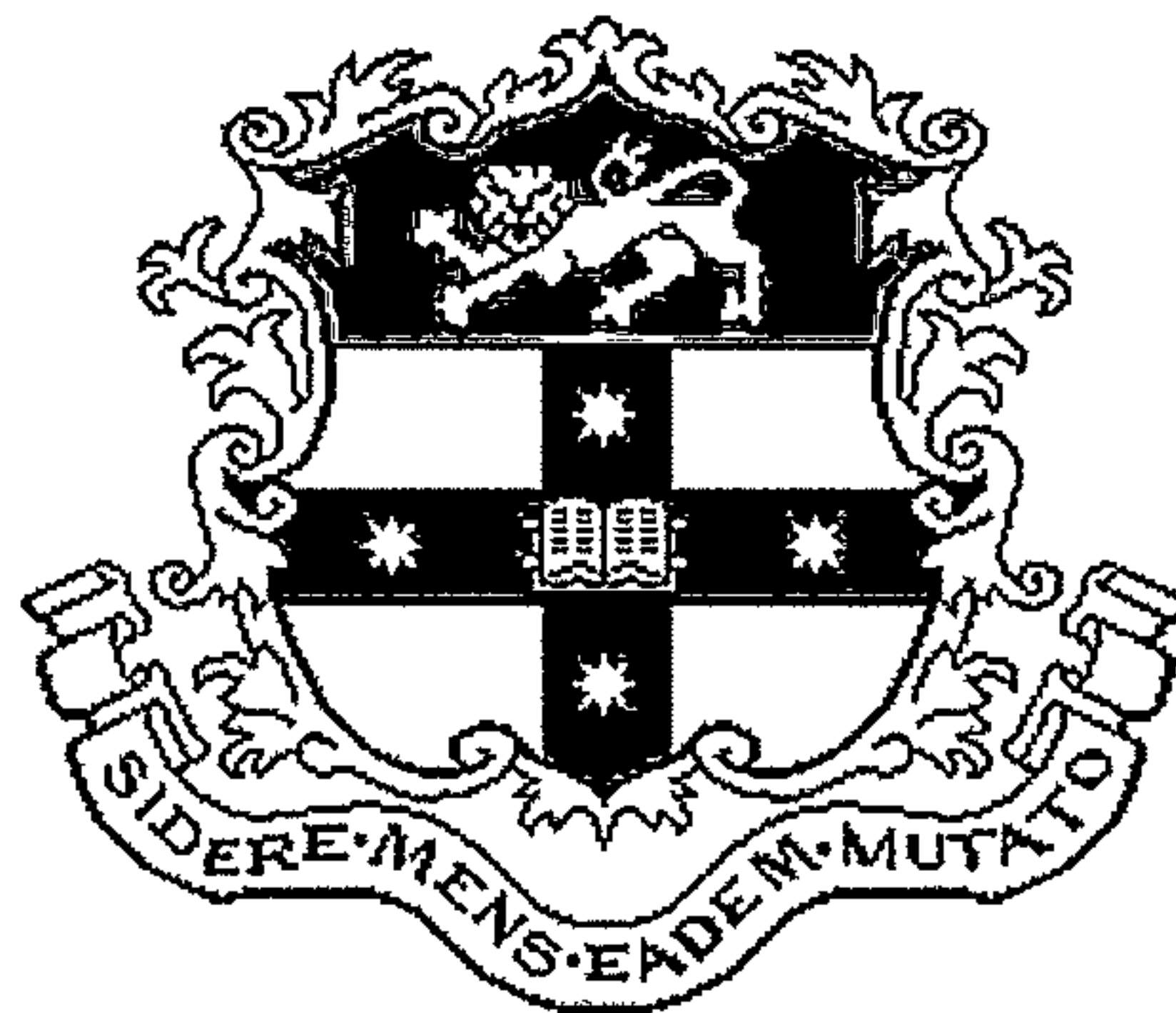


NOVEL EXPRESSION OF FGF8 AND SHH
DURING CHIRALITY RESOLUTION IN THE FUSING RAT PALATE



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Thesis

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DECLARATION

In accordance with the by laws of the University of Sydney, I declare that this thesis describes original research performed by myself, except where due acknowledgement has been made in the text, within the Discipline of Orthodontics and Department of Pathology, at the University of Sydney, and the Institute of Dental Research at Westmead Centre for Oral Health, Westmead Hospital.

To the best of my knowledge, this thesis does not contain any material previously written or published by another person except where duly referenced.

This work has not been submitted for any other degree at this or any other institution.

James Kyung-Wook Choi

2003

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Choi, J.K.W., Gibbins, J.R., Darendeliler, M.A., and Kharbanda, O.P. (2002) Role of FGFR2 in EMT during embryonic midline palatal fusion. University of Sydney Research Conference, Cell to Society 2. Leura, NSW, Australia.

ABBREVIATIONS

AER	Apical ectodermal ridge
ASE	Asymmetric enhancer element
dpc	Days post coitum
Car	Caronte
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal Transition
FGFs	Fibroblast Growth Factors
FGF8	Fibroblast Growth Factor 8
GAGs	Glycosaminoglycans
GFP	Green fluorescent protein
HSPGs	Heparan sulphate proteoglycans
KGF	Keratinocyte Growth Factor
IHC	Immunohistochemistry
LAP	Latency Associated Protein
LPM	Lateral Plate Mesoderm
LRD	Left Right Dynein
MEE	Medial edge epithelium
MES	Medial epithelial seam
MET	Mesenchymal-Epithelial Transition
mRNA	Messenger RNA
PM	Paraxial mesoderm
PCD	Programmed cell death
SHH	Sonic hedgehog
TGF β	Transforming Growth Factor β

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SUMMARY

Expression of the specific ligands, fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) was investigated to elucidate mechanisms involved in the resolution of chirality during the secondary palatal fusion process. Sprague-Dawley rats were mated overnight and timed embryos removed 14.5 to 17.5 days post coitum (dpc) to obtain tissue samples prior to, during and immediately following palatal fusion. Immunohistochemistry (IHC) for FGF8 and SHH were performed on 5 μ m paraffin sections of rat embryo maxillas fixed in 4% paraformaldehyde.

Asymmetrical FGF8 expression was detectable initially localized to the medial edge epithelium (MEE) of the right palatal shelf immediately after the elevation and rotation of the palatal processes above the tongue. Immediately prior to and following contact of the MEE of each of the shelves in the midline, localized regions of bilateral FGF8 expression were confined to the MEE in the contra-lateral shelf. SHH expression was not detected until contact of the MEE of each of the shelves in the midline. SHH expression was also confined to the MEE in the contra-lateral shelf in the zone of contact. No FGF8 or SHH expression was detected in the epithelium of the medial epithelial seam (MES) formed by the integration of the MEE on each palatal shelf.

These results are interpreted as indicating that asymmetrical right FGF8 expression in the MEE may play a critical role in chirality resolution to ensure subsequent merging of the palatal shelves, and that epithelial cells in the MEE are specifically primed to upregulated expression of both FGF8 and SHH genes

prior to MEE contact and MES integration. Specific expression of FGF8 and SHH during palatal fusion in the rat embryo may be analogous to the role played by FGF8 in the determination of handedness in the chick primitive streak.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Symmetry around a midline axis underlies the whole of embryonic development and is dependent on the successful completion of midline fusion (Shuler 1995). Midline fusion events are critical to normal embryonic development and their disturbance is a major contributor to congenital deformity (Cohen 2001) (Fig.1-3). Midline fusion events frequently involve trans-differentiation of cells from epithelial to mesenchymal (EMT) or mesenchymal to epithelial (MET) (Hay 1995; Hay et al. 1995).

The most frequently disturbed fusions occur during faciomaxillary development and this has fuelled investigations into the normal formation and fusion of the secondary palate (Ferguson 1988, Ferguson 2000). While morphogenetic events occurring around the midline require resolution of the chirality initiated at the stage of the primitive streak, the fusion of the palatal processes could be considered as the enforced midline union of two processes which have become highly polar, that is which have a high degree of handedness. The mechanisms underlying palatal fusion have been studied in a variety of species ranging from reptiles, through birds and rodents to man (Ferguson 1988). The studies reported in this thesis have been confined to the laboratory rat.

A**B**

Figure 1. Congenital deformities associated with disturbances in midline fusion (Cohen, 2001).

A. Unilateral anophthalmia.

B. Single nostril.

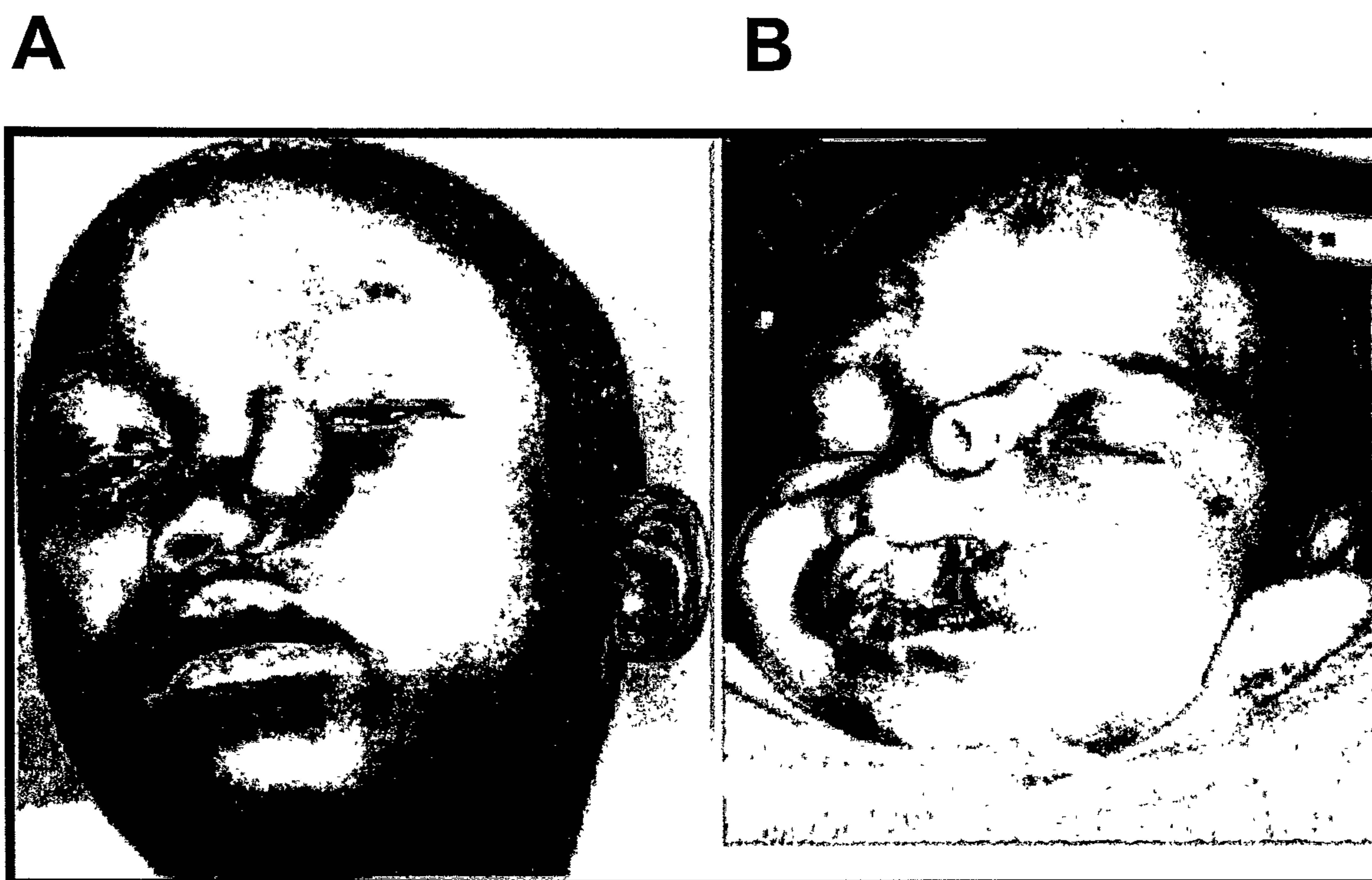


Figure 2. Congenital deformities associated with disturbances in midline fusion (Cohen, 2001).

- A. Single nostril and lateral nasal proboscis.
- B. Accessory proboscis, cleft lip/palate and two nostrils.

A**B**

Figure 3. Congenital deformities associated with disturbances in midline fusion (Cohen, 2001).

A. Eye located in temporal region.

B. Accessory mouth in temporal region.

1.2 Secondary Palate Development

In the rat embryo, morphologically identifiable stages of palatal development begin at 15 dpc with fusion completed by 18 dpc (Fig. 4) (Gibbins et al. 1999; Gibbins et al. 2000). At 15 dpc, the palatal processes are developing as tapered extensions of the maxilla lateral to and on either side of the tongue. At about 16 dpc, the palatal processes begin to reorientate to a position above (rostral) to the tongue. The palatal shelves then grow towards each other and by 17 dpc the palatal shelves adhere, and then begin to fuse with fusion completed by 18 dpc (Ferguson 1978).

The palatal shelves, which initially grow lateral to the developing tongue, arise from the maxillary prominence of the first brachial arch. At this stage, each palatal process is a simple structure covered by a two or three cell layered epithelium consisting of mesenchymal cells embedded in a loose extracellular matrix (ECM) with no specialized structures evident. The secondary palate is one of the latest embryonic structures to form, and when normal fusion of the palatal shelves fails, palatal clefting is the result (Ferguson 1988).

Before they meet in the midline, the re-orientation of the two palatal processes to a position rostral to the tongue appears to be restricted to a precise stage during embryonic development when the shelves become prepared for the subsequent fusion events. Earlier studies focused on factors likely to be important in determining this rotation of the palatal shelves such as craniofacial growth (Diewert 1983) and embryo movements leading to removal of the tongue from between the palatal shelves (Humphrey 1969, Walker 1969). Other

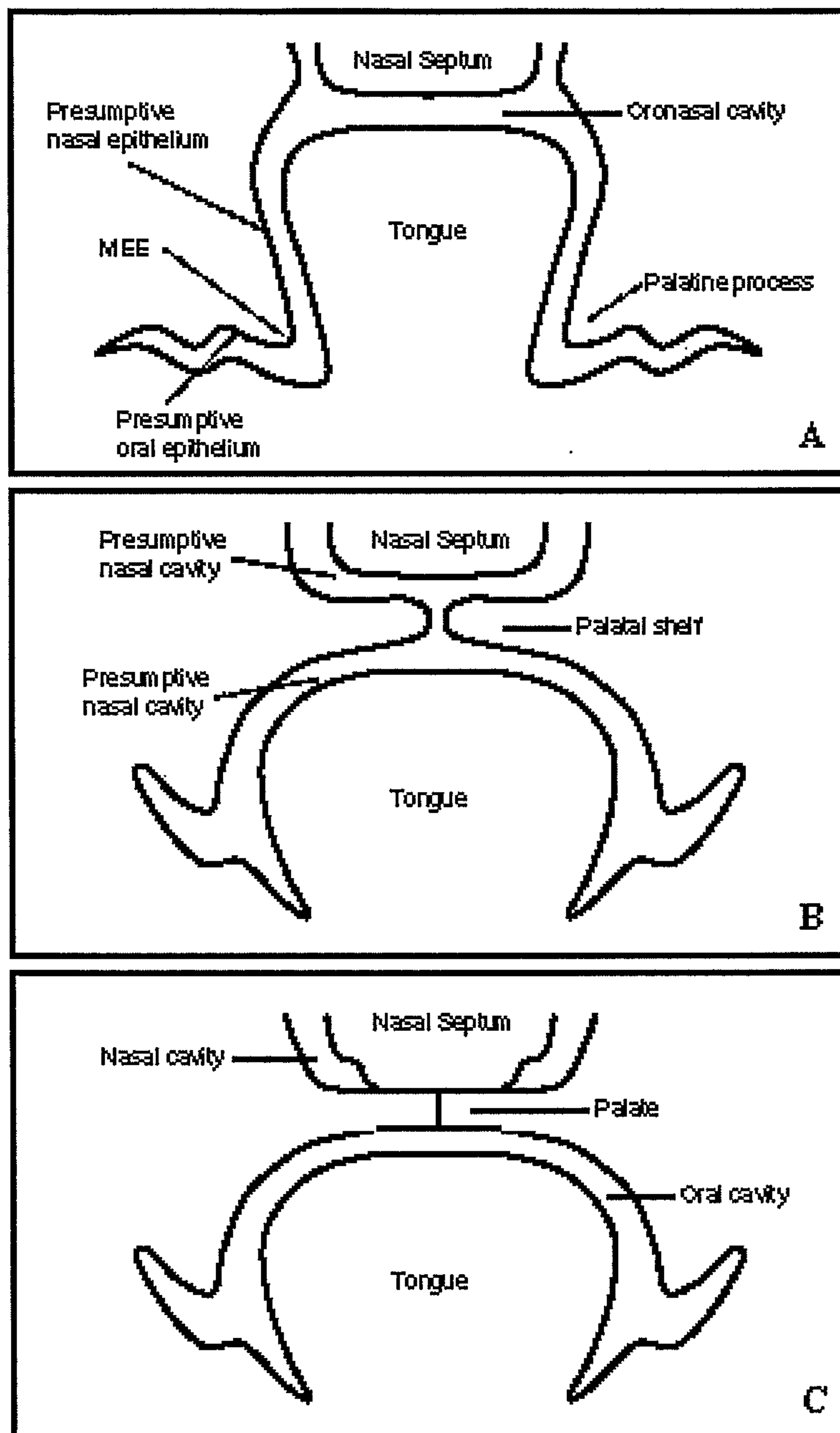


Figure 4. Schematic illustration of coronal section of the head taken through the developing secondary palate in the rat embryo.

- A. At 15 dpc, the palatal processes are lateral to the tongue.
- B. At 16 dpc, the palatal processes have elevated and rotated to the position above the tongue to become the palatal shelves. The MEE surfaces opposes each other.
- C. By 17 dpc, the palatal shelves begins to fuse to form the upper palate. The nasal septum also fuses to the palate.

studies focused on the shelf “force” involved in shelf elevation, examining the possible involvement of various intercellular substances (Brinkley et al. 1984), neurotransmitters (Wee et al. 1979), and other factors.

The epithelial cells covering the tips of the palatal shelves are considered to provide the initial adherence between the two palatal shelves (Meller 1980). The region of the epithelium on each palatal shelf which come into contact with the opposing shelf and is ultimately eliminated from the fusion zone is referred to as the medial edge epithelium (MEE). The contact and the adhesion of the MEEs of the two palatal shelves results in the formation of the medial epithelial seam (MES). The MES must be removed from the palatal midline to allow mesenchymal confluence to become established. Numerous studies have been concerned with the fusion of the palatal shelves in culture (Idoyaga-Vargas et al. 1972, Lewis et al. 1980, Carette et al. 1992, Watanabe et al. 1995, Taya et al. 1999). Various combinations of palatal shelf epithelia and mesenchyme between species with fusing (e.g. rodents) and those with non-fusing (e.g. chicken) palates showed that the mesenchyme was responsible for the epithelial changes that allow fusion to occur (Ferguson 1988).

1.2.1 Down growth of the palatal shelves

The palatal shelves begin to grow down lateral to the tongue at day 45 of gestation in humans, day 12 in mice, and day 14.5 in the laboratory rat (Ferguson 1988). At this stage, the palatal processes contain densely packed mesenchymal cells and the embryo is markedly flexed, just beginning to straighten with the tongue lying back in the posterior two-thirds of the oronasal cavity. By 15.5 dpc in the rat, the posterior one-fifth of the palate has already started to grow horizontally whereas the anterior four-fifth is still growing progressively lateral to the tongue. By 16.5 dpc in the rat the entire palatal shelves have elevated and rotated to a position above (rostral to) the tongue.

1.2.2 Shelf elevation

Numerous theories have been advanced to account for the elevation of palatal processes (Ferguson 1978). The role of continuing growth of the laryngeal and craniofacial region resulting in three-dimensional changes that change the spatial relationships of the tongue and the palatal shelves as development progresses has been attributed to the progressive displacement of the tongue downward and forward in the oronasal cavity both permitting and facilitating palatal elevation (Diewert 1980). It was shown that altering aspects of craniofacial growth with teratogens delayed shelf elevation and resulted in cleft palate (Diewert 1980).

Neuronal stimulation and movement of the tongue during palatogenesis were studied by Ferguson (1978). The finding that the palatal shelves in dead animals with tongues removed still underwent palatal elevation effectively eliminated any hypothesis favouring a positive effect of tongue movement. The ability of dissected palates to re-orientate strongly suggests that forces intrinsic to the palate must be responsible for elevation of the shelves (Brinkley et al. 1975, Ferguson 1978).

The mucopolysaccharide theory attributes the source of the intrinsic forces of shelf elevation to the accumulation of glycosaminoglycans, particularly hyaluronic acid (Ferguson 1981, Knudsen et al. 1985). The regionally specific accumulation of this highly electrostatically charged, open-coiled molecule, capable of binding up to ten-times its own weight in water, creates a situation where small changes in hyaluronic acid can result in large changes in osmotic pressure thus giving the palate a driving force for elevation which forces the

shelves upwards (Knudsen et al. 1985, Ferguson 1988, Sharpe et al. 1988). Unique, region-specific patterns of hyaluronic acid distribution and relative concentration evident as intensity of staining were identified prior to and during shelf reorientation that became homogeneous after reorientation supported the role of hyaluronic acid in shelf reorientation (Brinkley et al. 1987). Further support for the role of hyaluronic acid was provided by incubating fetal heads in hyaluronidase which abolished shelf elevation (Ferguson 1988).

