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ORIGINAL ARTICLE

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Bmp4 gene is expressed at the putative site of fusion in the midfacial region

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Abstract The molecular mechanisms by which the primordia of the midface grow and fuse to form the primary palate portion of the craniofacial region are not well characterized. This is in spite of the fact that failure of growth and/or fusion of these primordia leads to the most common craniofacial birth defect in humans (i.e. clefts of the lip and/or palate). *Bmp4* plays a critical role during early embryonic development and has previously been shown to play a role in epithelial-mesenchymal interactions in the craniofacial region of chicks. We analyze the expression of *bmp4* in mouse as the midfacial processes undergo fusion to form the primary palate. We show that *bmp4* is expressed in a very distinct manner in the three midfacial processes (lateral nasal, LNP, medial nasal, MNP, and maxillary processes, MxP) that ultimately fuse to form the midface. Prior to fusion of the midfacial processes, *bmp4* is expressed in the ectoderm of the LNP, MNP, and MxP in a distinct spatial and temporal manner near and at the site of fusion of the midface. *Bmp4* appears to demarcate the cells in the LNP and MNP that will eventually contact and fuse with each other. As fusion of the three prominences proceeds, some *bmp4* expressing cells are trapped in the fusion line. Later, the expression of *bmp4* switches to the mesenchyme of the midface underlying its initial expression in the ectoderm. The switch occurs soon after fusion of the three processes. The pattern of expression in the midfacial region implicates the important role of *bmp4* in mediating the fusion process, possibly through apoptosis of cells in the putative site of fusion, during midfacial morphogenesis.

Key words lateral nasal prominence · medial nasal prominence · maxillary prominence · nasal pit · primary palate fusion · clefts of lip and palate · mouse development · *in situ* hybridization · craniofacial development · face · midface

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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 due to alterations in growth and/or fusion of the midfacial primordia during early embryonic development. Soon after the normal formation of the nasal or olfactory placodes on the ventral side of the forebrain, the placodes invaginate to form the nasal pit (Vermeij-Keers, 1990; Ferguson, 1991). This is followed by growth of two prominences on the medial and lateral sides of the pits to form the medial and lateral nasal prominences or processes (MNP and LNP, respectively). As the facial primordia reach the appropriate size, fusion of the midfacial processes is initiated; the MNP and LNP contact at the posterior end of the nasal pits, where fusion between these two prominences starts and proceeds anteriorly (terms of orientation are taken from Trasler (1968). This postero-anterior "zipping up" fusion process results in the formation of the primary palate, reinforced subsequently by the MxP (reviewed in Kosaka et al., 1985, and references therein) (Fig. 1A). During fusion, the epithelial layers of the facial processes contact each other to form what has been termed the epithelial seam, a bilayered epithelium that increases in size and eventually disintegrates to allow penetration of the mesenchyme

