signaling through Alk-3 and Alk-6, whereas Smad2 and Smad3 mediate the TGF-βs and activin signaling through Alk-5 (Heldin et al., 1997; Shi and Massague, 2003; Waite and Eng, 2003). Several lines of evidence suggest that the biological responses initiated by *Tgf*β*3* during secondary palatogenesis are mediated by this particular pathway. For example, embryonic secondary palatal tissue contains functional types I and II Tgfβ receptors (Linask et al., 1991; Cui and Shuler, 2000) that when activated, elicit changes such as cell proliferation (Linask et al., 1991) and the synthesis of extracellular matrix components (D'Angelo and Greene, 1991; D'Angelo et al., 1994); the involvement of specific Smad proteins such as Smad2 in *Tgf*β-initiated signaling in cells of the embryonic palate (Greene et al., 2003); and the Alk-5/Smad pathway mediates *Tgf*β*3* induced palatal fusion (Dudas et al., 2004). During primary palate development, it is conceivable that there is more than one molecule that mediates fusion, and that should one molecule be absent or non-functional, the others step up to the plate. The speculation of there being more than one player involved in mediating fusion in the primary palate is supported by an interesting observation with null mutant mice: of the numerous phenotypes generated from knock-out mice, very few have clefts of the lip. Functional studies of various kinds will need to be performed to test out the hypothesis of several players mediating fusion of the primary palate.

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