As in the mammal, avian palatal shelves begin as outgrowths from the maxillary process but they do not continue to grow vertically. They initially develop horizontally above the dorsum of the tongue, approximate and contact each other, but do not adhere or fuse. Instead medial edge epithelium becomes keratinised, producing a natural cleft palate (Sun et al. 1998). This is a similar scenario to the reptilian family for all except the crocodilians. They also have an intact fused 'mammal-like' palate (Ferguson 1984).

1.2.3 Adhesion of the Palatal Shelves

Following elevation, the shelves contact each other, the MEE on each shelf adheres and rapidly integrates to form the MES. During this process of epithelial fusion, the majority of the superficial 'periderm-like' layer of the epithelium is sloughed off. This sloughing exposes the cells which are the critical cell population of MEE and is thought to be necessary to allow the MEE to integrate (Fitchett et al. 1989). The MEE of each palatal shelf adheres to each other by means of numerous desmosomes (Ferguson 1978, Shah 1979). Observations of the MEE surface in as near as natural state as possible by environmental scanning electron microscopy found bulging epithelial cells on the MEE just prior to fusion (Martinez-Alvarez et al. 2000). Vinculin, involved in the linking of actin filaments to integrins that attach cells to each other and the ECM (Drenckhahn et al. 1988), and chondroitin sulphate proteoglycans were found in the bulging MEE cells when they were establishing contact in the midline (Martinez-Alvarez et al. 2000). A similar pattern of expression has been found to be important for epithelial adhesion during neural tube closure (Trasler et al. 1991, Alonso et al. 1998). Cellular adhesion molecules such as E-cadherin and β -catenin were found to be present all around the surface of the MEE cells (Martinez-Alvarez et al. 2000).

1.2.4 Formation of the Medial Epithelial Seam (MES)

The original thickness of arising from the integration of the two MEE layers to form the MES of two cell layers changes rapidly to a thin MES only one cell thick in the centre. Superiorly, at the nasal surface and inferiorly at the oral surface of the MES, the epithelium is expanded into a triangular area (Hayward 1969). The cells in this area contain numerous electron dense granules, which was shown to be positive for high levels of acid phosphatase (Hanker et al. 1973). This triangular area of epithelial cells in the MES also stained strongly for L-fucose (as terminal sugar) on glycoconjugate carbohydrates (Zschabitz et al. 1994). Lysosomal bodies found throughout the MES, were rarely seen in the oral or nasal epithelial surfaces (Mato et al. 1975). Initially the breakdown of MES was not correlated with a breakdown of the basement membrane (Hayward 1969), however the importance of the disruption and degradation of the basement membrane of MES in the final stages before the breakdown of MES has become recognised in more recent studies (Shuler et al. 1992).

1.2.5 Medial Epithelial Seam Elimination

The MES is rapidly eliminated from the midline and as epithelium disappears mesenchymal confluence is established. There is still controversy surrounding the actual mechanism responsible for the MES elimination in fusing palatal shelves. Currently, there are three mechanisms for which substantial evidence has been accumulated and are generally accepted as contributing to the removal of MES from the midline. The three mechanisms are: programmed cell death (Clarke 1990, Mori et al. 1994), epithelial-mesenchymal transformation (EMT) (Fitchett et al. 1989, Shuler et al. 1991, Hay 1995), and migration of the cells of MES in to the nasal or oral epithelium of the palate (Carette et al. 1992, Calkhoven et al. 1996).

1.2.5.1 *Programmed Cell Death*

Programmed cell death (PCD) involves a defined population of cells undergoing a specific pattern of cell death and cell destruction without cell lysis, and thus is different to necrosis which affects cells randomly, without discrimination of cell type and thus can affect any tissue (Kerr 1993). PCD is defined by a pattern involving cell shrinkage and fragmentation eliminating cell contents to adjacent tissue, as opposed to cell necrosis which involves swelling, cell lysis and release of cell contents locally. In this way, there is no stimulation of the inflammatory response with PCD, and thus no tissue scarring (Kerr 1993). The terms Apoptosis and PCD are often used interchangeably, however there are some differences which distinguish these seemingly identical processes, the most important being the predictability of the population of cells targeted for removal in PCD relative to apoptosis. PCD was defined in and applies more accurately to embryonic processes (Horvitz 1999) whereas apoptosis was first defined in pathological processes in adult tissues (Kerr 1993).

Shapiro and Sweney (Shapiro et al. 1969) were the first to claim that there was evidence to suggest that the cells of the MEE were eliminated by undergoing a targeted cell death. Cells of the MEE appeared to cease DNA synthesis 24 to 36 hours before shelf elevation and contact, and displayed subsequent morphological signs of necrosis. They discussed the importance of enzyme systems to indicate cells targeted for destruction. They proposed that low levels of oxidative enzymes in the pentose phosphate pathway observed in the MEE would be indicative of decreased DNA and RNA synthesis (Pratt et al. 1976). Cessation of tritiated thymidine incorporation into DNA in the cells of the MEE was considered to indicate an arrest in cell replication and a harbinger of cell

death while expression of new genes or mRNA synthesis was not detected at all (Greene et al. 1976).

1.2.5.2 *Epithelial-Mesenchymal Transformation (EMT)*

The tissue phenotype of the earliest stages of embryogenesis in animalia is epithelial. A second tissue type, mesenchyme, is formed from the primordial epithelium via the process of epithelial-mesenchymal transition (EMT) (Gibbins et al. 1969, Tilney et al. 1969). In chordates, the cells migrate from the primitive streak to differentiate into the mesoderm and endodermal epithelium. The specialised mesenchymal tissues, such as connective tissue and muscle, have their origin the primitive epithelium, having been derived by the process of EMT (Griffith et al. 1992).

Fitchett & Hay (1989) proposed a model for MEE dispersion in which EMT was responsible for the removal of the epithelium from the midline with PCD playing a secondary role in the loss of epithelium in the fusing palate. The opposing epithelial cells adhered to one another and transdifferentiated into mesenchymal cells, melding into the adjacent mesenchyme. The loss of basement membrane as the epithelium dispersed was attributed to extensions of filopodia and then pseudopodia by the epithelial cells into the adjacent connective tissue. Furthermore, MES cells exposed to the matrix showed loss of epithelial type cell junctions and acquired the morphology of fibroblasts (Fitchett et al. 1989). These cells were also reported to lose keratin expression and acquire vimentin (Shuler et al. 1991) and down-regulate expression of epithelial-type intercellular adhesion molecules (Sun et al. 1998).

Expression of vimentin mRNA has also been found in the epithelial cells of the MEE, which were also expressing keratin 5/6 mRNA, just prior to contact with the opposing palatal shelf (Gibbins et al. 1999). The upregulation

of vimentin mRNA, an intermediate filament expressed in the cells of the mesenchyme in epithelial cells was interpreted as indicating 'priming' of cells in the MEE in anticipation of EMT.

The fate of the MES has also been followed by the use of replication-defective helper-free retroviral vector CXL which carries the *E.coli* lacZ gene introduced into the MEE before formation of the MES (Martinez-Alvarez et al. 2000). β -galactosidase activity in cells of the MES during fusion and in the mesenchyme following dispersion of the epithelium was interpreted as demonstrating that the epithelial cells in the seam transformed into mesenchyme during palatal fusion (Martinez-Alvarez et al. 2000).

1.2.5.3 *Migration*

Experiments were also conducted by Carette and Ferguson (1992) where cells in the MEE were labeled with DiI and analysed using confocal laser scanning microscopy. Their interpretation of their results differed from the similar experiments outlined above. Their preferred interpretation was that the labeled cells adopted a conserved migratory phenotype. They argued that the cells of the MES migrated to the oral and nasal epithelium, eventually becoming part of these epithelia. The migratory mechanism of palatal closure was also felt to confer greater strength to the newly fused palate, ensuring a more stable structure with greater resistance to post-fusion dehiscence than would be possible if a zone of epithelial cell death occurred in the midline. The epithelial triangles observed at the extreme ends of the MEE were proposed to represent the cells migrating to the surface of the palate (Carette et al. 1992).

1.3 Dispersion of the Medial Epithelial Seam

A consensus has not yet been achieved on a mechanism for the dispersion of the MES during palatal fusion. Epithelial-mesenchymal transition as a mechanism for the dispersal of the MES in palatal fusion is supported by an impressive body of evidence from experiments carried out in several laboratories, but the question of the significance of EMT relative to the other proposed mechanisms in the process of palatal fusion has not been resolved. One reason for this reticence is that it has not been possible to elucidate any specific role for the cells that have transdifferentiated or to determine their eventual fate. In other situations where EMT has been studied, such as in the generation of neural crest cells when the neural tube is formed in early embryogenesis (Le Douarin et al. 1994) or in the cells of the cardiac cushion during cardiac development (Markwald et al. 1996), the fate of the cells has been followed and their contribution to subsequent developmental events defined. The knowledge gained from tracing the cells derived from the neural crest has been one of the most enlightening areas of developmental biology, providing insights into diverse areas from craniofacial development to the development of the neural and pigmentary systems.

1.3.1 Intermediate Filaments

Microtubules, intermediate filaments, and microfilaments are the major cytoskeletal elements in the eukaryotic cell. The most complex of these filamentous structures are the intermediate filaments which form a superfamily of 10 nm fibres, composed of α -helical polypeptides that intertwine in a coiled-coil fashion to form the subunit structure (Steinert 1993, Fuchs et al. 1994). Members of the intermediate filament superfamily exhibit cell-type specificity and complex patterns of expression. This specificity is reflected in their varied genetic sequence. Intermediate filament proteins share about 30 to 50% nucleotide sequence identity, in contrast to microtubules and microfilaments which are composed of tubulin and actin respectively, and are highly conserved (Shoeman et al. 1993).

The most important function of intermediate filaments appears to be formation of a mechanical framework for the cell. However, the tissue-specific expression of these proteins suggests that intermediate filaments may also be involved in more specialised roles of the cells and tissues (Steinert 1993, Fuchs et al. 1994).

Currently, there are nearly 50 known human intermediate filaments that are differentially expressed in different tissues. These have been classified into 4 major groups, which are further subclassified into 6 types.

The first group are the keratins, the largest and the most complex group of the intermediate filaments. Type I keratins are acidic, and type II keratins are neutral-basic, and their size ranges from 40 to 67 kD. Keratin filaments occur

as heteropolymers of one type I chain and one type II chain. Pairs of keratin type I and type II assemble together at the same time, but the different pairs are expressed differentially in most epithelial cells at various stages of development and differentiation (Fuchs et al. 1994).

The second major group (Types III and IV) contains intermediate filaments that can be constructed from a single protein subunit, that is, they exist as homopolymers. The type III intermediate filament proteins include vimentin, desmin, glial fibrillary acidic protein, and peripherin and occur in mesenchymal cells, myogenic cells, glial cells & astrocytes, and peripheral neurons respectively. Type IV intermediate filament proteins, the neurofilament and α -internexin filament constituents, are expressed in most neurons (Fuchs et al. 1994).

The third group contains type V intermediate filament proteins. These are proteins found in the nuclear lamina, the lamins. Lamins form a fibrous meshwork on the inner surface of the nuclear membrane where their function appears to be to provide framework for the nucleus (Fuchs et al. 1994).

A fourth group contains type VI intermediate filament proteins. These proteins, called nestins, are the most recently discovered neuronal-specific intermediate filaments. The expression of nestin is restricted to neuroepithelial stem cells (van de Klundert et al. 1993, Fuchs et al. 1994).

1.3.2 *TGF- β*

The TGF- β family of polypeptides includes three structurally and functionally related mammalian isoforms, TGF- β_1 , TGF- β_2 , and TGF- β_3 , each encoded by different genes located on different chromosomes (Massague 1990), with multifunctional actions controlling the growth, differentiation and function of a broad range of target cells of both epithelial and mesenchymal derivation (Derynck et al. 1985, Massague 1990). They are expressed early in embryogenesis and their tissue-specific and developmentally dependent expression is strongly suggestive of an essential role in particular morphogenetic and histogenetic events (Roberts et al. 1990). TGF- β s also influence cell proliferation, differentiation and extracellular matrix (ECM) production (Moses 1990). Cell adhesion, skeletal development, haematopoiesis, inflammatory response, and wound healing are also affected (Massague 1990).

The TGF- β s show a high degree of sequence conservation. TGF- β_1 /TGF- β_2 have 74% amino acid homology, TGF- β_2 /TGF- β_3 have 82% homology and TGF- β_1 /TGF- β_3 have 78% homology (Derynck et al. 1985, Massague 1990). The TGF- β homodimers is composed of two 112 amino acid chains, containing nine cysteine residues (Schlunegger et al. 1993). X-ray diffraction studies of TGF- β_2 crystals have revealed that of these nine cysteines, only one from each monomer (residue 77) is involved in forming an inter-chain disulfide bond linking the two monomers, whereas the other eight cysteines form intra-chain disulfide bonds (Daopin et al. 1992, Schlunegger et al. 1992).

TGF- β s are synthesised as a large precursor proteins, 390 amino acids per monomer for β_1 (Gentry et al. 1988), and 414 for β_2 (Brunner et al. 1992),

and 412 for β_3 (Denhez et al. 1990) containing the amino-terminal pro-domain consisting of a signal sequence and Latency Associated Protein (LAP). LAP is a fundamental component of TGF- β that is required for its efficient secretion, preventing it from binding to ubiquitous cell surface receptors, and maintains its availability in a large extracellular reservoir that is readily accessed by activation (Barcellos-Hoff 1996). The precursor also has sites for glycosylation, integrin binding (RGD), and a tetrabasic amino acid (RKKR) site for cleavage of the precursor peptide to the biologically active protein; a mature carboxy-terminal subunit of 112 amino acids (Kondaiah et al. 1990).

In general, the major biological activities of TGF- β s can be categorised into three main groups, with some overlapping effects (Lawrence 1996). TGF- β s inhibit or stimulate growth of cells, exert immunosuppressive effects and enhance deposition of ECM components, such as collagens, fibronectin, tenascin, glycosaminoglycans and proteoglycans (Lawrence 1996). Chemotactic effects of TGF- β also have been described, with evidence as a chemo attractant for human fibroblasts (Postlethwaite et al. 1987), monocytes (Wahl et al. 1987), and neutrophils (Brandes et al. 1991). The TGF- β s in their mature activated forms *in vitro* have similar activities qualitatively, but show some quantitative differences which may depend on cell type (Roberts et al. 1990). However, from studies of the expression levels of the TGF- β isoforms during embryogenesis and histological analysis of organs and tissues from TGF- β_1 and TGF- β_3 knockout mice, there is evidence that each isoform has specific functions *in vivo* (Shull et al. 1992, Kaartinen et al. 1995, Proetzel et al. 1995).

1.3.2.1 *TGF- β receptors*

Chemical cross-linking of radiolabeled TGF- β to cell surface TGF- β proteins have revealed the existence of up to nine different “receptors”. While a number of soluble proteins are known to bind the TGF- β s, most of the biological effects of TGF- β s are exerted via specific interactions involving three major membrane-bound receptors. The high-affinity species are designated type I (55 to 65 kD), type II (70 to 85 kD), and type III/betaglycan (200 to 280 kD) receptors that are present in most normal cells. TGF- β_1 , TGF- β_2 , and TGF- β_3 bind to all three receptor types (Lyons et al. 1991, Segarini 1991). The type III receptors are functionally different from type I and type II receptors. Type I and type II receptors are both serine/threonine kinases and appear to be preponderant in TGF- β signal transduction. The type III receptor is required for binding to all three TGF- β isoforms, but its enzymatic potential is not yet known and seems not to be directly involved in signal transduction, but may effect signal transduction indirectly by its ability to present ligand to the type II receptor (Lopez-Casillas et al. 1993).

During palatal fusion type II receptor is expressed by all palatal epithelial cells (Cui et al. 1998) and therefore cannot localize TGF- β_3 responsiveness. The expression pattern of type III and type I receptor in the developing palate in mice *in vivo* and *in vitro* by immunohistochemistry revealed that type III receptor expression was temporo-spatially restricted to the MEE during palatal fusion, while the expression of type I was primarily localized in all palatal epithelia (Cui et al. 2000), consistent with the expression patterns of Type II receptor and TGF- β_3 (Cui et al. 1998).

1.3.2.2 *Signal Transduction of TGF- β s*

TGF- β isoforms signal via the same heteromeric receptor complex, consisting of a ligand binding TGF- β receptor type II, and a type I receptor (Wrana et al. 1992). Signal transduction from the receptor to the nucleus is mediated by intracellular molecules called SMADs (Baker et al. 1997). SMAD2 and SMAD3 are structurally highly similar and mediate TGF-beta signals. SMAD4 is distantly related to SMADs 2 and 3, and forms a heteromeric complex with SMAD2 after TGF-beta or activin stimulation (Kawabata et al. 1998). SMAD2 and SMAD3 interact with the kinase-deficient TGF- β type I receptor after it is phosphorylated by type II receptor kinase. It had been suggested that TGF- β induces heteromeric complexes of SMADs 2, 3 and 4, and the SMAD complexes translocate into the nucleus, where they act as transcription factors (Nakao et al. 1997) and control the expression of target genes in a cell-type-specific manner (ten Dijke et al. 2000).

SMAD signaling is also not merely determined by activation of the class of TGF-beta receptors, but is also regulated through cross-talk with other kinase-signaling cascades. This signaling cross-talk might explain the complexity of the responses to TGF- β and related factors (Zhang et al. 1999). SMADs, characterized by an astonishingly complex array of molecular and functional interactions with other signaling pathways, appear to function not only as nuclear effectors for TGF- β family members, but as signal integrators within an extensive intracellular network (ten Dijke et al. 2000). These emerging patterns of signaling cross-talk involving SMAD proteins suggest a dynamic profile of positive or negative transmodulation of TGF- β signaling, depending on the cellular context (Schiffer et al. 2000).

1.3.2.3 *TGF- β s in Embryogenesis*

TGF- β expression begins early in development. Each TGF- β -isoform shows a distinct spatial and temporal expression pattern, with the distribution of two TGF- β isoforms usually overlapping. During development, TGF- β expression is found in cartilage (Thompson et al. 1989), bone (Heine et al. 1987), teeth (Chai et al. 1994), heart (Akhurst et al. 1990, Nakajima et al. 1997), blood vessels (Kurihara et al. 1989, Sauvage et al. 1998), haematopoietic cells (Hampson et al. 1989, Keller et al. 1990), lung (Pelton et al. 1991, Kaartinen et al. 1995), kidney (Thompson et al. 1988), bladder (Baskin et al. 1996), liver (Bissell et al. 1995), eye (Hales et al. 1994), skin (Thompson et al. 1989), and the nervous system (Johnson et al. 1993). Marked TGF- β expression is generally observed in areas undergoing morphogenetic events, those involving epithelial-mesenchymal interactions or differentiation. In many locations, mRNA and protein expression of TGF- β is maintained throughout postnatal life.

1.3.2.4 *TGF- β in Palatogenesis*

The three TGF- β isoforms have been shown to be differentially expressed within the developing palate (Gehris et al. 1992). Study of the expression profiles of TGF- β s during development of the secondary palate in the mouse found that TGF- β_3 mRNA is localised in the epithelial component of the vertical palatal shelf (Fitzpatrick et al. 1990). This expression continues in the horizontal palatal shelf, predominantly in the medial edge epithelium, and is lost as the epithelial seam disrupts during palatal shelf fusion. TGF- β_1 mRNA is expressed with the same epithelial pattern as TGF- β_3 but is not detectable until the horizontal palatal shelf stage, and TGF- β_2 mRNA is localised to the palatal mesenchyme underlying the medial edge epithelia in the horizontal shelves and in the early post-fusion palate (Fitzpatrick et al. 1990).

TGF- β_1 stimulated the accumulation of type III collagen in murine embryonic palate mesenchymal (MEPM) cells *in vitro* (D'Angelo et al. 1991). Immunohistochemical analysis confirmed that type III collagen was predominant in the mesenchyme of the embryonic palate, whereas type I collagen was ubiquitous throughout palatal epithelium and mesenchyme suggesting an involvement of TGF- β in the regulation of the amount of collagen present in embryonic palatal tissue at the level of synthesis and degradation (D'Angelo et al. 1994).

Most importantly, the large body of evidence accumulated *in vivo* in TGF- β_3 -null mutant mice (Karttinen et al. 1995, Proetzel et al. 1995), and *in vitro* in TGF- β_3 antisense deoxynucleotide experiments targeting TGF- β_3 mRNA during secondary palatal fusion (Brunet et al. 1995) clearly indicate the

absolute requirement of TGF- β 3 for successful adhesion and dispersion of the MES during fusion. *In vitro*, TGF- β 3 antisense deoxynucleotide or neutralising antibodies to TGF- β 3 resulted in the inhibition of the fusion process, while palatal fusion occurred in treatments with antisense deoxynucleotides or neutralising antibodies to TGF- β 1 and TGF- β 2 (Brunet et al. 1995, Kaartinen et al. 1997). TGF- β 3 has been proposed to be involved in disintegration of the MEE basement membrane (Kaartinen et al. 1997), induction of EMT in MEE cells (Sun et al. 1998), and induction of apoptosis during fusion (Martinez-Alvarez et al. 2000). It is clear that TGF- β 3 has multiple biological effects, but the exact functions and the mechanism by which TGF- β 3 exerts its effect in the fusing secondary palate is yet to be understood fully.