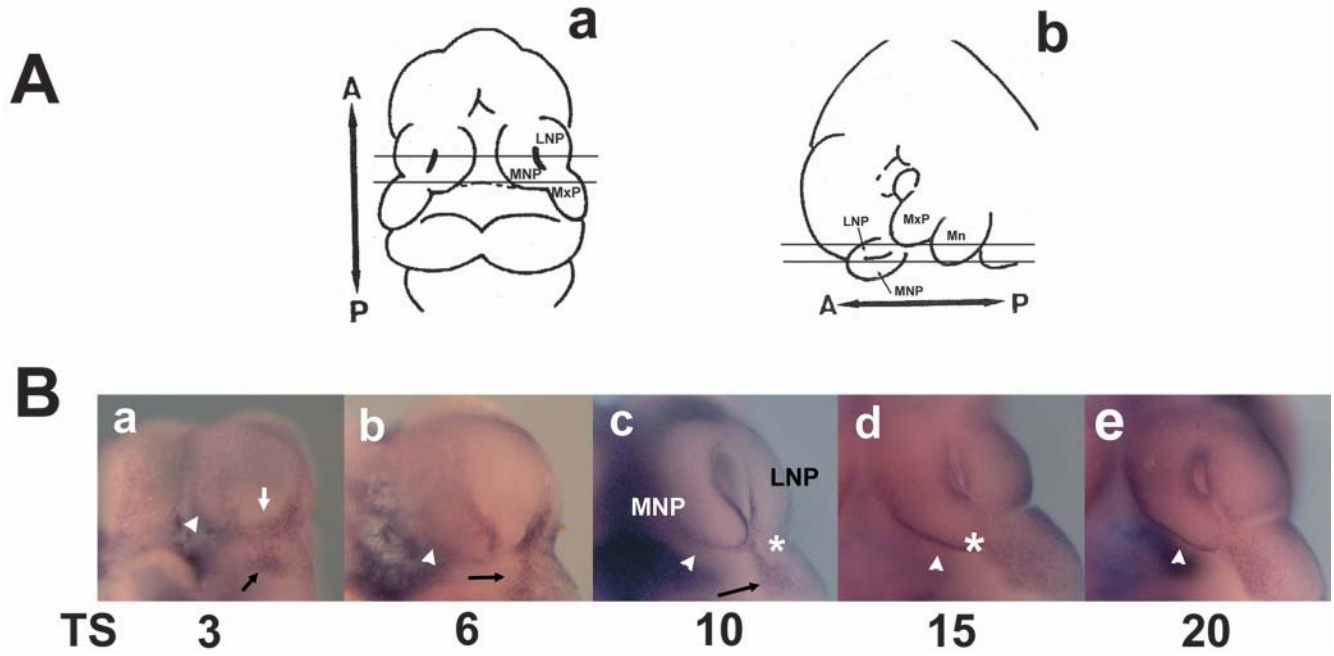


Fig. 1 (**A**) Schematic diagram of the midface of 10.5 dpc embryos from infero-frontal (**a**) and lateral (**b**) views. Transverse (**a** – *horizontal lines*) and frontal (**b** – *horizontal lines*) sections for analysis by *in situ* hybridization were collected. (Figs. adapted from Kosaka et al., 1985). (**B**) Infero-frontal views of embryos at different tail somite (TS) stages, hybridized with *bmp4* riboprobe. Embryos **a** = TS 3, **b** = TS 6, **c** = TS 10, **d** = TS 15, and **e** = TS 20. The three facial processes, LNP, MNP, and MxP exhibit increasing degrees of fusion (site of fusion denoted by *). *Bmp4* is expressed at the edges of the MNP and LNP at the posterior half of the nasal pit in early embryos from TS 3–10. *A-P*: antero-posterior plane, *LNP*: lateral nasal process, *MNP*: medial nasal process, *MxP*: maxillary process, *Mn*: mandibular primordia.

(Wang et al., 1995). Failure of the growth and/or fusion of the three facial primordia results in the formation of clefts of the lip with or without clefts of the palate.

Very little information is currently available on the molecular basis of fusion in the midfacial region. In this present study, we show that *bmp4* is expressed in a very distinctive pattern in the ectodermal layer of the LNP and MNP as they fuse, implicating a possible role of this gene in the important event of fusion of the midfacial processes.

Methods

Mouse embryos

C57Bl/6 male and female mice were obtained from Charles River (MA) and mated as described in Gong et al. (2000). Embryos were removed at 10 and 11 days post-coitum (dpc) and further sub-staged by tail somite count. Stages that represent the different stages of fusion of the MNP and LNP were collected: tail somite (TS) 2–6 (representing pre-fusion stage), TS 10 (early fusion), TS 14 (mid-fusion stage), and TS 18 (late fusion) stages. The embryos were fixed in 4% paraformaldehyde (in PBS buffer) overnight at 4^0C. The next day, fixed embryos were processed either for whole-mount or section *in situ* hybridization. For whole-mount *in situ* hybridization, embryos were washed in PBTX (PBS, pH 7.4, and 0.1% Triton X-1000) and stored in methanol at 20^0C until ready for hybridization.

For section *in situ* hybridization, embryos were washed three times in PBS, and cryoprotected in 30% sucrose/0.1 M phosphate buffer overnight at 4^0C. The embryos were then equilibrated in a 50:50 mixture of 30% sucrose/OCT (Tissue Tek 4583) for at least 1 hour on a rocking platform. Whole heads of embryos were then removed and embedded in 2 different planes, frontal and transverse across the midfacial region. Sections were made, 14 μm thick, and mounted onto Superfrost (Fisher-Scientific) and air-dried overnight. The slides were stored at -20^0C until needed.

In situ hybridization

Digoxigenin riboprobes were prepared by linearizing *bmp4* gene-specific DNA plasmid (gift of Genetics Institute, Inc., MA) followed by *in vitro* transcription.