1.4 Left-Right Asymmetry Determination (Chirality)

An important question in the normal development of the faciomaxillary structures is the underlying molecular mechanisms determining left-right (L-R) sidedness (chirality) and the suppression of sidedness (resolution of chirality) that must occur to allow midline fusion of tissues to occur during embryonic growth (Vogel et al. 1996; Lewandoski et al. 2000). Asymmetrical expression of laterality genes begins just after node formation at the anterior end of the primitive streak and before development of the notochord. Determination of L-R asymmetry has been linked to the molecular determinates, fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) signaling molecules (Riddle et al. 1993; Heikinheimo et al. 1994; Levin et al. 1995; Yost 1995; Levin 1997; Beddington et al. 1999; Meyers et al. 1999; Capdevila et al. 2000; Mercola et al. 2001).

The human body shows profound left-right asymmetries in the placement and looping of the heart and visceral organs, and in the structure of the cardiovascular system, which occur systematically throughout the population. Complete mirror-image reversals of asymmetry (*situs inversus totalis*) are not harmful. By contrast, the incomplete reversal of *situs*, or heterotaxia, leaves some tissues normally oriented and others reversed and is a situation associated with severe medical consequences. In addition to understanding such syndromes, the study of L-R axis formation directly addresses several basic issues such as how cells in large sheets or tubes, and in several tissue layers, precisely orchestrate their morphogenesis.

1.4.1 Asymmetry Generation

The generation of L-R asymmetry can be imagined as comprising several distinct phases. The global breaking of embryonic symmetry is followed by the stabilization of asymmetry through the induction of unilateral gene expression patterns, and the L-R information is then transmitted, either globally or organ specifically, to the organ precursors where effector programs lead to differential morphogenesis. The right-sided gene expression of activin receptor and ligand (Fig. 5) in chicks precedes the asymmetric SHH and *nodal* expression and is the earliest molecular asymmetry so far reported in vertebrates, but details on how this pattern is set up remain obscure. In fact, two of the principal unsolved problems are when and how symmetry is initially broken in any vertebrate and if L-R axis specification in all tissues and organs is a result of the same initiating event.

The most spectacular data involved the demonstration of physiological connections between asymmetric electrical potentials and L-R determination in the chick, and evidence of conservation of this mechanism in frog embryos. A refreshingly stimulating model based on these data proposes that a type of electrical flow through gap junctions, with a zone of isolation at the presumptive embryonic midline acting as an insulator, might drive the accumulation of a small "left-right specifier" molecule on one side of the embryo. Because this is one of the only mechanisms that may function in widely divergent species (birds and amphibians), an important challenge will be to identify this molecule and characterize the biochemical linkage in other processes in the pathways. This difference, which might only have to be quite small, would then unfold into in chick, right sided activin signaling and, in frog, the asymmetric activity of a

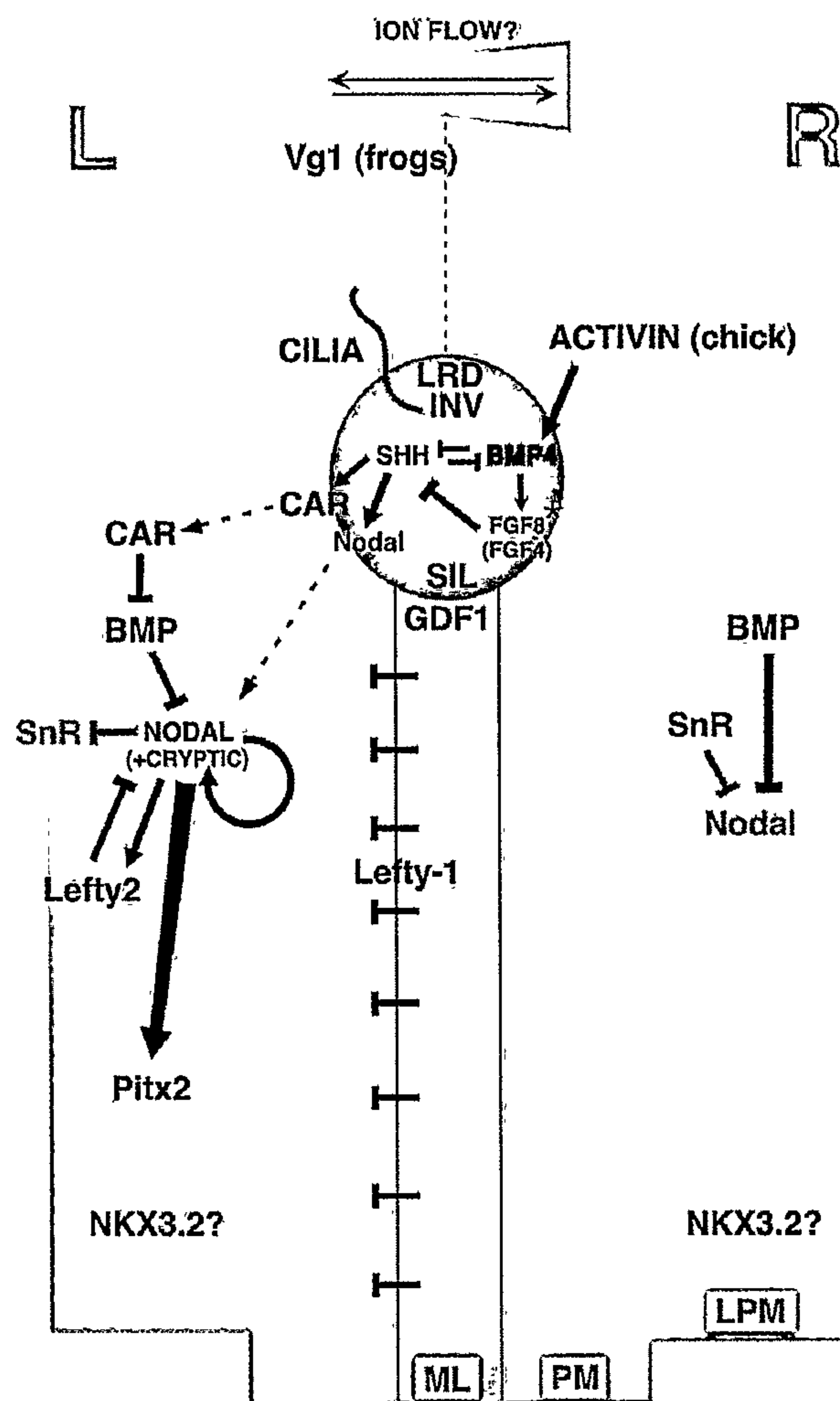


Figure 5. Diagrammatic Representation of Vertebrate L-R Axis Formation (Wright et al 2001).

Directional ion flow may produce a symmetry breaking L-R bias, stabilized through asymmetric gene expression. A left-right coordinator, Vg1, may be involved in frogs; right sided activin signaling occurs very early in chick embryos. Activin-like signaling may induce right-sided BMP4 expression, which engages in a mutually inhibitory relationship with SHH. Rightsided FGF8 inhibits SHH in chick. Caronte (CAR) has a putative transfer function in moving L cues from the node (darker disc) through paraxial mesoderm (PM), to lateral plate mesoderm (LPM), where it inhibits BMP4 extracellularly and derepresses Nodal expression. Nodal signaling induces Lefty-2, a feedback inhibitor. The Pitx2 homeobox gene is a component of the effector programs that direct asymmetric morphogenesis.

putative left-right coordinator, Vgl, (Hyatt and Yost, 1998).

1.4.2 *Gene expression asymmetries*

Beginning with data from chick embryos, the embryonic node has for some time held center stage in the L-R asymmetry field. In fact, the landmark discovery that left-sided SHH and *nodal* expression in the chick node is instructive for L-R axis specification (Levin et al., 1995) essentially led the way into molecular studies on this pathway.

Transplanted node tissue carries primary axis-inducing activity in all vertebrates tested. The mouse node, chick Hensen's node, the Spemann organizer in frogs and embryonic shield in zebrafish are all analogous structures based on gene expression criteria, and in all cases have primary embryonic axis-inducing activity when transplanted to other regions of the embryo. In the mouse (and very similarly in chick), the node is a bilaminar pit-like structure that forms at the anterior limit of the incipient primitive streak and from which the notochord extends forward. Elegant studies in chick embryos (Capdevila et al., 2000), however, show that the emergent node is initially L-R naive until the receipt of asymmetric influences from the surrounding blastodisc, including from quite distant regions (Levin and Mercola, 1999). Thereafter, the node seems to adopt the dominant role in dictating embryonic handedness and concurrently begins to display the asymmetric SHH-*nodal* expression pattern (Fig. 5). Monsoro-Burq showed that an additional determinant of the left sided SHH and right-sided FGF8 expression in the chick node is right-side expressed BMP4, which engages in a reciprocal inhibitory relationship with SHH (Monsoro-Burq et al, 2001).

More details arose on the mechanisms transferring L-R information from

the node to more lateral regions, adding to the reports that an asymmetrically expressed and diffusible BMP antagonist, Caronte, a Cerberus related protein, may be one intermediary (Capdevila et al., 2000) (Fig. 6). Based on new discoveries regarding the expression patterns of SHH response genes, it seems likely that asymmetric hedgehog signaling affects the ectoderm and mesoderm to different extents.

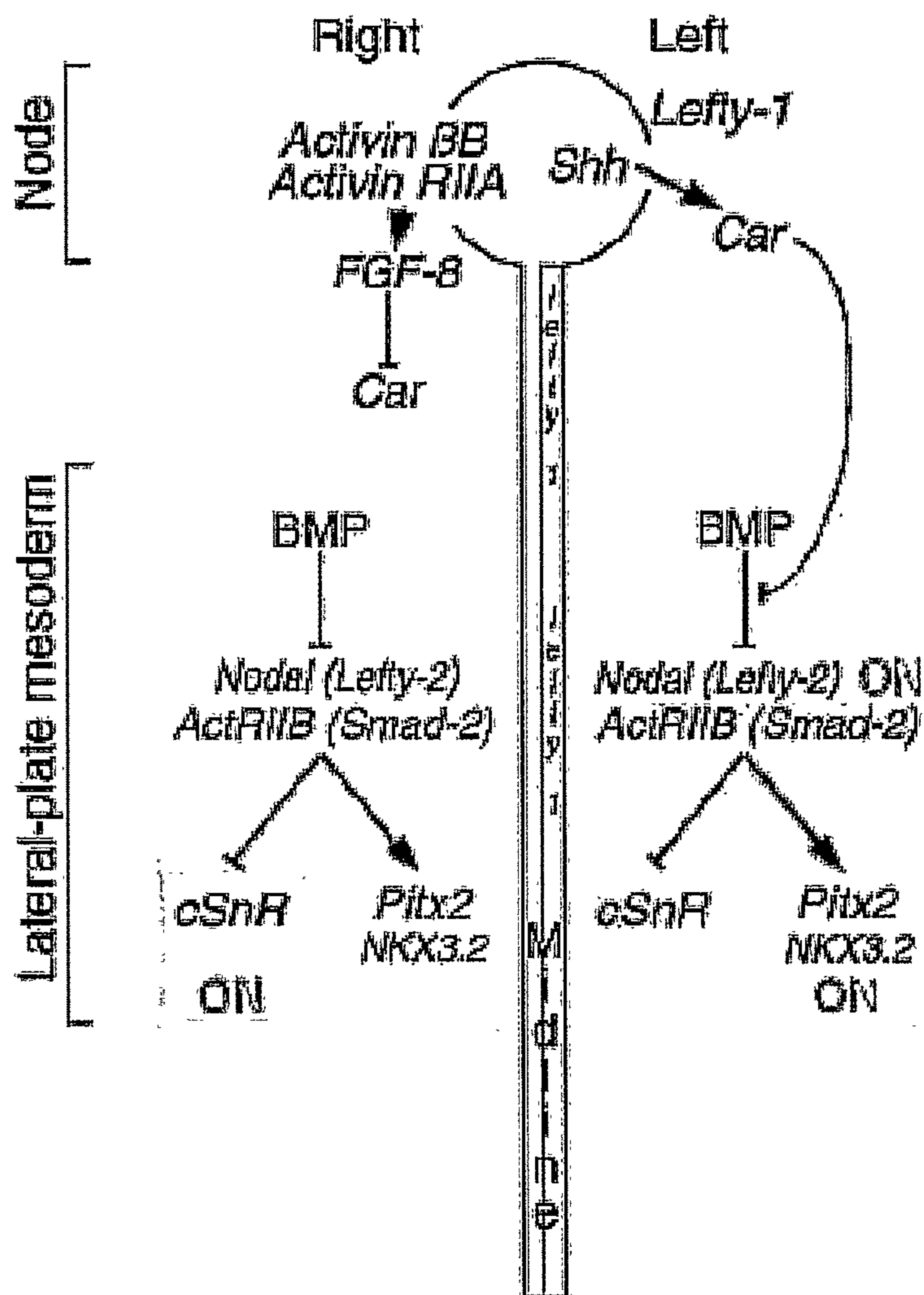


Figure 6. Role of Car in the genetic cascade that determines left-right development (Esteban et al 1999)

Control of Car expression by SHH and FGF8, together with a possible barrier mechanism elicited by Lefty-1, restricts Car activity to the left side of the embryo, where it can antagonize the repressive effect of BMPs on Nodal transcription. Nodal activates downstream left-specific genes and represses right-specific genes in the LPM. Green arrows indicate activation and red lines indicate repression; in the case of Car the red line indicates antagonism of BMPs by direct binding to them. This is a simplified model intended to illustrate the role of Car, and only some of the genes involved in left-right development are included.

1.4.3 Nodal flow

A “nodal flow model” for either the generation or transfer of L-R information within the embryo has recently gained favor, at least in mouse. In this model, the concerted unidirectional rotation of monocilia that are found on each cell of the ventral surface of the node acts to waft a left-specific morphogen across the node, enriching it on the left side. The inactivation of the *kif3A* or *kif3B* (heterotrimeric kinesin) motor protein genes leads to absent cilia and associated L-R defects, supporting the model (Capdevila et al., 2000). Furthermore, the rotation is perturbed in *iv* and *inv* mutant embryos, as discussed below. While the nodal flow model certainly has its attractions, much discussion centered on two items: first, the need for directly showing that this flow system can directionally move morphogen-sized molecules in addition the latex microbeads used so far; and second, whether the putative morphogen-wafting process represents a symmetry-breaking event, or propagates an earlier asymmetry. A compelling aspect of the former possibility is the potential direct link from molecular chirality, which determines the direction of ciliary rotation, to generating a small bias that eventually leads to L-R asymmetric gene expression.

The model also seems ripe for physical and theoretical investigation using the principles of hydrodynamics. An upright rotating cilium, which defines an “upturned cone” envelope, seems unlikely per se to generate unidirectional flow. It should be possible to determine whether the characteristic anatomy of the node and the rotational characteristics of the monocilia, in combination with patterned apical-basal and planar polarity of the ventral node cells, could synergistically result in unidirectional flow. Most importantly, however, it will be fascinating to fit these characteristics with future data on the actual L-R morphogen, whose

secretion and cell surface association properties may determine whether it enters the region of maximum nodal flow, which is presumably located at the distal ends of the cilia.

1.4.4 *Inversin and Left-Right Dynein*

Before knowledge of the Shh-nodal asymmetry in chick, the best hope for significant inroads into the L-R problem was pinned on studies of the genes represented by two classical mouse mutations, *iv* (*inversus viscerum*) and *inv* (*inversion of turning*). Homozygous *iv/iv* mice are essentially a 50:50 split between normal and situs inversus, but *inv/inv* mice are qualitatively different: almost all embryos show reversed situs. *iv* is now known to represent a mutation in the axonemal dynein-related gene, Left-Right Dynein (LRD), while *inv* corresponds to Inversin, a large ankyrin repeat-containing protein whose primary sequence does not provide obvious clues to its function. The nodal cilia are immotile in *lrd/iv* mutant embryos, while the cilia in *inv* mutants, although they appear at current levels of resolution to exhibit normal motility, can for some reason only drive a slow rate of flow. Proposals for how the randomized asymmetry in *iv/lrd* mutants is associated with bidirectional diffusion of the left-specifying morphogen(s), and how slow nodal flow may lead to the reproducible situs inversus of *inv* mutants, have been published previously (Okada et al., 1999).

The biochemical and cell biological function of LRD and Inversin is being investigated. Likely LRD and Inversin homologs have been isolated in several species and in some cases display intriguing expression patterns in established organizer tissues, which is therefore similar to mouse *lrd* and suggestive of a localized function in L-R determination. It is unknown if inversin or LRD are well conserved in animals below the vertebrates. If they can be found, it is possible that studies in simpler organisms will give clues to the basic functions of these proteins. While the radial symmetry of jellyfish precludes studies of

relevance to L-R asymmetry, the experimental subjugation of what may now be its most famous gene, GFP, has set the stage for future quantum leap advances in the study of Inversin and LRD. The goal is to follow functional GFP chimeras of these proteins in living embryos, real time, and at high resolution, to determine their subcellular and organellar localization. GFP in transgenic mouse embryos is broadly distributed but apparently assembled into cilia and centrosomes. Preliminary experiments include subcellular colocalization and biochemical tests of a direct physical LRD/Inv interaction based on their possible colocalization in transfected cultured cells. A definite advantage here is that an LRDGFP fusion protein seems to function normally in mouse embryos, which may enable the monitoring of LRD assembly into the cilia or other structures.

1.4.5 The Nodal Signaling Cassette

A recurring concept in embryogenesis is the genomic economy afforded by the reiterated use of gene regulatory cassettes, with the cellular response within the signal-receiving cells varying according to their developmental history. The Nodal signaling pathway is a case in point. The TGF related Nodal ligand binds to activin- like receptors with the aid of an EGF-CFC cofactor such as Cripto/OEP, which initiates a signal transduction cascade involving Smad2 phosphorylation and transcriptional regulation via the FAST winged helix protein. The *Nodal* gene can autoregulate itself, and one of its direct response genes, *lefty/antivin*, acts as a negative feed-back regulator by competitive inhibition at the receptor and interrupts the Nodal autoregulatory loop.

A down-stream target gene of Nodal signaling is the *Pitx2* homo-box gene. The Nodal signaling cassette is required for mesendoderm induction (Yeo and Whitman, 2001) and is also activated during L-R specification processes (Fig. 5), not only in the lateral plate mesoderm (LPM) but also in the brain. Moreover, in contrast to the uncertainty over the extent of conservation of early steps of the L-R pathway, there is much evidence suggesting that the expression and function of the Nodal cassette is constant in all vertebrates studied so far. That left-sided *nodal* expression is essential for visceral asymmetry in the mouse had been suggested previously via inactivation of its cofactor, Cryptic (Yan et al., 1999), but has now been more directly demonstrated by analyzing hypomorphic *nodal* alleles that, unlike the null condition, allow gastrulation to proceed and the effects on L-R asymmetry to be assessed (Lowe et al., 2001).

Regulatory sequence deletion experiments in the mouse confirm the role

of *Lefty-2* as essential to limit the intensity and duration of Nodal signaling in the left LPM. Removal of a Nodal/FAST dependent asymmetric enhancer element (ASE) from *Lefty-2*, preventing its left-side LPM expression but not its earlier pattern: this causes increased and sustained Nodal expression in the lateral tissues and associated *situs* defects (Meno et al., 2001). An interesting corollary from this experiment was the activation of Nodal-responsive genes far away from Nodal-expressing cells, in particular in contralateral tissues in the posterior regions of the embryo. Together with data on Nodal signaling in zebrafish (Chen and Schier, 2001), these findings challenge previous notions that Nodal-related signals do not act at long range. But, furthermore, it raises the possibility that Nodal itself could be the left-specifying morphogen in the nodal flow model, or participates directly in transferring asymmetric L-R signals from a node/midline source upward to the left LPM.

This idea may explain the almost simultaneous activation of *Lefty-2* and *Nodal* expression in the LPM (*Lefty-2* induction via LPM-derived Nodal signals would be expected to produce a temporal delay). Again, the technical breakthrough of GFP tagging may be an important key to several of these issues. Similar to the finding that DPPGFP rescues *Drosophila decapentaplegic* mutant phenotypes, types, inductive properties are preserved in a NodalGFP fusion protein, and this may allow the unraveling of the details of ligand production and processing, as well as secretion and transfer, e.g., by diffusion versus transcytosis.

Once an asymmetric signal is activated, while it must spread rapidly to the appropriate tissues during their period of L-R competence, its intensity and

duration must then be limited in order to allow the effector programs to work properly. The hypothesis that the self-activation loop of the Nodal inducer and its negative feedback inhibitor Lefty (Figure 5) resembles an inducer/inhibitor reaction-diffusion system that serves to propagate and then inactivate the Nodal signal as a trigger of L-R asymmetric development. This Nodal-Lefty relationship is conserved in the vertebrates, and provides a very satisfying link back to the proposal that Turing-style reaction-diffusion would be involved in L-R axis formation. It will be interesting to determine if the diffusion / transport characteristics of Nodal and Lefty fulfill a central tenet of classic reaction-diffusion models: that the inhibitor travels faster than the inducer.