Whole-embryo *in situ* hybridization experiments were performed as described in Gong (2001). For section *in situ* hybridization, sections were removed from storage and warmed to room temperature. The riboprobe was diluted in hybridization buffer (0.2 M NaCl, Tris HCl, pH 7.5, 1 mM Tris base, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.05 M EDTA, 50% formamide, 10% dextran sulfate, 1 mg/ml rRNA, 1× Denhardt's). The probe was denatured at 70^0C for 10 minutes, vortexed, and pipetted onto each slide. After coverslipping, the slides were placed in a box containing 2 sheets of 3 mm Whatman paper wet with 50% Formamide/1× salt. The box was sealed with tape and incubated overnight at 60^0C. The next day, the slides were washed in wash buffer (1× SSC, 50% formamide, 0/1% Tween) at 60^0C for 15 minutes with rocking. The wash was repeated twice with new buffer for 30 minutes. After this, the slides were washed in 1× MABT and incubated at room temperature for 30 minutes, this was repeated. Blocking solution (20% heat-inactivated sheep serum/2% blocking reagent in 1×MABT) was added to sections and incubated at room temperature for at

least 1 hour in a humidified box saturated with PBS. The blocking solution was then removed and about 120 µl of anti-DIG antibody diluted 1:1500 was added and coverslipped. This was placed in the humidified chamber at 4^0C overnight. The next day, the slides were transferred to a rack and washed 5 times for 20 minutes each in 1 × MABT at room temperature with rocking. The sections were then equilibrated in staining buffer (without NBT and BCIP) two times at 10 minutes each, with rocking at room temperature. Color reaction was performed by incubating the slides in staining buffer with NBT (4.5 µl/ml) and BCIP (3.5 µl/ml) in the dark at RT. The staining reaction was stopped by washing the slides several times in PBS.

Results

Expression of *bmp4* at pre-fusion stage of the MNP and LNP (TS 2–6)

To determine the expression pattern of *bmp4* in the midfacial region of normal mouse embryos, mouse embryonic craniofacial regions at specific stages of development were subject to *in situ* hybridization with the *bmp4* gene. Embryos were staged by tail somite count such that embryos spanning different stages of fusion of the midfacial region were obtained. The heads of these embryos were embedded to obtain transverse (Fig. 1Aa, horizontal lines) and frontal (Fig. 1Ab, horizontal lines) sections.

In embryos at TS (tail somite) 3, prior to fusion of the MNP and LNP, *bmp4* expression is observed at the posterior half of the processes flanking the nasal pit, being concentrated in the posterior-most part of the region (Fig. 1B, a-c). In transverse sections through the nasal pit region, *bmp4* is clearly expressed in the thickened epithelial layer that forms the posterior-most border of the nasal pit (Fig. 2B, arrow). The expression in the posterior-most border of the nasal pit surrounds the ventral part of the midfacial primordia (LNP and MNP) and is also seen extending to the medial surface into the stomadeal area (Fig. 2B, asterisk). Further anterior along the nasal pit, *bmp4* expression becomes restricted to the growing tips of the LNP and MNP, and this expression tapers off and is not observed in the anterior half of the nasal pit (Fig. 1B, a-c; Fig. 2, C-F). In slightly older embryos (TS 6; just prior to the fusion of the facial processes), the pattern of expression in the LNP and MNP is maintained in a very similar manner to that

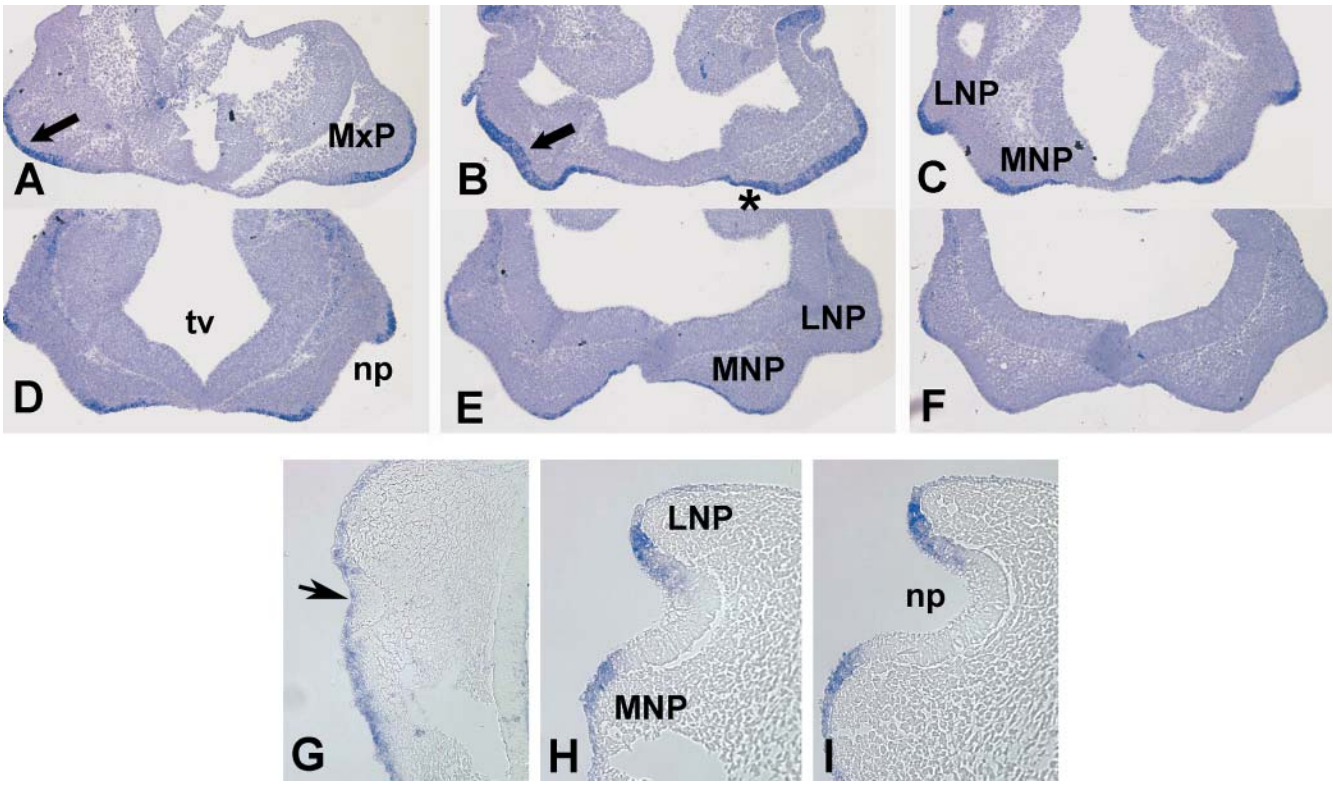


Fig. 2 Transverse sections (from posterior to anterior direction) of embryos at TS 2–3 (**A-F**) and TS 6 (**G-I**), prior to fusion of the midfacial processes. The expression of the *bmp4* gene is initially in a semicircular area around the maxillary prominence (**A**, *arrow*) and in the epithelial thickening of the nasal placode (**B**, *arrow*). More to the anterior, *bmp4* expression becomes restricted to the growing edges of the LNP and MNP, at the junction between the facial and nasal ectoderm. This expression pattern is maintained to TS 6, just shortly before fusion of the facial processes (**G-I**). *tv*: telencephelic vesicle, *np*: nasal pit.

observed in TS 3 embryos, being restricted to the growing tips in the anterior part of the nasal pit (Fig. 2H, I). At the posterior-most part of the nasal pit, however, the ectodermal layer has thickened further and eventually becomes the floor of the nasal pit (Fig. 2G, arrow); *bmp4* expression is absent or expressed at very low levels in this thickened ectodermal area compared to that of the earlier stage (compare Fig. 2B and 2G, arrows).

Expression of *bmp4* during fusion of LNP and MNP (TS 10–13)

At TS 10, fusion of the LNP and MNP can be observed, starting from a posterior to an anterior direction of the nasal pit (Fig. 1Bc). Fusion is initiated when the epithelial layers of the LNP and MNP first contact to form a bilayer epithelial structure referred to as the epithelial seam (Fig. 3C; Wang et al., 1995). The epithelial seam disintegrates to allow penetration of mesenchymal tissues across the site of fusion (Fig. 3B, E; Wang et al., 1995). Therefore, as one moves from a more posterior to anterior position along the nasal pit area, a decreasing degree of fusion of the LNP and MNP can be observed, i.e. complete mesenchymal penetration in the most posterior part of the nasal pit to epithelial seam formation to contact of the epithelial layers and finally free standing MNP and LNP more anteriorly in the nasal pit (Fig. 3A-D for TS 10; Fig. 3E-H for TS 13). In more posterior regions of the nasal pit (in embryos with TS > 9), the bilayer epithelial seam is breaking down and in some cases mesenchymal penetration has occurred; *bmp4* is expressed in the layer of oral ectoderm ventral to the site of fusion in these areas (arrows in Fig. 3B, E, F). In some sections, cells expressing *bmp4* appeared "trapped" in the epithelial layers of the epithelial seam (Fig. 3G, arrow). Further anterior along the nasal pit, the facial processes are in contact and ectodermal cells at the edges of the MNP and LNP express *bmp4* (Fig. 3C, H). Further anterior to this, where the MNP and LNP are not contacting, *bmp4* is expressed at the tips of the LNP and MNP, restricted to the ectodermal layer at the junction between facial and nasal ectoderm of the LNP and MNP (Fig. 3D). On the medial aspect of the MNP, *bmp4* expression is also apparent in the oral ectodermal layer (Fig.1B, a-e, white arrowheads; Fig. 3B, C, F).