Preliminary data is available on how the availability of TGF like signaling molecules (and maybe others) might be regulated by interactions with specific components of the extracellular matrix, in this case the proteoglycan Syndecan (Yost 1995). This work is beginning to be linked to other experimental evidence that L-R information is carried or modulated by regions of the embryo other than the presumptive axial tissues and mesoderm: in the case of the frog embryo, the ectoderm across which the mesoderm crawls during gastrulation.

Based on the maintenance of its asymmetric expression in organ primordia as they undergo asymmetric morphogenesis, *Pitx2* has been proposed as one of the main transcriptional effectors of differential L-R morphogenesis (Fig. 5-6). A clue that equally important genes are also involved comes from the finding that *Pitx2* mutants have fewer defects than expected if it was the sole effector. Conversely, Nodal may not be the only upstream activator of *Pitx2* or any additional effector genes. Some aspects of the spatiotemporal expression pattern

of *Pitx2* are inconsistent with its activation via Nodal, and complete abrogation of Nodal responsiveness in the zebrafish diencephalon (by removing the Nodal signals Cyclops and Squint, or the obligate cofactor Oep) does not fully block *Pitx2* expression.

Further evidence that the pathway is more complex than currently drawn out included the observation that asymmetric *Pitx2* expression can be uncoupled from the direction of heart looping in chick embryos. Undoubtedly, however, *Pitx2* is still a crucial player. Elaborate studies involving a “designer series” of mutant *Pitx2* alleles in the mouse are leading to the suggestion that the generation of L-R asymmetry in the lungs requires the highest *Pitx2* levels, with low levels being sufficient for normal heart looping.

1.5 SHH

The first defined asymmetrical signal in the L-R patterning pathway is SHH, which is initially expressed throughout the node but becomes restricted to the left side (Nusslein-Volhard et al. 1980; Echelard et al. 1993; Levin et al. 1995; Pagan-Westphal et al. 1998). SHH is essential for morphogenesis of the frontonasal and maxillary processes, which give rise to the mid and upper face. SHH induces polarized gene activities (left specific genes *nodal*, *lefty1*, and *lefty2*) in the left paraxial mesoderm (LPM) (Levin 1997; Logan et al. 1998; Meno et al. 1998; Monsoro-Burq et al. 2001). *Nodal* expression induces expression of *Pitx2*, a transcription factor thought to directly specify left identity during organ development (Ryan et al. 1998). The stabilization of asymmetry through its induction of unilateral gene expression patterns and the L-R information, is then transmitted either globally or organ specifically to the organ precursors where effector programs lead to differential morphogenesis (Fig. 5-6).

1.6 FGF8

FGF8 has been found in different areas involved in outgrowth, patterning and axis formation during embryogenesis (Heikinheimo et al. 1994). FGF8 expression pattern shows a medial-lateral progression, starting with the node and/or neural structures and notochord, moving to the intermediate mesoderm and finally reaching a maximum in the LPM. In limb development FGF8 signaling from the apical ectodermal ridge (AER) has been found to be essential for the initiation, outgrowth and patterning of the limb (Lewandoski et al. 2000). FGF8 has opposite roles in the specification of L-R identity in chick and mouse (Fig. 7). In chick, FGF8 is expressed asymmetrically on the right side, posterior to the node (Crossley et al. 1995). FGF8 transcription is induced by activin and the FGF8 protein inhibits the expression of nodal and pitx2 (Boettger et al. 1999; Rodriguez Esteban et al. 1999). FGF8 is one growth factor in the FGF family (Table 1).

An activin like signaling pathway has been shown to act upstream of SHH and nodal in the chick node. Activin signaling leads to the restriction of SHH expression to the left of the node and induces FGF8 on the right side of the node. SHH then activates nodal lateral to the node, whereas FGF8 acts to inhibit nodal expression on the right (Levin et al. 1995; Boettger et al. 1999). In mouse, FGF8 is required for the left asymmetric expression of nodal, lefty2, and pitx2.

Right sided inhibition of FGF8 signaling induces bilateral marker gene expression, demonstrating that in rabbits FGF8 acts as a right determinant like in chick (Fischer et al. 2002). Thus, FGF8 could act on the mesoderm by

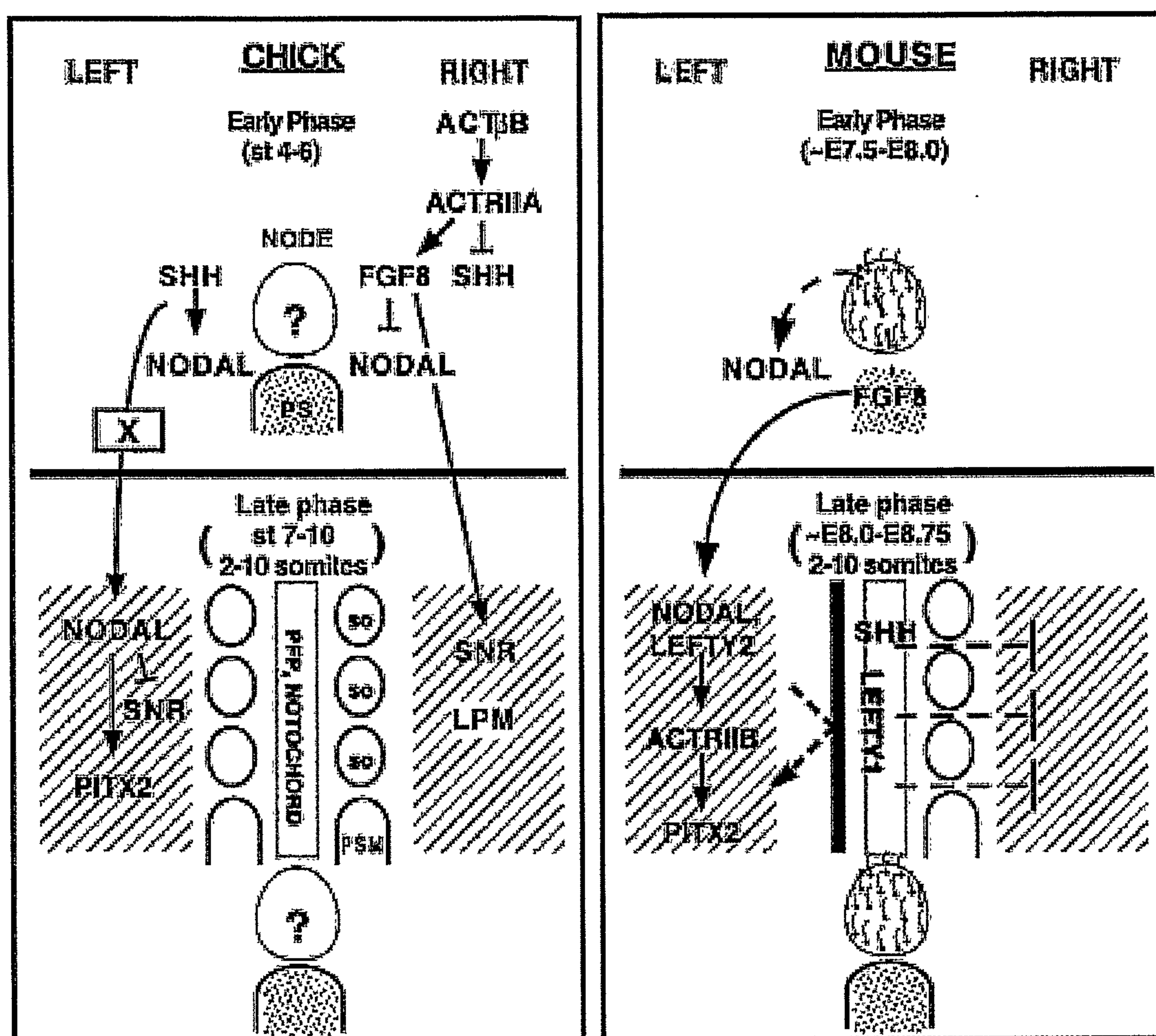


Figure 7. Comparison of LR asymmetry pathways in mouse and chick. (Meyers et al 1999)

The solid horizontal lines separate the pathways into two phases. FGF8 is expressed to the right of the chick node and functions to inhibit Nodal expression on the right. In contrast, in the mouse FGF8 is required for Nodal expression around the node during the early phase, and in the left LPM during the late phase. The dashed curved arrow indicates that FGF8 may be distributed to the left by the motion of the cilia in the node. Dashed symbols (- - -) indicate the possible production by midline structures of an active repressor of left determinants on the right. In the mouse SHH is not required to induce Nodal expression, but is required to provide midline barrier or repressor functions (or both) that prevent the expression of left determinants on the right. Abbreviations: PFP, prospective floor plate; PS, primitive streak; PSM, presomitic mesoderm; so, somites; X, a molecule required for induction of Nodal expression by SHH.

Table 1. Fibroblast Growth Factors (FGFs) 1-19

FGF	Gene or Protein	Chromosomal	Amino Acid	High Affinity	Signal	Associated
	Pseudonyms	Map Location	Length	Receptors	Sequence	Functions endothelial cell migration and proliferation, angiogenesis
FGF1	Acidic Fibroblast Growth Factor	5q31-33	155	FGFR1-4	no	
FGF2	Basic Fibroblast Growth Factor	4q25	155, 196, 201, 210	FGFR1-2	no	angiogenesis
FGF3	Oncogene int2	11q13	239, 271		yes	mouse mammary carcinoma
FGF4	Oncogene hst1	11q13	176	FGFR1-2	yes	human stomach cancer
FGF5	Oncogene fibroblast growth factor 5	4q21	267		yes	inhibit hair elongation
FGF6	Oncogene hst2	12p13	198		yes	oncogene
FGF7	Keratinocyte Growth Factor (KGF)		162	FGFR2b	yes	epidermal growth and wound healing
FGF8	Androgen-Induced Growth Factor (AIGF)		193, 246	FGFR2	yes	androgen dependant , limb induction, L-R
FGF9	Glial Activating Factor (GAF)		208		internal?	glial cell proliferation
FGF10	FGFR10		215		yes?	?
FGF11	FGF Homologous Factors (FHF) 1	3	244		no	nervous system development and function
FGF12	FHF 2	10	245		no	nervous system development and function
FGF13	FHF 3	17	225		no	nervous system development and function
FGF14	FHF 4	13	247		no	nervous system development and function
FGF15	FGF15		218		yes	nervous system development and function
FGF16	FGF16		207		no	development of brown adipose tissue, heart
FGF17	FGF17		216		yes	induction and patterning of embryonic brain
FGF18	FGF18		207		yes	lung development
FGF19	FGF19		216		yes	brain development

ectopically inducing some genes, which in turn, directly or indirectly, would regulate polarizing potential in both limb and flank mesoderm.

1.7 Chirality Resolution During Palatal Fusion

Embryonic midline tissues include the prechordal plate mesoderm anteriorly, the notochord and the spinal floor plate in the trunk. The palate is also a midline structure, with both secondary palatal shelves fusing in the midline. While morphogenetic events occurring around the midline require resolution of the chirality initiated at the stage of the primitive streak, the fusion of the palatal processes could be considered as the enforced midline union of two processes which have become highly polar, that is which have a high degree of handedness.

It is noteworthy that the midline fusion of the palatal processes, which are clearly the products of chiral morphogenesis, is related to the expression of specific ligands strongly implicated in the generation of chirality. Paradoxically, both the generation and resolution of chirality appear to employ overlapping mechanisms at least for this developmental sequence.

Studies in *xenopus* and mice have demonstrated that midline structures, including the notochord and the floor palate, are required for normal L-R identity and that the early response gene *SIL* blocks midline signaling (Izraeli et al. 1999). Studies have suggested apoptosis along the primitive streak midline as a novel mechanism involved in the regulation of L-R asymmetry during early embryogenesis (Kelly et al. 2002). FGF8 knockout embryos die during gastrulation, preventing the analysis of laterality defects in FGF8 null embryos (Sun et al. 1999). A conditional mouse knockout FGF8 displays a loss of nodal and *pitx2* expression, implicating FGF8 as a left determinant (Meyers et al.

1999). Interestingly, SHH mutant mice have also been found to have midline defects (Chiang et al. 1996).

1.8 Clinical Implications of Chirality Malfunction

Transient loss of SHH signaling in the embryonic face inhibits growth of the primordial and results in defects analogous to hypotelorism and cleft lip/palate, characteristics of the mild forms of holoprosencephaly. In contrast, excess SHH lead to a mediolateral widening of the frontonasal process and a widening between the eyes, a condition known as hypertelorism (Wall et al. 1995; Hu et al. 1999). Complications with midline and laterality identities lead to numerous congenital defects, such as cyclopia, situs inversus, and heteritaxia. L-R axis defects in humans occur at a frequency of 1 in 8500 live births, but account for many more deaths in utero (Brown et al. 1990; Casey 2001; Cohen 2001). Mutational analysis of the SHH gene in newborns with oral clefts found silent polymorphisms (Orioli et al. 2002).

Chromosomal 22 microdeletion (del22q11) syndrome presents with congenital cardiovascular defects and craniofacial anomalies, which include hypertelorism and cleft palates. It is the most common microdeletion syndrome occurring in an estimated 1 in 4000 live births and encompasses both DiGeorge and Velocardiofacial syndromes (Greenberg et al. 1984; Scambler et al. 1992; Lindsay 2001).

1.9 Aims of Study

1.9.1 General aims of this study

The aims of this study are to gain further insight into the molecular mechanisms determining left-right sidedness (chirality) and the suppression of sidedness (resolution of chirality) during fusion of the secondary palate in the embryonic rat.

1.9.2 Specific aims of this study

1. To investigate whether FGF8 and SHH are expressed during rat embryonic palatal development.
2. To define the expression profiles of FGF8 and SHH in the MEE and MES during rat midline palatal fusion.
3. To investigate the possible roles of FGF8 and SHH in the resolution of chirality during the palatal fusion process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

2.1.1 *Harvesting of embryos*

Time mated Sprague-Dawley female rats were obtained by placing 2 male rats into cages containing 4 female rats in the evening, and taking the males out 12 hours later on the following morning. Gestation times were determined from the morning following removal of the male rat as being half a dpc. The impregnated rats were given food and water *ad libitum*. The uterine horns of pregnant dams timed to be from 14.5 to 17.5 dpc were surgically removed under anesthesia and the embryos removed under sterile conditions. The embryos were removed from the uterine horn by transecting the uterus between each of the dilated regions containing an embryo and then separated the amnionic sac while immersed in the sterile cold PBS. The embryos were decapitated and the stage in the fusion process determined by inspection of the palates under a dissecting microscope (Gibbins et al. 1999; Gibbins et al. 2000).

2.1.2 Variation in development of individual embryos

The size of embryos increased dramatically over the 3 day period of collection (Fig. 8). On average, females 80 to 110 days old carried 12 to 15 embryos, and at 14.5 dpc the sizes of individual embryos carried by any one pregnant dam varied considerably, presumably reflecting differences in the time of implantation of the blastocyst being magnified by the rapid rate of size increase at this stage (Fig. 9).

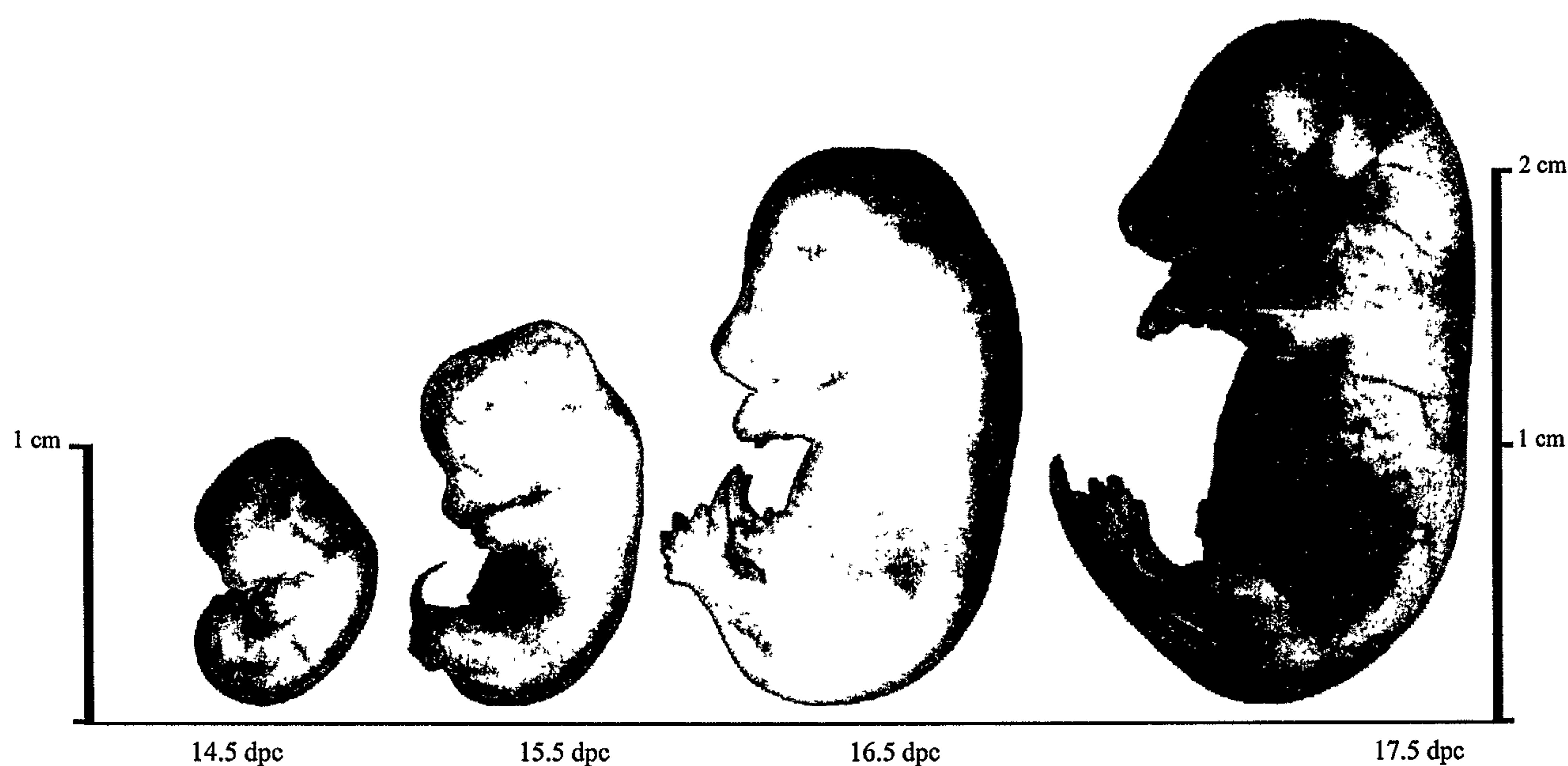


Figure 8. The developmental stages of Sprague-Dawley rat embryos.

At 14.5 dpc, the longest length of the embryo is 1 cm, and the developing hindbrain is almost translucent. The developing snout becomes more prominent in 15.5 dpc embryos, and by 16.5 dpc the embryo has doubled in length relative to 14.5 dpc. The intestine which develop externally is internalised by 17.5 dpc, and by this stage the secondary palate has almost completely fused.

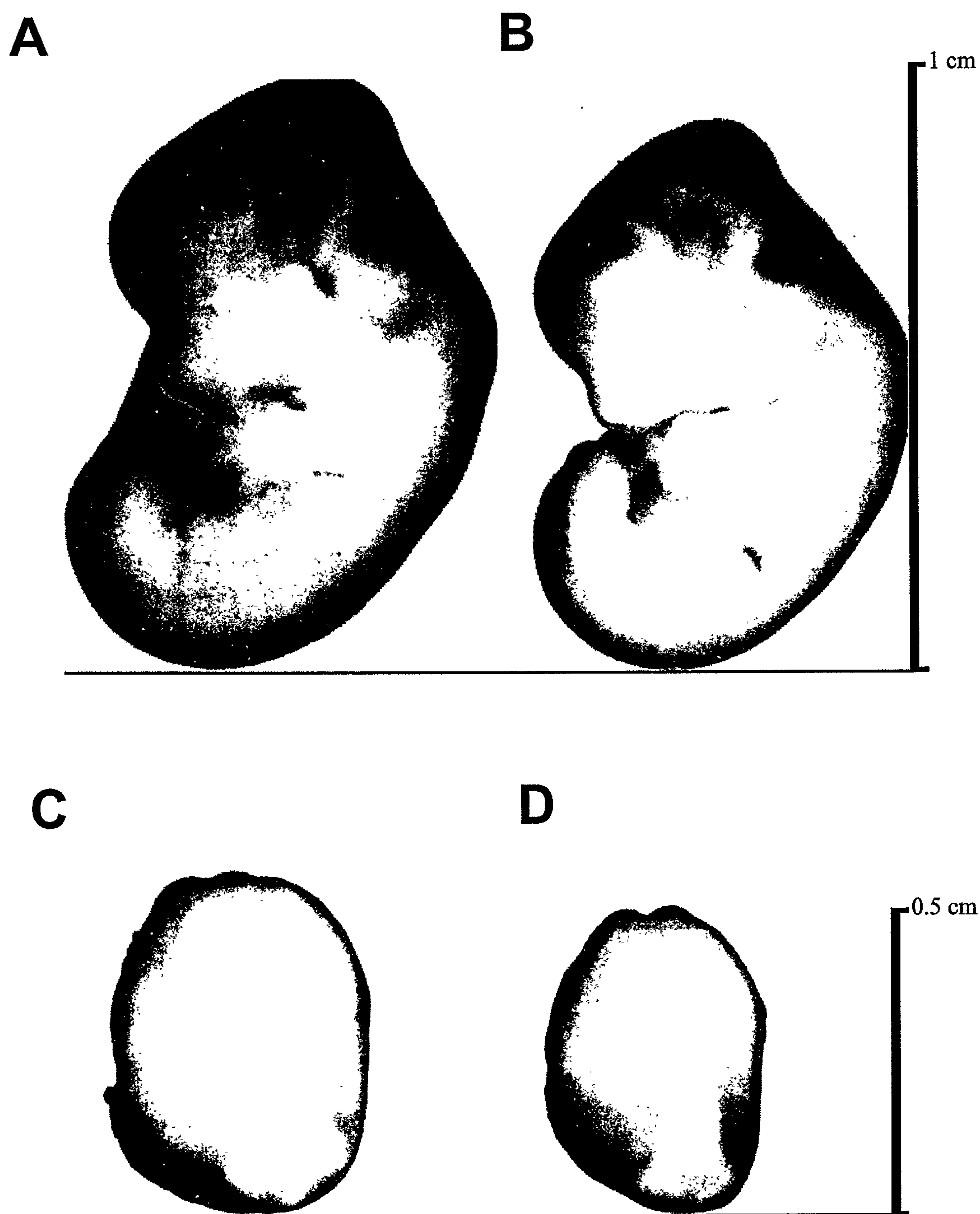


Figure 9. Size variation in 14.5 dpc embryos taken from the one pregnant female.