Expression of *bmp4* after the fusion of LNP and MNP (TS > 15)

In embryos older than TS 15, the midface has developed such that the MNP, LNP, and MxP have fused to a greater extent (compared to earlier embryos with TS < 15) with a corresponding decrease in the size of the nasal pit outline (compare outlines of nasal pits in Fig. 1B, c-e). Expression of the *bmp4* gene in the midfacial region is not as pronounced as before and appears to be localized to the rim of the nasal pit. Sections through the midface of TS 17 indicate there is total mesenchymal ingrowth of the nasal fin area and that expression of *bmp4* has shifted to the mesenchyme in both the LNP and MNP (Fig. 4A; b, d-f).

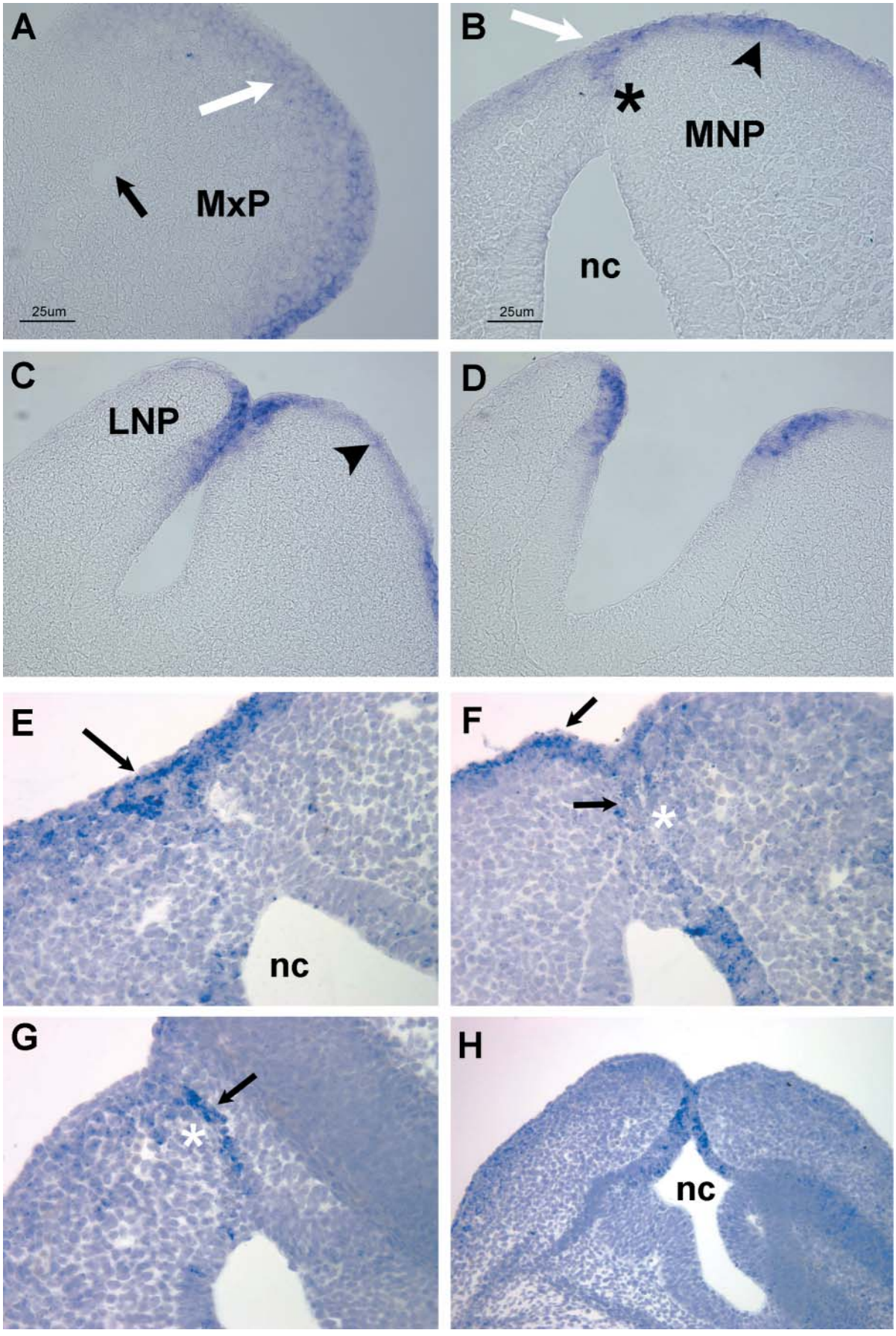
Expression of *bmp4* in the maxillary primordia