(A) is larger in size and more developed than (B).

(C) and (D) are superior view of the heads of (A) and (B) respectively.

2.2 Fixation and Tissue Processing

2.2.1 *Paraffin embedded tissues*

Timed rodent embryos prior to, during and immediately following palatal fusion (14.5 to 17.5 dpc) were removed, fixed in 4% paraformaldehyde / PBS for a minimum of 6 hours and washed three times in 70% ethanol. Tissues were placed in tissue processing cassettes and processed in an Vacuum Infiltration Processor Model 4618 (Miles Scientific), going through the following changes: 40 minutes each in 50% ethanol, 70% ethanol, 80% ethanol, then 2x 60 minutes each in 95% ethanol, and 100% ethanol, followed by 2x 90 minutes in Xylene then taken through 2x 60 minutes of paraffin followed by a further 90 minutes in paraffin before mounting and orientating the specimens in cassettes. Paraffin embedded tissues were sectioned at 5 μ m then sections were dewaxed and rehydrated (Gibbins et al. 1999; Gibbins et al. 2000).

2.3 Immunohistochemistry

A total of 12 embryos, consisting of 3 embryos from each time sequence (14.5, 15.5, 16.5 and 17.5 dpc) were utilized in this study. Sections at different time stages were blocked in 20% normal horse serum in PBS for 1 hour at RT, then reacted for 1 hour at room temperature with either a 1:50 dilution of goat polyclonal anti-FGF8 or anti-SHH antibody (Santa Cruz Biotechnology SC-6958 & SC-1194) in 10% FCS in PBS. After three 10 minutes washes in PBS, the sections were reacted with 1:100 dilution of rabbit antigoat horseradish peroxidase antibody in 10% FCS in PBS at room temperature for one hour. The slides were then washed with three 10 minutes washes in PBS. The specific binding of the antibodies were visualized with 3' dimaminobenzidine tetrahydrochloride (DAB) causing a colored precipitate to form on the slide at the location of the antigen.

2.4 Controls

Controls for the experiments were run in parallel with goat anti-mouse (GAM-b) polyclonal antibody and both FGF8 and SHH blocking peptides (Santa Cruz Biotechnology SC-6958P and SC-1194P) (Refer to Appendix).

CHAPTER 3

RESULTS

Results

3.1 FGF8 and SHH are not detected when the palatal processes are lateral to the tongue (14.5 to 15.5 dpc).

At 15 dpc, the developing tongue fills both the presumptive nasal and the oral cavities. The tissues that will form the secondary palate can be recognized as tapering plates occupying the space between the lateral surface of the tongue and the buccal mucosa, with the epithelium on the respective surfaces in close contact. FGF8 and SHH expression were not detected in the epithelium on the palatal processes, in the regions that would become the edges of the palatal shelves following rotation (Fig. 10), at this stage (14.5 to 15.5 dpc) when the palatal processes are lateral to the tongue. None of the epithelial cells covering palatal shelves in this stage showed evidence of FGF8 or SHH.

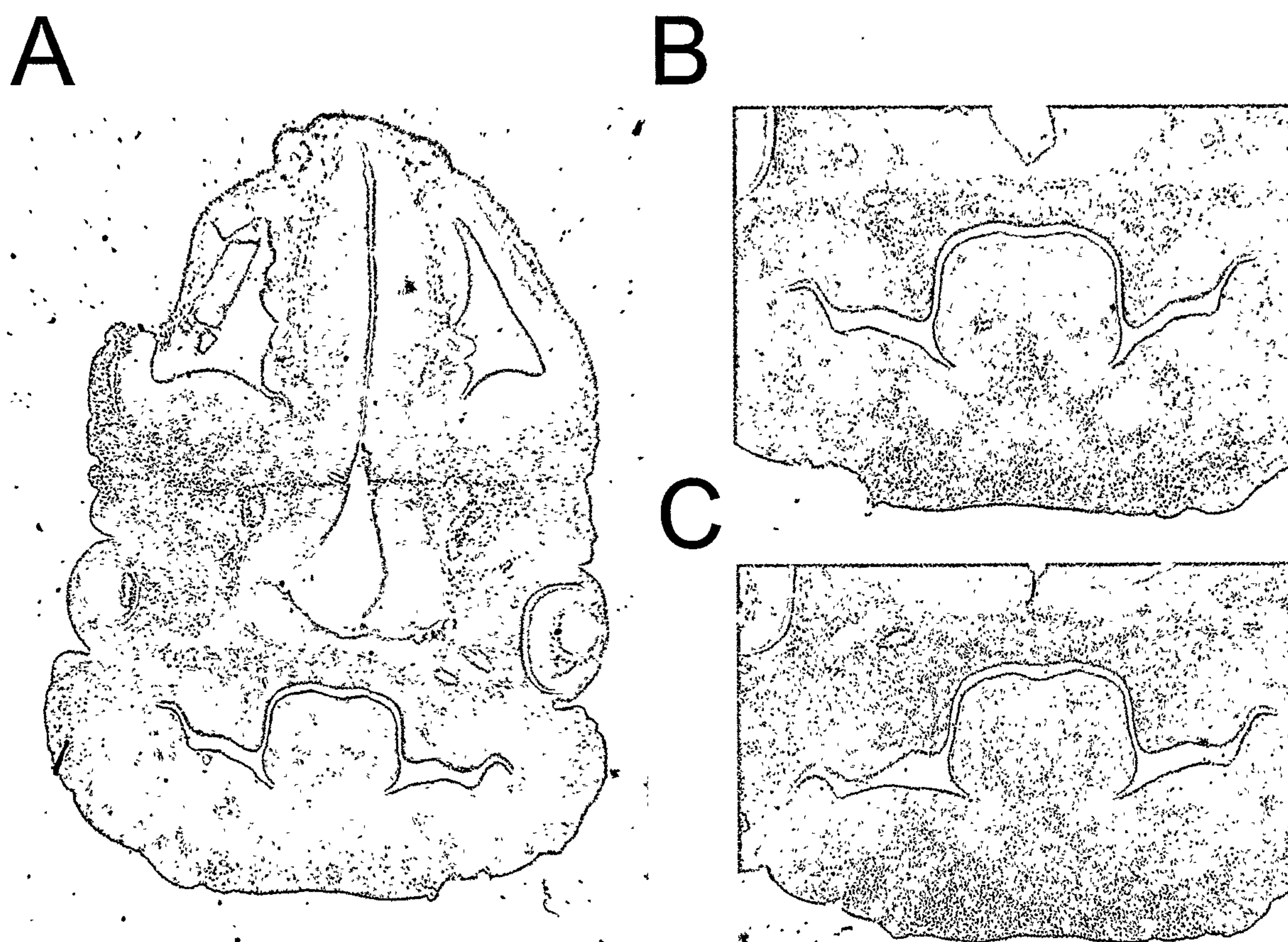


Figure 10. FGF8 and SHH were not detected in the palatal processes at 14.5 to 15.5 dpc.

A) A section of the whole embryo revealing no expression of FGF8 in the palatal processes. B) At low magnification, FGF8 is not detected in the palatal shelves when the palatal processes are lateral to the tongue. C) Similarly, SHH is not detected in the palatal shelves at this stage.

3.2 Asymmetrical FGF8 expression in the MEE of the right palatal process, immediately after elevation and rotation to a position above the tongue (16 dpc).

During the period from 15.5 to 16.5 dpc the tips of the palatal shelves move from lying lateral to the tongue to occupying a position directly opposite but separate from each other and rostral to the dorsal surface of the tongue. Asymmetrical expression of FGF8 was detected initially in the epithelium on the palatal processes in the regions that would become the edges of the palatal shelves, localized to the medial edge epithelium (MEE) of only the right palatal shelf (Fig. 11), immediately after elevation and rotation to a position above the tongue (16 dpc). FGF8 was evident initially in the simple epithelium on the presumptive tip of the right palatal shelf (MEE) and ceased abruptly in the region where the epithelium became columnar (Fig. 11 B&C). This was detected only in the pharyngeal region of the palatal shelves. All other structures, including the dorsal surface of the tongue did not reveal FGF8. SHH was not detected at this stage immediately after rotation and elevation of the palatal shelves to a position above the tongue (16 dpc) (Fig 12). Controls which included biotin conjugated goat anti-mouse (GAM-b) polyclonal antibody and blocking peptides, confirmed the specificity of staining (Fig. 2 D & E).

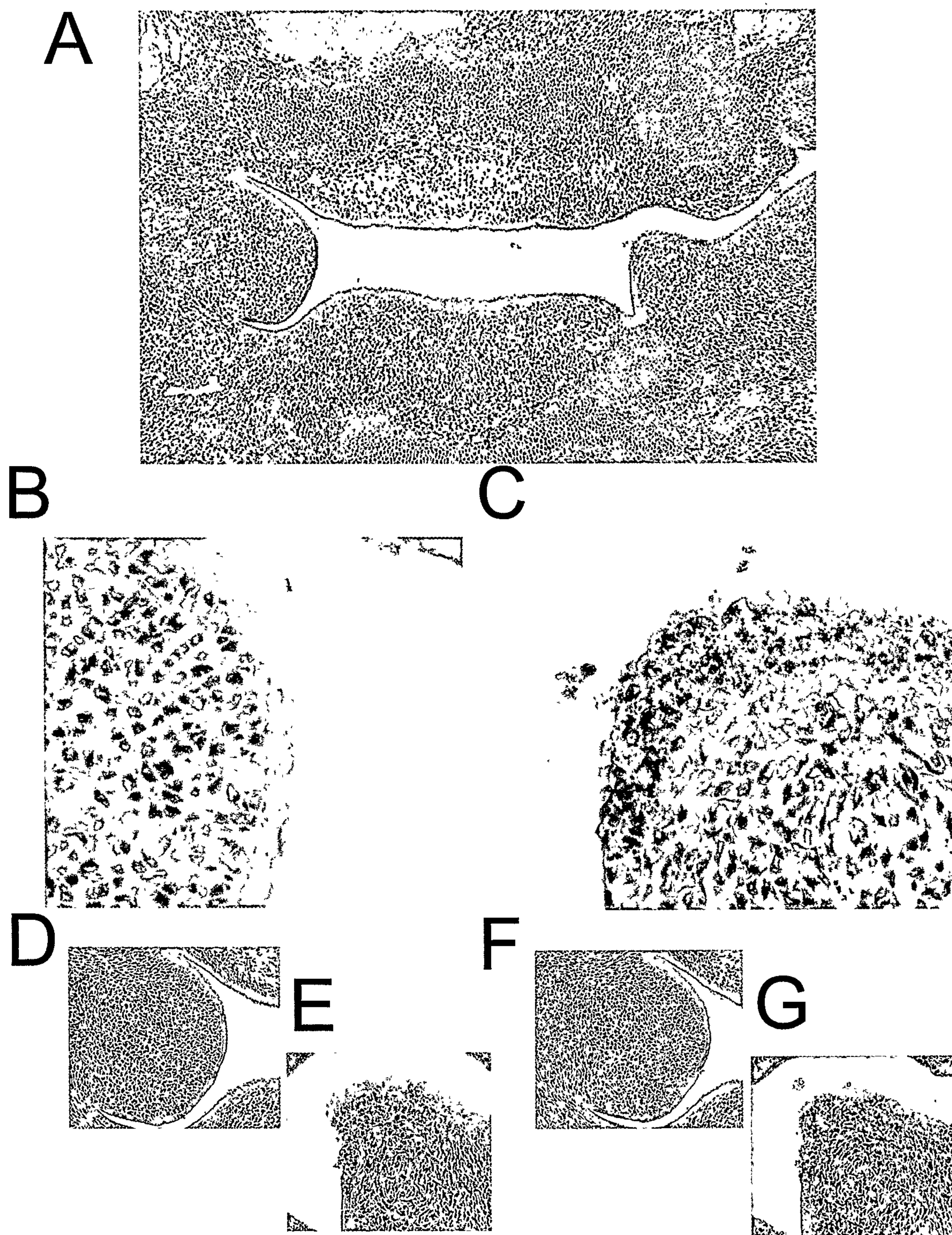


Figure 11. FGF8 detected in the MEE of the right palatal shelf at 16.0 dpc.

A) Section at low magnification, revealing asymmetrical FGF8 expression. B) At higher magnification, FGF8 was not detected in the MEE of the left palatal process. C) At higher magnification, FGF8 was detected in the MEE of the right palatal process, immediately after elevation and rotation to a position above the tongue. D&E) Control of primary antibody pre-incubated with FGF8 blocking peptide. E&F) Similarly, control GAM-b did not reveal any FGF8.

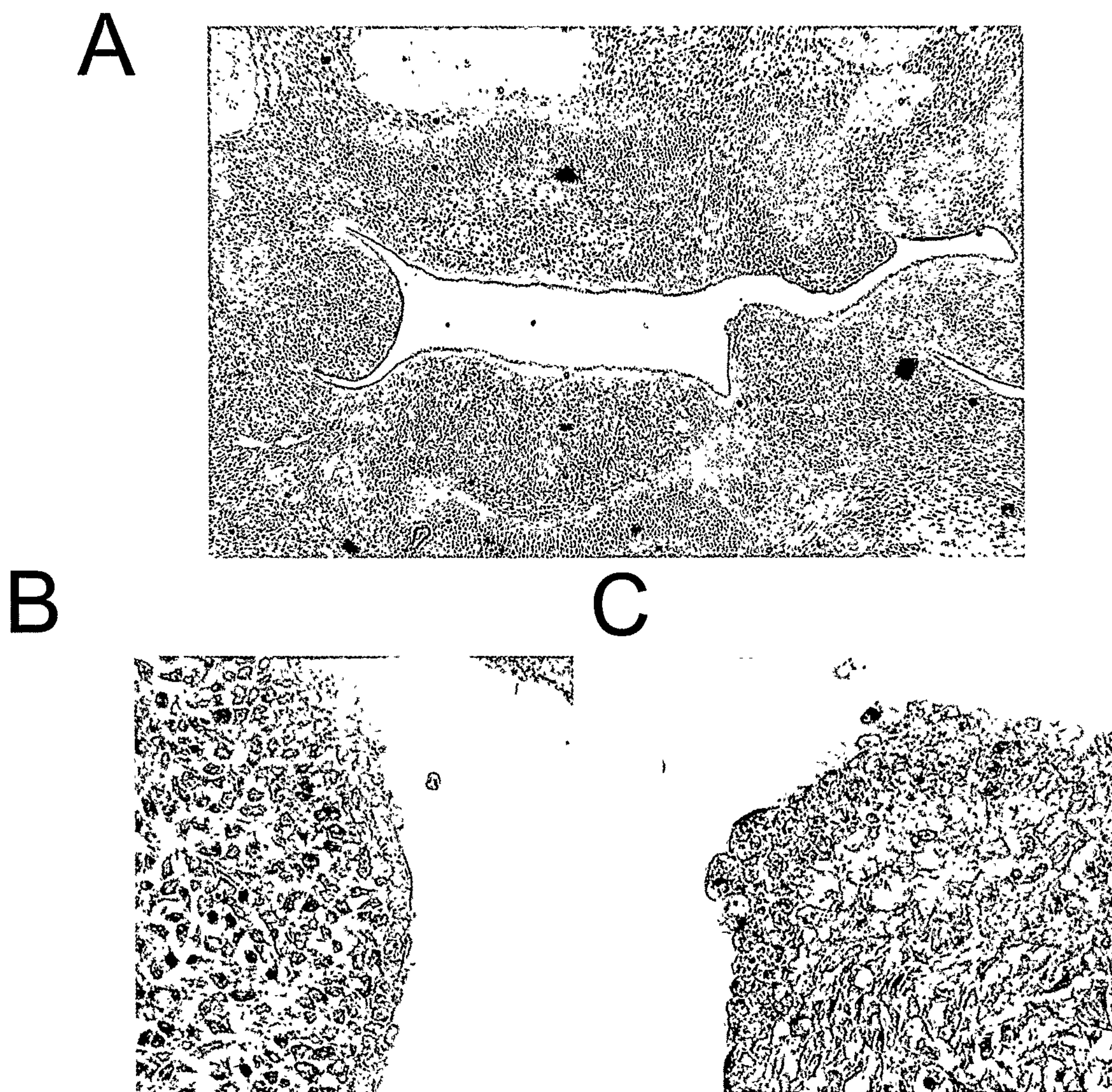


Figure 12. SHH was not detected in the MEE at 16.0 dpc.

A) Section at low magnification, revealing no SHH expression in the MEE of both the left and right palatal processes, immediately after elevation and rotation to a position above the tongue. B) At higher magnification, SHH is not detected in the MEE of the left palatal process. C) Similarly, at higher magnification SHH is not detected in the MEE of the right palatal process.

3.3 FGF8 is expressed bilaterally in the MEE prior to palatal fusion

FGF8 was detected in both left and right MEE prior to the contact of the tips of the palatal shelves in the midline (Fig 13). This FGF8 expression was restricted only to some specific cells in the MEE. FGF8 was not detected in the epithelial cells in the oral and nasal regions. Similarly, FGF8 was not detected in the mesenchymal cells of both left and right palatal processes.

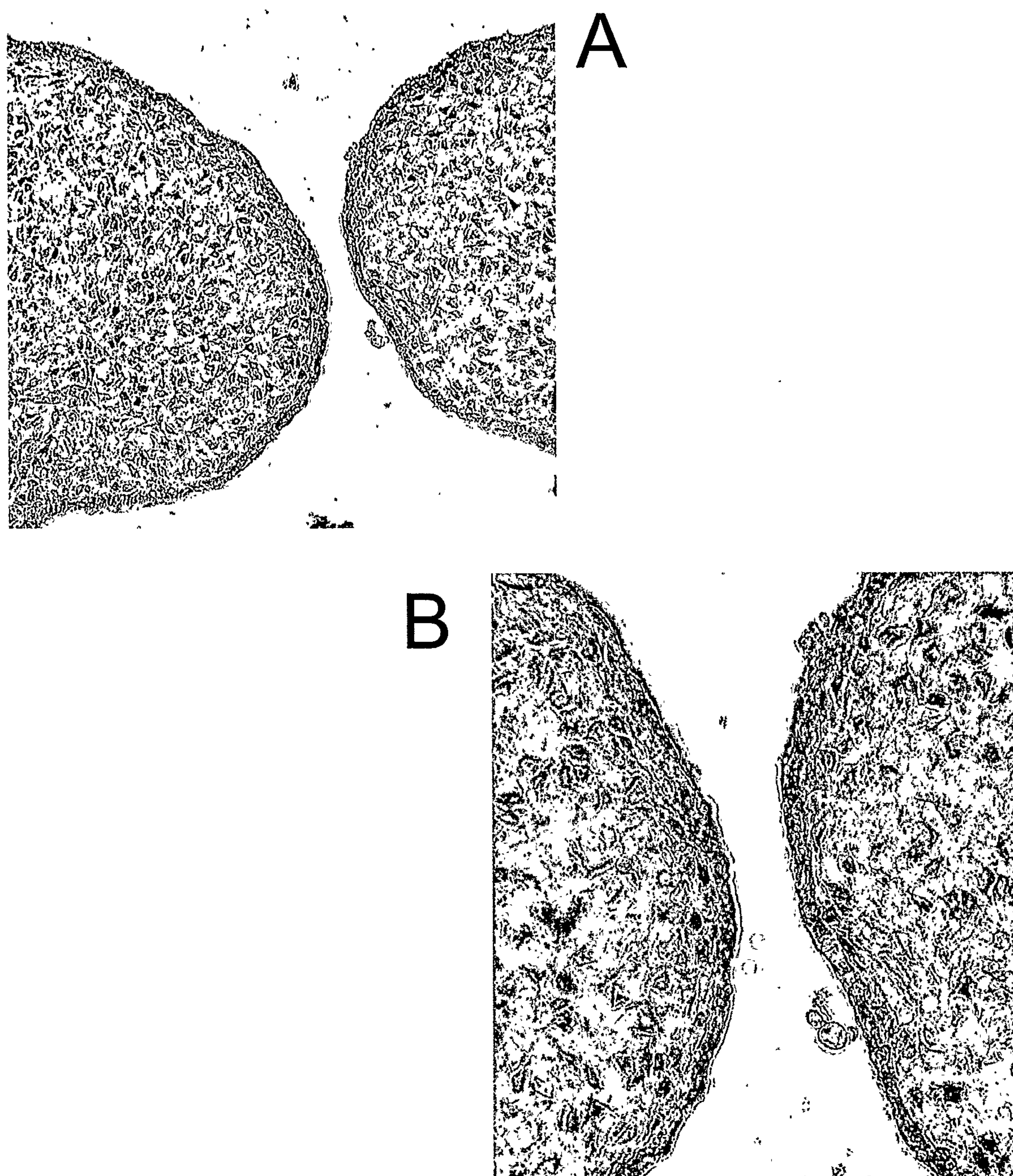


Figure 13. FGF8 was detected bilaterally in MEE prior to palatal contact.

A) Section at low magnification, revealing bilateral FGF8 expression in the MEE of both left and right palatal processes. B) Higher magnification reveals specific bilateral FGF8 detection in MEE cells. FGF8 was only detected in the cells of the medial edge of the palatal processes. FGF8 was not detected in epithelial cells of the oral and nasal regions.

3.4 Bilateral FGF8 and SHH expression in the pre-integrated MEE, immediately following midline palatal contact (16.5 to 17 dpc).

Following contact of the tips of the palatal shelves in the midline, the epithelial cells meld into an uniform medial epithelial seam (MES) by 16.5 dpc which then rapidly disperses leaving occasional islands of epithelial cells isolated in the mesenchyme, by 17.5 dpc. Bilateral specific expression of FGF8 and SHH were detected, localized to the pre-integrated MEE, immediately prior to midline palatal contact (Fig. 14 & 15). For both ligands the pattern was restricted to scattered cells of the MEE but excluding the periderm. In the MES regions no FGF8 nor SHH were detected. The columnar nasal epithelium and basal columnar oral epithelial cells contiguous with the MEE and MES did not show evidence of FGF8 or SHH. Interestingly, FGF8 was detected in the mesenchymal cells of both left and right palatal processes. Controls which included GAM-b polyclonal antibody and blocking peptides, confirmed the specificity of staining (Fig. 14 E & F, 15C).

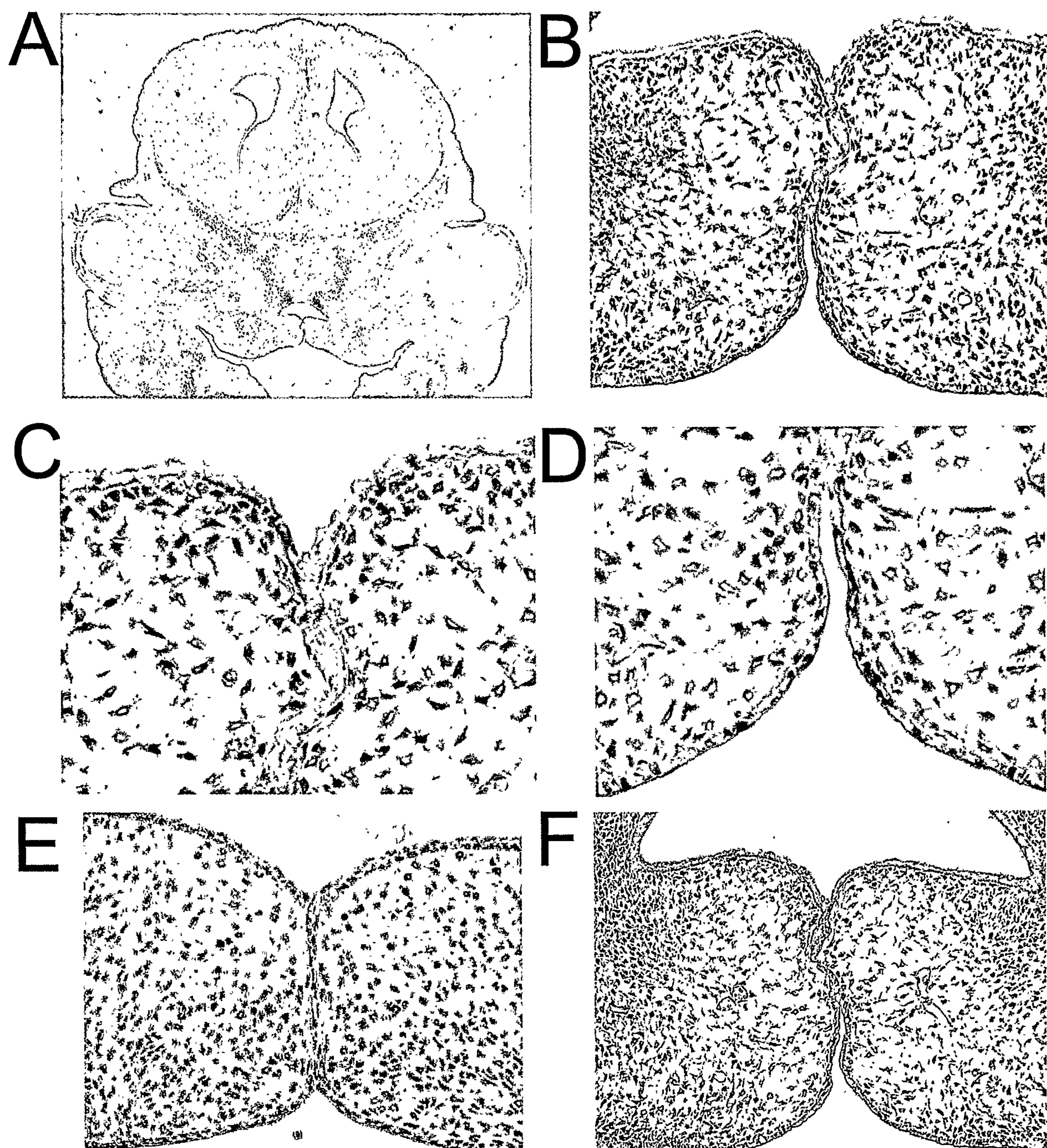
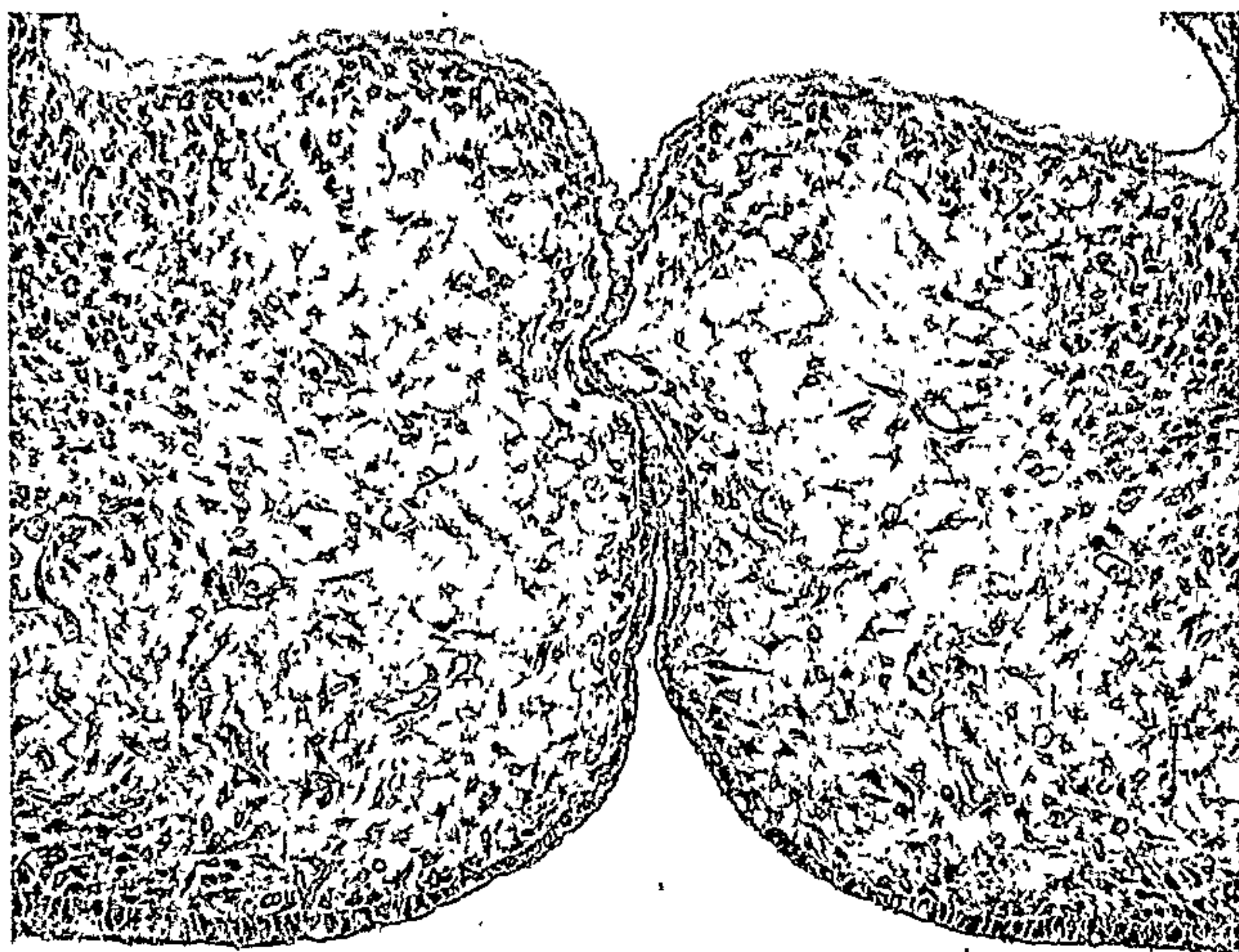


Figure 14. FGF8 was detected bilaterally in the pre-integrated MEE at palatal contact (16.5 to 17.5 dpc).

A&B) Sections at low magnification, revealing FGF8 expression in the embryo and fusing palate. C) Higher magnification of the nasal region, reveals specific bilateral FGF8 detection in the pre-integrated MEE. FGF8 was not detected in the central MEE which is integrated and proceeding to MES. D) Similarly, in the oral region, FGF8 was detected in specific bilateral pre-integrated MEE. E) Control with FGF8 blocking peptide. F) Control GAM-b only.

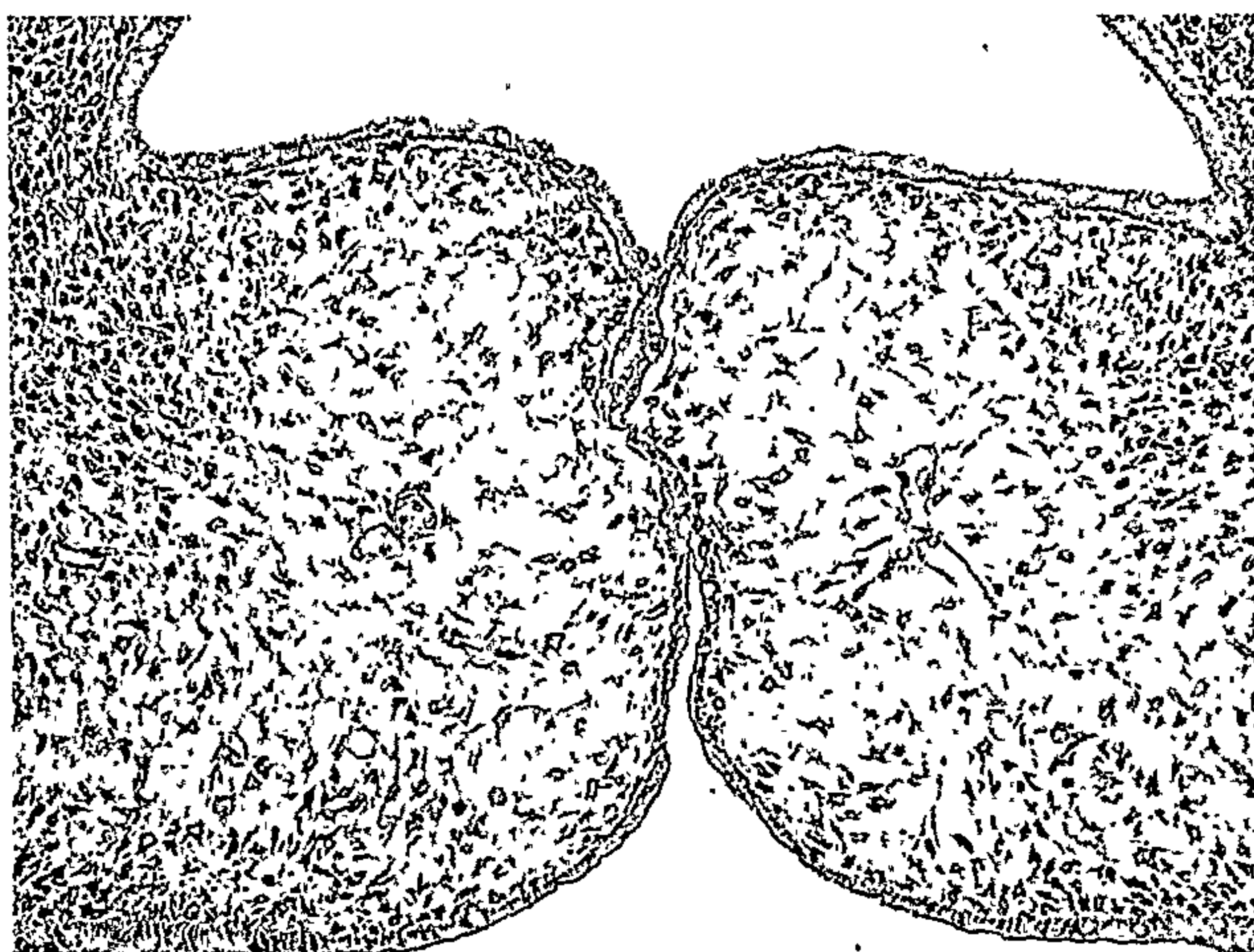
A



B



D



C

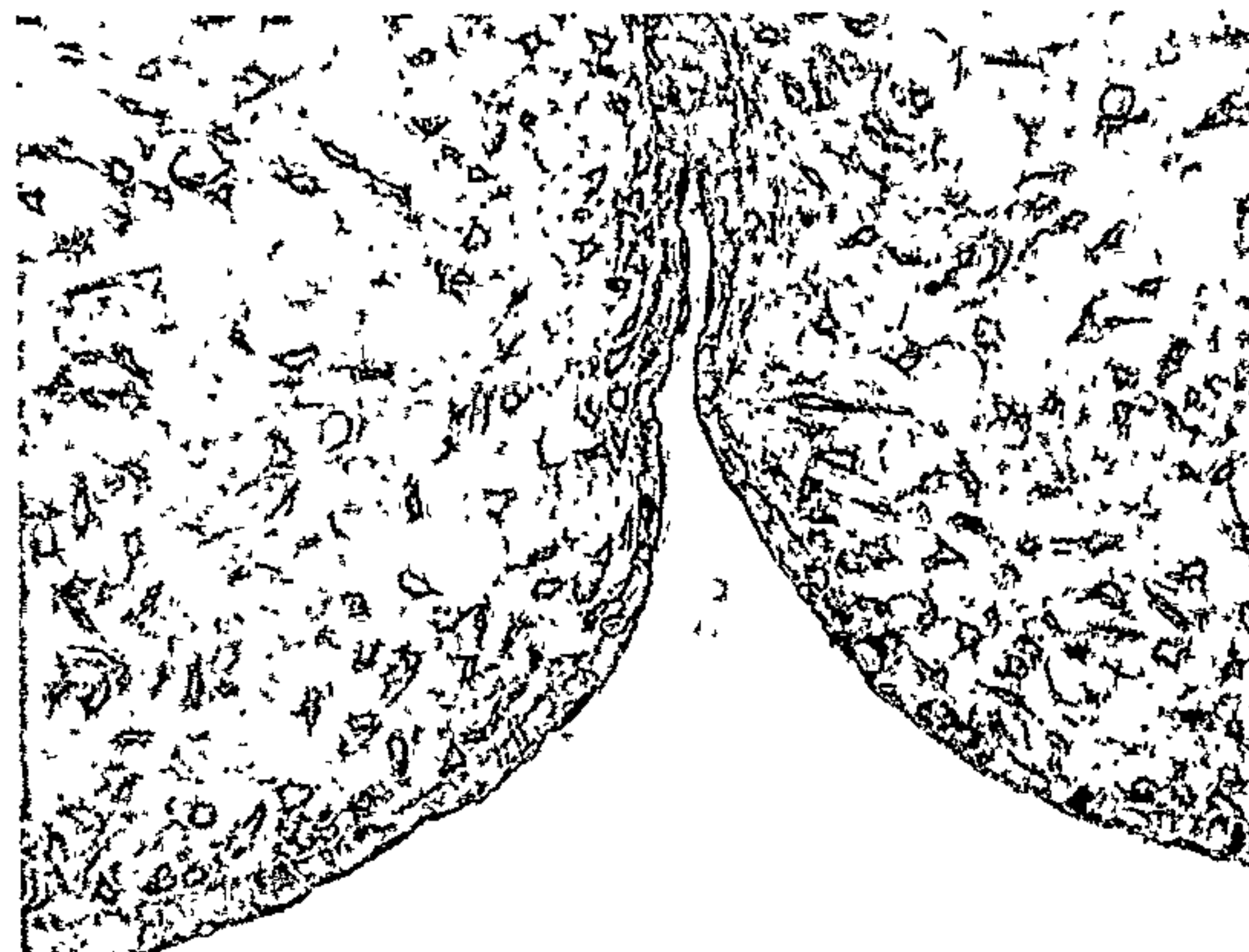


Figure 15. SHH was detected bilaterally in the pre-integrated MEE at palatal contact (16.5 to 17.5 dpc).

A) Section at low magnification, revealing bilateral SHH expression. B) Higher magnification of the nasal region, reveals specific bilateral SHH detection in the pre-integrated MEE. SHH was not detected in the central MEE which is in contact and proceeding to MES. C) Similarly, in the oral region, specific bilateral detection of SHH occurs only in the pre-integrated MEE. D) Control GAM-b only.

3.5 MES remnants express FGF8 at the completion of palatal fusion (18 dpc).

On completion of palatal fusion, the MES dispersed, leaving some remnants of the MES on the oral and nasal epithelial cells. FGF8 was detected in both the oral and nasal epithelial cells on the remnants of the MES (Fig. 16) at 18 dpc, prior to complete dispersion of the MES at both the nasal and oral surfaces. Controls GAM-b polyclonal antibody sections confirmed the specificity of staining (Fig. 16D).

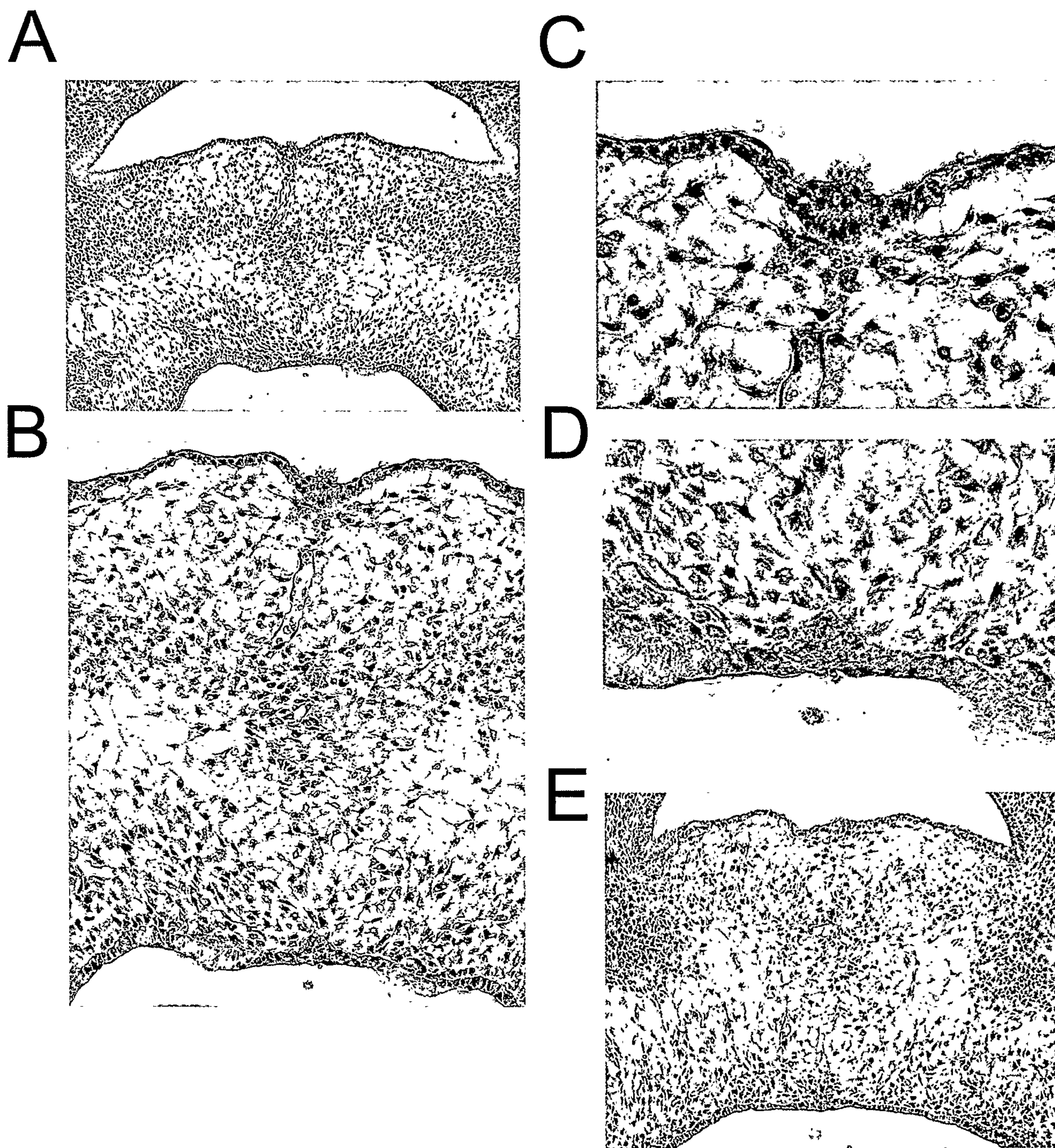


Figure 16. FGF8 was detected in the MEE remnants at completion of palatal fusion.