In the maxillary primordia prior to fusion of the three midfacial processes, *bmp4* is expressed in the ectoderm in a slight semicircular area (Fig. 1B, a-b, black arrows; Fig. 2A); this pattern of expression is visible just posterior to the putative site of fusion of the facial processes. Slightly later in development, the gene is expressed in a more restrictive band in the ectoderm of the maxillary primordia immediately posterior to the site of fusion of the three facial prominences (Fig. 1Bc and Fig. 2A, black arrows). However, some expression is observed in the mesenchyme soon after fusion of the MNP and LNP (Fig. 3A, white arrow). The shift of expression of *bmp4* in the maxillary primordia to the mesenchyme persists such that, at later stages, this gene is expressed highly in the mesenchyme (TS 12; Fig. 4Bb). The expression of *bmp4* remains high in a localized region of the ectoderm in a site that demarcates the future tooth bud (Fig. 4Ba, arrow).

Discussion

This is one of the first demonstrations of expression of a molecule in the putative site of fusion of the midfacial

Fig. 3 Distribution of *bmp4* transcripts in adjacent transverse sections of TS 10 (**A-D**) and TS 13 (**E-H**) embryos from a posterior to an anterior direction through the nasal pit region. (**A**) In the maxillary primordia, *bmp4* expression has shifted to the mesenchyme underlying the ectoderm (*white arrow*). Part of the nasal cavity is slightly visible (*black arrow*). (**B-H**) Where mesenchymal ingrowth has occurred, *bmp4* is expressed in the ectodermal layer of the oral or facial surface (* site of fusion; **B**, **F**, **G**). Further anterior along the site of fusion where a thin layer of epithelial seam formation is still evident, cells expressing *bmp4* are trapped in the fusion site (**F**, **G**, *arrows*). *Bmp4* expression is present in the transitional layer between nasal and oral ectoderm in areas where the MNP and LNP are contacting (**C**, **H**) or are no longer in contact (**D**). *Nc* = nasal cavity.