A&B) Sections at low magnification, revealing FGF8 expression at the completion of palatal fusion. C) Higher magnification of the nasal region, reveals specific FGF8 detection in the remnant MEE cells, prior to integration. The central MES is integrated and FGF8 is not detected. D) Similarly, in the oral region, specific FGF8 expression is detected only in the remnant MEE cells, prior to integration. E) Control GAM-b only.

CHAPTER 4

DISCUSSION

Discussion

It is noteworthy that the midline fusion of the palatal processes, which are apparently products of chiral morphogenesis, is related to the expression of specific ligands strongly implicated in the generation of chirality. Paradoxically, both the generation and resolution of chirality appear to employ overlapping mechanisms, at least for this developmental sequence.

The epithelial cells of the MEE in the pharyngeal region asymmetrically expressed FGF8 immediately after elevation and rotation to a position above the tongue. Interestingly, during pharyngeal arch patterning, FGF8 was also found to be expressed in the anterior endoderm and the overlying ectoderm (Wall *et al.* 1995; Graham *et al.* 2001).

FGF8 was detected in isolated epithelial cells of both the left and right MEE immediately prior to contact of the tips of the palatal shelves in the midline. Previously, vimentin mRNA expression was found to be specifically and uniformly up-regulated in the MEE, prior to midline contact (Gibbins *et al.* 1999). Uniform temporally and spatially restricted expression of *R-twist* mRNA was also observed in the MEE. *R-twist* transcripts were detected in a localised epithelial area at the tip of the shelves prior to fusion (Bloch-Zupan *et al.* 2001). It could be postulated that the epithelial cells in the MEE are specifically primed for midline contact. The observed restriction of FGF8 expression to isolated cells implies programming at the level of the cell rather than a tissue domain.

The MES is formed by the integration of the opposing epithelia covering

the tips of the palatal shelves (MEE) (Farbman 1969; Hayward 1969). At midline palatal contact, MES regions (which had integrated) did not show any expression of FGF8 or SHH. Marked reduction in staining possibly related to down-regulation of expression was noted in zones of cell contact between opposing MES indicating that contact could trigger this response. However, in the pre-integrated epithelia (MEE), there was bilateral, cell specific detection of both FGF8 and SHH. In confirmation of the site-specificity of expression, nasal and oral epithelial cells contiguous with the MEE and MES did not stain for FGF8 or SHH. The bilateral expression of FGF8 and SHH in specific relationship to regions of anticipated MEE integration is characteristic and should be considered in relation to the rapidity with which this process must necessarily occur. It could be postulated that both FGF8 and SHH expression at the protein level play a role in MEE and MES epithelial integration.

During MES dispersion, epithelial cells accumulate at the oral and nasal aspects to form epithelial triangles (Carette *et al.* 1992). FGF8 was detected in isolated cells of the epithelial triangles (remnants of the MES) at both the oral and nasal regions. Fusion of the tips of palatal shelves with the lateral surface of the tongue has been reported during mis-directed MEE integration in mice homozygous for *Jag2* deletion (Jiang *et al.* 1998). The unique priming of the MEE prior to fusion is also indicated by the critical and possibly controlling role played by TGF β 3 in the fusion of the murine secondary palate both *in vitro* (Brunet *et al.* 1995; Kaartinen *et al.* 1997) and *in vivo* (Proetzel *et al.* 1995; Kaartinen *et al.* 1997).

Studies in *xenopus* and mice have demonstrated that midline structures,

including the notochord and floor palate, are required for normal L-R differentiation. Apoptosis along the primitive streak midline has been proposed as a mechanism to prevent L-R communication (Kelly *et al.* 2002). Also, the early response gene *sil* has been found to block midline signalling (Izraeli *et al.* 1999). FGF8 knockout embryos die during gastrulation, preventing the analysis of laterality defects in FGF8 null embryos (Sun *et al.* 1999). A conditional mouse knockout FGF8 displays a loss of *nodal* and *pitx2* expression, implicating FGF8 as a left determinant (Meyers *et al.* 1999). Interestingly, SHH mutant mice have also been found to have midline defects (Chiang *et al.* 1996).

Transient loss of SHH signalling in the embryonic face results in defects, analogous to hypotelorism and cleft lip/palate, and which are characteristic of the mild forms of holoprosencephaly. In contrast, excess SHH leads to hypertelorism, a medio-lateral widening of the frontonasal process (Wall *et al.* 1995; Hu *et al.* 1999). Perturbations associated with midline and laterality identities lead to numerous congenital defects, including cyclopia, situs inversus, and heteritaxia. L-R axis defects in humans occur at a frequency of 1 in 8500 live births, but account for many more deaths *in utero* (Brown *et al.* 1990; Casey 2001; Cohen 2001). Mutational analysis of the SHH gene in newborns with oral clefts indicated silent polymorphisms (Orioli *et al.* 2002). Chromosomal 22 micro-deletion (del22q11) syndrome presents with congenital cardiovascular defects and craniofacial anomalies, which include hypertelorism and cleft palate. It is the most common micro-deletion syndrome occurring in an estimated 1 in 4000 live births and encompasses both DiGeorge and Velocardiofacial syndromes (Greenberg *et al.* 1984; Scambler *et al.* 1992; Lindsay 2001).

The results reported in this paper provide evidence that the fusion of the rat secondary palate exhibits a pattern of FGF8 and SHH expression that supports an interpretation of L-R chirality resolution. Epithelial cells in the MEE are specifically primed to up-regulated expression of both FGF8 and SHH genes prior to MEE contact and MES integration. Expression of FGF8 and SHH during secondary palatal fusion of the rat embryo may be analogous to their role played in the determination of handedness in the chick, mouse and rabbit primitive streak. However, in the secondary palate, resolution of chirality is also needed for midline fusion to take place. Further studies will be required to determine the laterality genes which control these changes during palatal development.

4.1 Limitations of This Study

This is the first descriptive study linking FGF8 and SHH expression during L-R palatal development. However, further research is required to manipulate this process to gain further insights into specific mechanisms. In addition to protein expression, studies investigating mRNA expression will reveal additional information. Manipulation studies will also help determine the importance of FGF8 and SHH in palatal development. Finally, a study incorporating greater number of embryos may reveal more information on this process.

4.2 Future Directions

Further investigations are required to determine which laterality genes control L-R asymmetry changes during palatal development. This would require in situ hybridisation for mRNA expression of laterality genes (e.g. FGF8 and SHH). In addition protein inhibition studies could determine wheather palatal fusion could be inhibited by FGF8 and/or SHH inhibition. Other manipulation studies could target antisense to FGF8 and/or SHH to determine its effect on palatal fusion. Palates which have been inhibited from fusion by TGFB3 antisense treatment could also be analysed for FGF8 and/or SHH expression. Investigations are also required to determine the role played by other laterality geens (e.g. lefty, nodal, BMP, Car) in the paltal fusion process.

Clinically, syndromic or cleft patients containing midline and/or clefts defects could be analysed for FGF8 and/or SHH mutations/polymorphisms. Future studies could also investigate the timing, modification and inhibition of “chirality” gene expression. Other studies could also identify “master” genes involved in regulating “chirality”.

CHAPTER 5

REFERENCES

References

- Akhurst, R. J., S. A. Lehnert, A. Faissner and E. Duffie (1990). TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. *Development* **108**(4): 645-56.
- Alonso, M. I., A. Gato, J. A. Moro and E. Barbosa (1998). Disruption of proteoglycans in neural tube fluid by beta-D-xyloside alters brain enlargement in chick embryos. *Anatomical Record* **252**(4): 499-508.
- Baker, J. C. and R. M. Harland (1997). From receptor to nucleus: the Smad pathway. *Current Opinion in Genetics & Development* **7**(4): 467-73.
- Barcellos-Hoff, M. H. (1996). Latency and activation in the control of TGF-beta. *Journal of Mammary Gland Biology & Neoplasia* **1**(4): 353-63.
- Baskin, L. S., S. W. Hayward, P. Young and G. R. Cunha (1996). Role of mesenchymal-epithelial interactions in normal bladder development. *Journal of Urology* **156**(5): 1820-7.
- Beddington, R. S. and E. J. Robertson (1999). Axis development and early asymmetry in mammals. *Cell* **96**(2): 195-209.
- Bissell, D. M., S. S. Wang, W. R. Jarnagin and F. J. Roll (1995). Cell-specific expression of transforming growth factor-beta in rat liver. Evidence for autocrine regulation of hepatocyte proliferation. *Journal of Clinical Investigation* **96**(1): 447-55.
- Bloch-Zupan, A., N. Hunter, A. Manthey and J. Gibbins (2001). R-twist gene expression during rat palatogenesis. *Int J Dev Biol* **45**(2): 397-404.
- Boettger, T., L. Wittler and M. Kessel (1999). FGF8 functions in the specification of the right body side of the chick. *Curr Biol* **9**(5): 277-80.

-
- Boyer, A. S., C. P. Erickson and R. B. Runyan (1999). Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGFbeta signal transduction mechanisms. *Developmental Dynamics* **214**(1): 81-91.
- Brandes, M. E., U. E. Mai, K. Ohura and S. M. Wahl (1991). Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta. *Journal of Immunology* **147**(5): 1600-6.
- Brinkley, L., G. Basehoar, A. Branch and J. Avery (1975). A new in vitro system for studying secondary palate development. *Journal of Embryology & Experimental Morphology* **34**(2): 485-95.
- Brinkley, L. L. and J. Morris-Wiman (1984). The role of extracellular matrices in palatal shelf closure. *Current Topics in Developmental Biology* **19**: 17-36.
- Brinkley, L. L. and J. Morris-Wiman (1987). Computer-assisted analysis of hyaluronate distribution during morphogenesis of the mouse secondary palate. *Development* **100**(4): 629-35.
- Brown, N. A. and L. Wolpert (1990). The development of handedness in left/right asymmetry. *Development* **109**(1): 1-9.
- Brunet, C. L., P. M. Sharpe and M. W. J. Ferguson (1995). Inhibition Of Tgf-Beta(3) (But Not Tgf-Beta(1) or Tgf-Beta(2)) Activity Prevents Normal Mouse Embryonic Palate Fusion. *International Journal of Developmental Biology* **39**(2): 345-355.

-
- Brunner, A. M., M. N. Lioubin, H. Marquardt, A. R. Malacko, W. C. Wang, R. A. Shapiro, M. Neubauer, J. Cook, L. Madisen and A. F. Purchio (1992). Site-directed mutagenesis of glycosylation sites in the transforming growth factor-beta 1 (TGF beta 1) and TGF beta 2 (414) precursors and of cysteine residues within mature TGF beta 1: effects on secretion and bioactivity. *Molecular Endocrinology* **6**(10): 1691-700.
- Calkhoven, C. F. and G. Ab (1996). Multiple steps in the regulation of transcription-factor level and activity. *Biochemical Journal* **317**(Part 2): 329-342.
- Capdevila, J., K. J. Vogan, C. J. Tabin and J. C. Izpisua Belmonte (2000). Mechanisms of left-right determination in vertebrates. *Cell* **101**(1): 9-21.
- Carette, M. J. and M. W. Ferguson (1992). The fate of medial edge epithelial cells during palatal fusion in vitro: an analysis by DiI labelling and confocal microscopy. *Development* **114**(2): 379-88.
- Casey, B. (2001). Genetics of human situs abnormalities. *Am J Med Genet* **101**(4): 356-8.
- Chai, Y., A. Mah, C. Crohin, S. Groff, P. Bringas, Jr., T. Le, V. Santos and H. C. Slavkin (1994). Specific transforming growth factor-beta subtypes regulate embryonic mouse Meckel's cartilage and tooth development. *Developmental Biology* **162**(1): 85-103.
- Chiang, C., Y. Litington, E. Lee, K. E. Young, J. L. Corden, H. Westphal and P. A. Beachy (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**(6599): 407-13.
- Clarke, P. G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anatomy & Embryology* **181**(3): 195-213.

-
- Cohen, M. M., Jr. (2001). Asymmetry: molecular, biologic, embryopathic, and clinical perspectives. *Am J Med Genet* **101**(4): 292-314.
- Crossley, P. H. and G. R. Martin (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**(2): 439-51.
- Cui, X. M., D. Warburton, J. Zhao, D. L. Crowe and C. F. Shuler (1998). Immunohistochemical localization of TGF-beta type II receptor and TGF-beta3 during palatogenesis in vivo and in vitro. *International Journal of Developmental Biology* **42**(6): 817-20.
- Cui, X. M. and C. F. Shuler (2000). The TGF-beta type III receptor is localized to the medial edge epithelium during palatal fusion. *International Journal of Developmental Biology* **44**(4): 397-402.
- D'Angelo, M. and R. M. Greene (1991). Transforming growth factor-beta modulation of glycosaminoglycan production by mesenchymal cells of the developing murine secondary palate. *Developmental Biology* **145**(2): 374-8.
- D'Angelo, M., J. M. Chen, K. Ugen and R. M. Greene (1994). TGF beta 1 regulation of collagen metabolism by embryonic palate mesenchymal cells. *Journal of Experimental Zoology* **270**(2): 189-201.
- Daopin, S., K. A. Piez, Y. Ogawa and D. R. Davies (1992). Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. *Science* **257**(5068): 369-73.
- Denhez, F., R. Lafyatis, P. Kondaiah, A. B. Roberts and M. B. Sporn (1990). Cloning by polymerase chain reaction of a new mouse TGF-beta, mTGF-beta 3. *Growth Factors* **3**(2): 139-46.

-
- Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn and D. V. Goeddel (1985). Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316**(6030): 701-5.
- Dickson, M. C., J. S. Martin, F. M. Cousins, A. B. Kulkarni, S. Karlsson and R. J. Akhurst (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**(6): 1845-54.
- Diebold, R. J., M. J. Eis, M. Yin, I. Ormsby, G. P. Boivin, B. J. Darrow, J. E. Saffitz and T. Doetschman (1995). Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. *Proceedings of the National Academy of Sciences of the United States of America* **92**(26): 12215-9.
- Diewert, V. (1980). The Role of Craniofacial Growth in Palatal Shelf Elevation. *Current Research Trends in Prenatal Craniofacial Development*.
- Diewert, V. M. (1983). A morphometric analysis of craniofacial growth and changes in spatial relations during secondary palatal development in human embryos and fetuses. *American Journal of Anatomy* **167**(4): 495-522.
- Drenckhahn, D., M. Beckerle, K. Burrige and J. Otto (1988). Identification and subcellular location of talin in various cell types and tissues by means of [125I]vinculin overlay, immunoblotting and immunocytochemistry. *European Journal of Cell Biology* **46**(3): 513-22.
- Duband, J. L., F. Monier, M. Delannet and D. Newgreen (1995). Epithelium-mesenchyme transition during neural crest development. *Acta Anatomica* **154**(1): 63-78.

-
- Echelard, Y., D. J. Epstein, B. St-Jacques, L. Shen, J. Mohler, J. A. McMahon and A. P. McMahon (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**(7): 1417-30.
- Farbman, A. I. (1969). The epithelium-connective tissue interface during closure of the secondary palate in rodent embryos. *J Dent Res* **48**(5): 617-24.
- Ferguson, M. W. (1978). Palatal shelf elevation in the Wistar rat fetus. *J Anat* **125**(3): 555-77.
- Ferguson, M. W. (1981). Developmental mechanisms in normal and abnormal palate formation with particular reference to the aetiology, pathogenesis and prevention of cleft palate. *British Journal of Orthodontics* **8**(3): 115-37.
- Ferguson, W. J. (1984). Epithelial-mesenchymal interactions during vertebrate palatogenesis. *Current Topics in Developmental Biology* **19**: 137-64.
- Ferguson, M. W. (1987). Palate development: mechanisms and malformations. *Irish Journal of Medical Science* **156**(11): 309-15.
- Ferguson, M. W. (1988). Palate development. *Development* **103** [Suppl]: 41-60.
- Ferguson, M. W. (2000). A hole in the head. *Nat Genet* **24**(4): 330-1.
- Fischer, A., C. Viebahn and M. Blum (2002). FGF8 acts as a right determinant during establishment of the left-right axis in the rabbit. *Curr Biol* **12**(21): 1807-16.
- Fitchett, J. E. and E. D. Hay (1989). Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Developmental Biology* **131**(2): 455-74.

-
- Fitzpatrick, D. R., F. Denhez, P. Kondaiah and R. J. Akhurst (1990). Differential expression of TGF beta isoforms in murine palatogenesis. *Development* **109**(3): 585-95.
- Fuchs, E. and K. Weber (1994). Intermediate filaments: structure, dynamics, function, and disease. [Review]. *Annual Review of Biochemistry* **63**(345): 345-82.
- Gehris, A. L. and R. M. Greene (1992). Regulation of murine embryonic epithelial cell differentiation by transforming growth factors beta. *Differentiation* **49**(3): 167-73.
- Geiser, A. G., Q. Q. Zeng, M. Sato, L. M. Helvering, T. Hirano and C. H. Turner (1998). Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene. *Bone* **23**(2): 87-93.
- Gentry, L. E., M. N. Lioubin, A. F. Purchio and H. Marquardt (1988). Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Molecular & Cellular Biology* **8**(10): 4162-8.
- Gibbins, J. R., L. G. Tilney and K. R. Porter (1969). Microtubules in the formation and development of the primary mesenchyme in *Arbacia punctulata*. I. The distribution of microtubules. *Journal of Cell Biology* **41**(1): 201-26.
- Gibbins, J. R., T. R. Nicholson, R. J. Vozab, B. Ying and K. R. Messerle (1991). Different malignant phenotypes induced in a stable, subdiploid, benign epithelial clone by DNA transfection. *Anticancer Research* **11**(1): 129-37.

-
- Gibbins, J. R., A. Manthey, Y. M. Tazawa, B. Scott, A. Bloch-Zupan and N. Hunter (1999). Midline fusion in the formation of the secondary palate anticipated by upregulation of keratin K5/6 and localized expression of vimentin mRNA in medial edge epithelium. *International Journal of Developmental Biology* **43**(3): 237-44.
- Gibbins, J. R., S. Brent, M. Srivastava, N. Garibotto, Y. M. Tazawa, A. Cameron and N. Hunter (2000). Rapid disappearance of the medial epithelial seam during palatal fusion occurs by multifocal breakdown that is preceded by expression of alpha smooth muscle actin in the epithelium. *International Journal of Developmental Biology* **44**(2): 223-31.
- Graham, A. and A. Smith (2001). Patterning the pharyngeal arches. *Bioessays* **23**(1): 54-61.
- Greenberg, F., W. E. Crowder, V. Paschall, J. Colon-Linares, B. Lubianski and D. H. Ledbetter (1984). Familial DiGeorge syndrome and associated partial monosomy of chromosome 22. *Hum Genet* **65**(4): 317-9.
- Greene, R. M. and R. M. Pratt (1976). Developmental aspects of secondary palate formation. *Journal of Embryology & Experimental Morphology* **36**(2): 225-45.
- Griffith, C. M. and E. D. Hay (1992). Epithelial-mesenchymal transformation during palatal fusion: Carboxyfluorescein traces cells at light and electron microscopic levels. *Development* **116**: 1087-1099.
- Hales, A. M., M. W. Schulz, C. G. Chamberlain and J. W. McAvoy (1994). TGF-beta 1 induces lens cells to accumulate alpha-smooth muscle actin, a marker for subcapsular cataracts. *Current Eye Research* **13**(12): 885-90.

-
- Hampson, J., I. L. Ponting, N. Cook, L. Vodinelich, S. Redmond, A. B. Roberts and T. M. Dexter (1989). The effects of TGF beta on haemopoietic cells. *Growth Factors* 1(2): 193-202.
- Hanker, J. S., A. D. Dixon and G. R. Smiley (1973). Acid phosphatase in the golgi apparatus of cells forming extracellular matrix of hard tissues. *Histochemie* 35(1): 39-50.
- Hartshorne, D. J. (1987). Physiology of the Gastrointestinal Tract. New York, Raven Press.
- Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anatomica* 154(1): 8-20.
- Hay, E. D. and A. Zuk (1995). Transformations between epithelium and mesenchyme - Normal, pathological and experimentally induced. *American Journal of Kidney Diseases* 26(4): 678-690.
- Hayward, A. F. (1969). Ultrastructural changes in the epithelium during fusion of the palatal processes in rats. *Archives of Oral Biology* 14(6): 661-78.
- Heikinheimo, M., A. Lawshe, G. M. Shackleford, D. B. Wilson and C. A. MacArthur (1994). Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mech Dev* 48(2): 129-38.
- Heine, U., E. F. Munoz, K. C. Flanders, L. R. Ellingsworth, H. Y. Lam, N. L. Thompson, A. B. Roberts and M. B. Sporn (1987). Role of transforming growth factor-beta in the development of the mouse embryo. *Journal of Cell Biology* 105(6 Pt 2): 2861-76.