A
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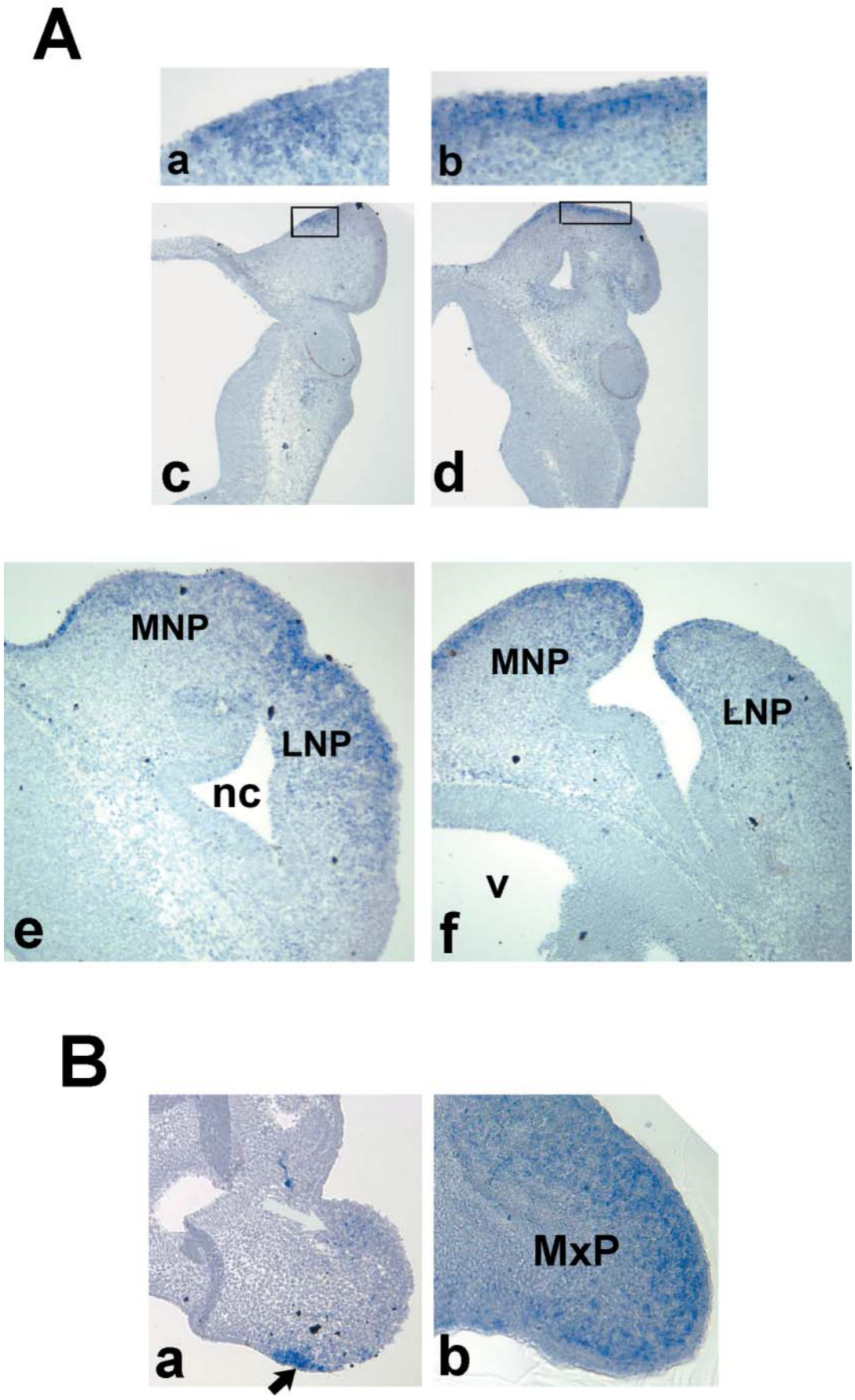
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Fig. 4 Distribution of *bmp4* transcripts in adjacent transverse sections of TS 17 embryos. (**A**) Mesenchymal expression of *bmp4* in embryos at TS 17. At this stage, MNP and LNP have fused postero-anteriorly, enclosing a nasal cavity, *nc* (**e**). **a** and **b** are magnified views of the boxed areas in **c** and **d**, respectively. *Bmp4* expression is clearly in the mesenchyme of all the facial processes. In the anterior part of the MNP and LNP where fusion is not present, *bmp4* expression has also shifted to the mesenchyme (**f**). (**B**) Expression in the maxillary primordia. By this stage, *bmp4* marks the tooth bud formation (*arrow*, **a**). Further anterior along the maxilla, expression of the gene is distinctly in the mesenchyme (**b**).

primordia during mouse embryonic development. In addition to the expression of *bmp4* in the medial aspect of the MNP, one striking feature is its expression pattern in the superficial outer layers of cells at the junction of the oral ectoderm and nasal ectoderm in the LNP and MNP anterior to the site of fusion. Cells in this area have previously been examined at the light and electron microscopic level and found to possess distinctive histological features (e.g. these cells have large nuclei, abundant cytoplasm, and well-developed junctional complexes) (Kosaka et al., 1985). In addition, occasional dense granules that are candidates for programmed cell death have also been identified in these cells (Kosaka et al., 1985). The spatial and temporal distribution of the gene suggests that *bmp4* marks the cells that will eventually meet and fuse, i.e. cells situated in the so-called putative site of fusion of the LNP and MNP.

Studies in the chick system using TUNEL staining and transmission electron microscopy have confirmed that it is this outermost layer, the so-called periderm, of the two-layered embryonic epithelium of each facial primordia that is removed by apoptosis shortly before they fuse (Sun et al., 2000). An important role for *bmp4*, therefore, is possibly in mediating the process of programmed cell death or apoptosis of cells in the superficial peridermal cells which have to be removed before the underlying basal layers are able to come into contact and adhere, leading to lip fusion and confluence of epithelial seams through epithelial-mesenchymal transformation (Sun et al., 2000). Interestingly, Sun et al. (2000) also found an occasional trapped peridermal cell positive for TUNEL staining, corresponding to the epithelial cells in the early fusion site, in a pattern very similar to what we observed with trapped *bmp4* cells at the site of fusion. *Bmp4* can therefore be envisioned to be critical in lip fusion by mediating the apoptotic activity of cells in the region of the facial processes that will eventually fuse.

The importance of apoptosis in mediating the process of fusion of other facial primordia has also been observed during the development of the secondary palate. As the secondary palatal shelves elevate from a vertical to horizontal position and grow towards each other to fuse, contact occurs first at the leading growing borders of the shelves in a region known as the medial edge epithelium (MEE) (Ferguson, 1988). Three general mechanisms have been proposed by different investigators to explain the fate of the MEE as the shelves fuse: programmed cell death, (Pourtois, 1966; Farbman, 1969; Clarke, 1990), epithelial-mesenchymal transformation (Fitchett and Hay, 1989; Shuler et al., 1991; Hay, 1995), and migration to palatal oral and nasal epithelial triangles and epithelia (Carette and Ferguson, 1992). Recent evidence suggests that all three mechanisms very likely contribute to the fate of the MEE as the secondary palatal shelves fuse (Martinez-Alvarez et al., 2000; Cuervo et al., 2002). Another recent study, however, implicated apoptosis as the critical event in palatal shelf fusion; without apoptosis of cells in the MEE, shelf fusion did not occur (Cuervo et al., 2002). Apoptosis of MEE was found to occur just prior to contact of shelves in the posterior palate, whereas contact in anterior palate was necessary before the MEE underwent apoptosis (Cuervo et al., 2002). The authors suggested that apoptosis was the regulatory event that determine fusion of the palate, failing which, clefts of the palate is observed *in vivo*.

Molecules such as *TGF-*β_3 and retinoic acids are involved in mediating apoptosis in the secondary palatal shelves (Martinez-Alvarez et al., 2000; Cuervo et al., 2002). *TGF-*β_3 induces apoptosis and morphological changes in the MEE indicative of motility that favors the contact, adherence, and intercalation of MEE seam cells (Martinez-Alvarez et al., 2000); it is expressed at relatively high levels in the MEE before fusion of the palatal shelves and disappears soon after fusion of the shelves; its blockade *in vitro* prevents palatal fusion and this blockade can be rescued by addition of *TGF-*β_3 to the culture medium (Brunet et al., 1995; Kaartinen et al., 1997); and *TGF-*β_3 null mice show cleft palate (Proetzel et al., 1995; Kaartinen et al., 1997). In addition, retinoic acid was shown to exert its teratogenic effect on palatal shelf fusion by a direct increase in MEE cell death (Cuervo et al., 2002). We envisage that apoptosis plays a similar role in mediating fusion of the primary palate and that one of the players that mediates the apoptotic event is *bmp4*. *Bmp4* is a member of the transforming growth factor superfamily, of which *TGF-*β_3 is a member. *Bmp4* has been shown to be a potent inducer of cell death in several model systems (Graham et al., 1994; Macias et al., 1997; Chen and Zhao, 1998). Functional and mechanistic analyses of the role of *bmp4* in the fusion of the midfacial region would lead to more definitive answers to the role of the gene during the fusion of the primary palate. As *bmp4* knockout mice die prior the development of the palate (Winnier et al., 1995), the utility of *in vitro* assays such as whole-embryo culture or midfacial organ culture will be needed to study the role of *bmp4* during primary palate formation. In addition, isolation of genes via the latest microarray

technology would eventually allow the identification of other genes that may be involved in the pathway of fusion of the primary palate.

The expression pattern of *bmp4* and *bmp2* had been analyzed in great detail in the chick midfacial region and found expressed in restricted domains in the epithelium in association with underlying regions of *bmp2*, *bmp7*, *msx1*, and *msx2* expression in the mesenchyme (Francis-West et al., 1994; Wall and Hogan, 1995; Barlow and Francis-West, 1997). There was, however, no mention of *bmp4* gene expression in the putative site of fusion in the chick system, possibly because the development of the chick midfacial region differs slightly from that of the mouse and humans. A similar switch of pattern of expression of *bmp4* from the epithelia to the mesecnhyme and subsequently in specific regions of mesenchyme at the distal tips of the primordia was noted in the chick system as we find in our study. The dynamic pattern of expression in the ectoderm and mesenchyme could be related to a second role of *bmp4* during primary palate development, i.e. in mediating outgrowth of the midface via epithelial-mesenchymal interactions and patterning in this region. Members of the *bmp* family have already been shown to play a role in patterning the identity of the midface, specifically that of the maxillary primordia as antagonist against the *bmps* and retinoic acid were able to transform the identity of the maxillary primordia into a frontonasal mass (Lee et al., 2001).

One would expect that there will be many players involved in the morphogenesis and patterning of a region as complicated as the midface. The identification of expression of molecules with distinct expression pattern in the midface represents one of the first steps in building a cascade of molecular pathways that define the growth and patterning of the midface such that any aberrations of such growth, e.g. clefts of the lip and palate can be better understood.

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