-
- Horvitz, H. R. (1999). Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. *Cancer Research* **59**(7 Suppl): 1701s-1706s.
- Hu, D. and J. A. Helms (1999). The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development* **126**(21): 4873-84.
- Humphrey, T. (1969). The relation between human fetal mouth opening reflexes and closure of the palate. *American Journal of Anatomy* **125**(3): 317-44.
- Idoyaga-Vargas, V., C. E. Nasjleti and J. M. Azcurra (1972). Cytodifferentiation of the mouse secondary palate in vitro: morphological, biochemical, and histochemical aspects. *Journal of Embryology & Experimental Morphology* **27**(2): 413-30.
- Izraeli, S., L. A. Lowe, V. L. Bertness, D. J. Good, D. W. Dorward, I. R. Kirsch and M. R. Kuehn (1999). The SIL gene is required for mouse embryonic axial development and left-right specification. *Nature* **399**(6737): 691-4.
- Jiang, R., Y. Lan, H. D. Chapman, C. Shawber, C. R. Norton, D. V. Serreze, G. Weinmaster and T. Gridley (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**(7): 1046-57.
- Johnson, M. D., M. T. Jennings, L. I. Gold and H. L. Moses (1993). Transforming growth factor-beta in neural embryogenesis and neoplasia. *Human Pathology* **24**(5): 457-62.
- Kaartinen, V., J. W. Voncken, C. Shuler, D. Warburton, D. Bu, N. Heisterkamp and J. Groffen (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nature Genetics* **11**(4): 415-21.

-
- Kaartinen, V., X. M. Cui, N. Heisterkamp, J. Groffen and C. F. Shuler (1997). Transforming growth factor-beta3 regulates transdifferentiation of medial edge epithelium during palatal fusion and associated degradation of the basement membrane. *Developmental Dynamics* **209**(3): 255-60.
- Kallapur, S., I. Ormsby and T. Doetschman (1999). Strain dependency of TGFbeta1 function during embryogenesis. *Molecular Reproduction & Development* **52**(4): 341-9.
- Kawabata, M., H. Inoue, A. Hanyu, T. Imamura and K. Miyazono (1998). Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. *EMBO Journal* **17**(14): 4056-65.
- Keller, J. R., I. K. McNiece, K. T. Sill, L. R. Ellingsworth, P. J. Quesenberry, G. K. Sing and F. W. Ruscetti (1990). Transforming growth factor beta directly regulates primitive murine hematopoietic cell proliferation. *Blood* **75**(3): 596-602.
- Kelly, K. A., Y. Wei and T. Mikawa (2002). Cell death along the embryo midline regulates left-right sidedness. *Dev Dyn* **224**(2): 238-44.
- Kerr, J. F. R. (1993). Definition of Apoptosis and Overview of its Incidence. Programmed Cell Death- The Cellular and Molecular Biology of Apoptosis. M. Lavin and D. Watters. N.Y., Harwood Academic Publishers. **1**: 1-15.
- Knudsen, T. B., R. F. Bulleit and E. F. Zimmerman (1985). Histochemical localization of glycosaminoglycans during morphogenesis of the secondary palate in mice. *Anatomy and Embryology (Berl)* **173**(1): 137-42.

-
- Kondaiah, P., M. J. Sands, J. M. Smith, A. Fields, A. B. Roberts, M. B. Sporn and D. A. Melton (1990). Identification of a novel transforming growth factor-beta (TGF-beta 5) mRNA in *Xenopus laevis*. *Journal of Biological Chemistry* **265**(2): 1089-93.
- Kuhn, E. M., B. S. Babiarz, J. L. Lessard and E. F. Zimmerman (1980). Palate morphogenesis. I. Immunological and ultrastructural analyses of mouse palate. *Teratology* **21**(2): 209-23.
- Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward and S. Karlsson (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proceedings of the National Academy of Sciences of the United States of America* **90**(2): 770-4.
- Kurihara, H., M. Yoshizumi, T. Sugiyama, F. Takaku, M. Yanagisawa, T. Masaki, M. Hamaoki, H. Kato and Y. Yazaki (1989). Transforming growth factor-beta stimulates the expression of endothelin mRNA by vascular endothelial cells. *Biochemical & Biophysical Research Communications* **159**(3): 1435-40.
- Lawrence, D. A. (1996). Transforming growth factor-beta: a general review. *European Cytokine Network* **7**(3): 363-74.
- Le Douarin, N. M., E. Dupin and C. Ziller (1994). Genetic and epigenetic control in neural crest development. *Current Opinion in Genetics and Development* **4**(5): 685-95.
- Levin, M. (1997). Left-right asymmetry in vertebrate embryogenesis. *Bioessays* **19**(4): 287-96.

-
- Levin, M., R. L. Johnson, C. D. Stern, M. Kuehn and C. Tabin (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**(5): 803-14.
- Lewandoski, M., X. Sun and G. R. Martin (2000). Fgf8 signalling from the AER is essential for normal limb development. *Nat Genet* **26**(4): 460-3.
- Lewis, C. A., L. Thibault, R. M. Pratt and L. L. Brinkley (1980). An improved culture system for secondary palatal elevation. *In Vitro* **16**(6): 453-60.
- Li, J., K. Foitzik, E. Calautti, H. Baden, T. Doetschman and G. P. Dotto (1999). TGF-beta3, but not TGF-beta1, protects keratinocytes against 12-O-tetradecanoylphorbol-13-acetate-induced cell death in vitro and in vivo. *Journal of Biological Chemistry* **274**(7): 4213-9.
- Lindsay, E. A. (2001). Chromosomal microdeletions: dissecting del22q11 syndrome. *Nat Rev Genet* **2**(11): 858-68.
- Logan, M., S. M. Pagan-Westphal, D. M. Smith, L. Paganessi and C. J. Tabin (1998). The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* **94**(3): 307-17.
- Lopez, C., M. Sanchez, A. Hidalgo and M. J. Garcia de Boto (2000). Mechanisms involved in UTP-induced contraction in isolated rat aorta. *European Journal of Pharmacology* **391**(3): 299-303.
- Lopez-Casillas, F., J. L. Wrana and J. Massague (1993). Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**(7): 1435-44.
- Luning, C., A. Rass, B. Rozell, J. Wroblewski and B. Obrink (1994). Expression Of E-Cadherin During Craniofacial Development. *Journal of Craniofacial Genetics & Developmental Biology* **14**(4): 207-216.

-
- Lyons, R. M., D. A. Miller, J. L. Graycar, H. L. Moses and R. Derynck (1991). Differential binding of transforming growth factor-beta 1, -beta 2, and -beta 3 by fibroblasts and epithelial cells measured by affinity cross-linking of cell surface receptors. *Molecular Endocrinology* **5**(12): 1887-96.
- Markwald, R., C. Eisenberg, L. Eisenberg, T. Trusk and Y. Sugi (1996). Epithelial-mesenchymal transformations in early avian heart development. *Acta Anatomica* **156**(3): 173-86.
- Martin, J. S., M. C. Dickson, F. M. Cousins, A. B. Kulkarni, S. Karlsson and R. J. Akhurst (1995). Analysis of homozygous TGF beta 1 null mouse embryos demonstrates defects in yolk sac vasculogenesis and hematopoiesis. *Annals of the New York Academy of Sciences* **752**: 300-8.
- Martinez-Alvarez, C., R. Bonelli, C. Tudela, A. Gato, J. Mena, S. O'Kane and M. W. Ferguson (2000). Bulging medial edge epithelial cells and palatal fusion. *International Journal of Developmental Biology* **44**(3): 331-5.
- Martinez-Alvarez, C., C. Tudela, J. Perez-Miguelsanz, S. O'Kane, J. Puerta and M. W. Ferguson (2000). Medial edge epithelial cell fate during palatal fusion. *Developmental Biology* **220**(2): 343-57.
- Massague, J. (1990). The transforming growth factor-beta family. *Annual Review of Cell Biology* **6**: 597-641.
- Mato, M. and Y. Uchiyama (1975). Ultrastructures of glosso-palatal fusion after treatment of meclozine-hydrochloride. *Virchows Archiv. A, Pathological Anatomy & Histology* **369**(1): 7-17.
- Meller, S. (1980). Morphological Alterations in the Perfusion Human Palatal Epithelium. *Current Research Trends in Prenatal Craniofacial Development*.

-
- Meno, C., A. Shimono, Y. Saijoh, K. Yashiro, K. Mochida, S. Ohishi, S. Noji, H. Kondoh and H. Hamada (1998). *lefty-1* is required for left-right determination as a regulator of *lefty-2* and *nodal*. *Cell* **94**(3): 287-97.
- Mercola, M. and M. Levin (2001). Left-right asymmetry determination in vertebrates. *Annu Rev Cell Dev Biol* **17**: 779-805.
- Meyers, E. N. and G. R. Martin (1999). Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. *Science* **285**(5426): 403-6.
- Monsoro-Burq, A. and N. M. Le Douarin (2001). BMP4 plays a key role in left-right patterning in chick embryos by maintaining Sonic Hedgehog asymmetry. *Mol Cell* **7**(4): 789-99.
- Moses, H. L. (1990). The biological actions of transforming growth factor beta. Growth Factors: From Genes to Clinical Application. V. R. Sara. New York, Raven Press: 141-155.
- Nakajima, Y., V. Mironov, T. Yamagishi, H. Nakamura and R. R. Markwald (1997). Expression of smooth muscle alpha-actin in mesenchymal cells during formation of avian endocardial cushion tissue: a role for transforming growth factor beta3. *Developmental Dynamics* **209**(3): 296-309.
- Nakao, A., T. Imamura, S. Souchelnytskyi, M. Kawabata, A. Ishisaki, E. Oeda, K. Tamaki, J. Hanai, C. H. Heldin, K. Miyazono and P. ten Dijke (1997). TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO Journal* **16**(17): 5353-62.
- Nusslein-Volhard, C. and E. Wieschaus (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**(5785): 795-801.

-
- Orioli, I. M., A. R. Vieira, E. E. Castilla, J. E. Ming and M. Muenke (2002). Mutational analysis of the Sonic Hedgehog gene in 220 newborns with oral clefts in a South American (ECLAMC) population. *Am J Med Genet* **108**(1): 12-5.
- Pagan-Westphal, S. M. and C. J. Tabin (1998). The transfer of left-right positional information during chick embryogenesis. *Cell* **93**(1): 25-35.
- Pelton, R. W., M. D. Johnson, E. A. Perkett, L. I. Gold and H. L. Moses (1991). Expression of transforming growth factor-beta 1, -beta 2, and -beta 3 mRNA and protein in the murine lung. *American Journal of Respiratory Cell & Molecular Biology* **5**(6): 522-30.
- Postlethwaite, A. E., J. Keski-Oja, H. L. Moses and A. H. Kang (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *Journal of Experimental Medicine* **165**(1): 251-6.
- Pratt, R. M. and R. M. Greene (1976). Inhibition of palatal epithelial cell death by altered protein synthesis. *Developmental Biology* **54**(1): 135-45.
- Proetzel, G., S. A. Pawlowski, M. V. Wiles, M. Y. Yin, G. P. Boivin, P. N. Howles, J. X. Ding, M. W. J. Ferguson and T. Doetschman (1995). Transforming Growth Factor-Beta-3 Is Required For Secondary Palate Fusion. *Nature Genetics* **11**(4): 409-414.
- Riddle, R. D., R. L. Johnson, E. Laufer and C. Tabin (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**(7): 1401-16.
- Rodriguez Esteban, C., J. Capdevila, A. N. Economides, J. Pascual, A. Ortiz and J. C. Izpisua Belmonte (1999). The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. *Nature* **401**(6750): 243-51.

-
- Ryan, A. K., B. Blumberg, C. Rodriguez-Esteban, S. Yonei-Tamura, K. Tamura, T. Tsukui, J. de la Pena, W. Sabbagh, J. Greenwald, S. Choe, D. P. Norris, E. J. Robertson, R. M. Evans, M. G. Rosenfeld and J. C. Izpisua Belmonte (1998). Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* **394**(6693): 545-51.
- Roberts, A. B., K. C. Flanders, U. I. Heine, S. Jakowlew, P. Kondaiah, S. J. Kim and M. B. Sporn (1990). Transforming growth factor-beta: multifunctional regulator of differentiation and development. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* **327**(1239): 145-54.
- Robinson, S. D., G. B. Silberstein, A. B. Roberts, K. C. Flanders and C. W. Daniel (1991). Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development* **113**(3): 867-78.
- Sanford, L. P., I. Ormsby, A. C. Gittenberger-de Groot, H. Sariola, R. Friedman, G. P. Boivin, E. L. Cardell and T. Doetschman (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124**(13): 2659-70.
- Sauvage, M., N. Hinglais, C. Mandet, C. Badier, F. Deslandes, J. B. Michel and M. P. Jacob (1998). Localization of elastin mRNA and TGF-beta1 in rat aorta and caudal artery as a function of age. *Cell & Tissue Research* **291**(2): 305-14.

-
- Scambler, P. J., D. Kelly, E. Lindsay, R. Williamson, R. Goldberg, R. Shprintzen, D. I. Wilson, J. A. Goodship, I. E. Cross and J. Burn (1992). Velo-cardio-facial syndrome associated with chromosome 22 deletions encompassing the DiGeorge locus. *Lancet* **339**(8802): 1138-9.
- Schiffer, M., G. Von Gersdorff, M. Bitzer, K. Susztak and E. P. Bottinger (2000). Smad proteins and transforming growth factor-beta signaling. *Kidney International* **58**(Suppl 77): 45-52.
- Schlunegger, M. P. and M. G. Grutter (1992). An unusual feature revealed by the crystal structure at 2.2 Å resolution of human transforming growth factor-beta 2. *Nature* **358**(6385): 430-4.
- Schlunegger, M. P. and M. G. Grutter (1993). Refined crystal structure of human transforming growth factor beta 2 at 1.95 Å resolution. *Journal of Molecular Biology* **231**(2): 445-58.
- Segarini, P. R. (1991). TGF-beta receptors. *Ciba Foundation Symposium* **157**: 29-40; discussion 41-50.
- Shah, R. M. (1979). A cellular mechanism for the palatal shelf reorientation from a vertical to a horizontal plane in hamster: light and electron microscopic study. *Journal of Embryology & Experimental Morphology* **53**: 1-13.
- Shapiro, B. L. and L. Sweney (1969). Electron microscopic and histochemical examination of oral epithelial- mesenchymal interaction (programmed cell death). *Journal of Dental Research* **48**(5): 652-60.
- Sharpe, P. M. and M. W. Ferguson (1988). Mesenchymal influences on epithelial differentiation in developing systems. *Journal of Cell Science Supplementary* **10**: 195-230.

-
- Shoeman, R. and P. Traub (1993). Assembly of Intermediate Filaments. *BioEssays* **15**(9): 605-611.
- Shuler, C. F., Y. Guo, A. Majumder and R. Y. Luo (1991). Molecular and morphologic changes during the epithelial-mesenchymal transformation of palatal shelf medial edge epithelium in vitro. *International Journal of Developmental Biology* **35**(4): 463-72.
- Shuler, C. F., D. E. Halpern, Y. Guo and A. C. Sank (1992). Medial edge epithelium fate traced by cell lineage analysis during epithelial mesenchymal transformation *in vivo*. *Developmental Biology* **154**: 318-330.
- Shuler, C. F., D. E. Halpern, Y. Guo and A. C. Sank (1992). Medial edge epithelium fate traced by cell lineage analysis during epithelial mesenchymal transformation *in vivo*. *Developmental Biology* **154**(2): 318-330.
- Shuler, C. F. (1995). Programmed cell death and cell transformation in craniofacial development. *Critical Reviews in Oral Biology & Medicine* **6**(3): 202-17.
- Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel and D. Calvin (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**(6397): 693-9.
- Sim, S. S., H. J. Baek, S. H. Yoon, D. J. Rhie, S. J. Hahn, Y. H. Jo and M. S. Kim (1997). Regulation of protein kinases in steady-state contraction of cat gastric smooth muscle. *European Journal of Pharmacology* **324**(2-3): 205-10.
- Smiley, G. R. and W. E. Koch (1975). A comparison of secondary palate development with different in vitro techniques. *Anatomical Record* **181**(4): 711-23.

-
- Steinert, P. M. (1993). Structure, function, and dynamics of keratin intermediate filaments. [Review]. *Journal of Investigative Dermatology* **100**(6): 729-34.
- Sun, D., K. R. McAlmon, J. A. Davies, M. Bernfield and E. D. Hay (1998). Simultaneous loss of expression of syndecan-1 and E-cadherin in the embryonic palate during epithelial-mesenchymal transformation. *International Journal of Developmental Biology* **42**(5): 733-6.
- Sun, D., C. R. Vanderburg, G. S. Odierna and E. D. Hay (1998). TGFbeta3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. *Development* **125**(1): 95-105.
- Sun, X., E. N. Meyers, M. Lewandoski and G. R. Martin (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* **13**(14): 1834-46.
- Taya, Y., S. O'Kane and M. W. Ferguson (1999). Pathogenesis of cleft palate in TGF-beta3 knockout mice. *Development* **126**(17): 3869-79.
- ten Dijke, P., K. Miyazono and C. H. Heldin (2000). Signaling inputs converge on nuclear effectors in TGF-beta signaling. *Trends in Biochemical Sciences* **25**(2): 64-70.
- Thompson, K. L., R. Assoian and M. R. Rosner (1988). Transforming growth factor-beta increases transcription of the genes encoding the epidermal growth factor receptor and fibronectin in normal rat kidney fibroblasts. *Journal of Biological Chemistry* **263**(36): 19519-24.
- Thompson, N. L., K. C. Flanders, J. M. Smith, L. R. Ellingsworth, A. B. Roberts and M. B. Sporn (1989). Expression of transforming growth factor-beta 1 in specific cells and tissues of adult and neonatal mice. *Journal of Cell Biology* **108**(2): 661-9.

-
- Tilney, L. G. and J. R. Gibbins (1969). Microtubules and filaments in the filopodia of the secondary mesenchyme cells of *Arbacia punctulata* and *Echinarachnius parma*. *Journal of Cell Science* **5**(1): 195-210.
- Trasler, D. G. and G. Morriss-Kay (1991). Immunohistochemical localization of chondroitin and heparan sulfate proteoglycans in pre-spina bifida splotch mouse embryos. *Teratology* **44**(5): 571-9.
- van de Klundert, F. A. J. M., J. M. Raats and H. Bloemendal (1993). Intermediate filaments: regulation of gene expression and assembly. *European Journal of Biochemistry* **214**(2): 351-66.
- Vogel, A., C. Rodriguez and J. C. Izpisua-Belmonte (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**(6): 1737-50.
- Wall, N. A. and B. L. Hogan (1995). Expression of bone morphogenetic protein-4 (BMP-4), bone morphogenetic protein-7 (BMP-7), fibroblast growth factor-8 (FGF-8) and sonic hedgehog (SHH) during branchial arch development in the chick. *Mech Dev* **53**(3): 383-92.
- Wahl, S. M., D. A. Hunt, L. M. Wakefield, N. McCartney-Francis, L. M. Wahl, A. B. Roberts and M. B. Sporn (1987). Transforming growth factor type beta induces monocyte chemotaxis and growth factor production. *Proceedings of the National Academy of Sciences of the United States of America* **84**(16): 5788-92.
- Walker, B. E. (1969). Correlation of embryonic movement with palate closure in mice. *Teratology* **2**(3): 191-7.

-
- Watanabe, T., K. Dakshinamurti and T. V. Persaud (1995). Biotin influences palatal development of mouse embryos in organ culture. *Journal of Nutrition* **125**(8): 2114-21.
- Wee, E. L., B. S. Babiarz, S. Zimmerman and E. F. Zimmerman (1979). Palate morphogenesis. IV. Effects of serotonin and its antagonists on rotation in embryo culture. *Journal of Embryology & Experimental Morphology* **53**: 75-90.
- Wrana, J. L., L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X. F. Wang and J. Massague (1992). TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* **71**(6): 1003-14.
- Yost, H. J. (1995). Vertebrate left-right development. *Cell* **82**(5): 689-92
- Zhang, Y. and R. Derynck (1999). Regulation of Smad signalling by protein associations and signalling crosstalk. *Trends in Cell Biology* **9**(7): 274-9.
- Zschabitz, A. R., H. K. Biesalski, V. Krahm, H. J. Gabius, H. Weiser, A. Khaw, C. Hemmes and E. Stofft (1994). Distribution Patterns In Glycoconjugate Expression During the Development Of the Rat Palate. *Histochemical Journal* **26**(9): 705-720.

CHAPTER 6
MANUSCRIPT

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**Expression of FGF8 and SHH anticipates epithelial
integration during palatal fusion in the rat.**

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Abstract

Expression of fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) was investigated to elucidate mechanisms involved in the apparent resolution of chirality during formation of the secondary palate. Sprague-Dawley rats were mated overnight and timed embryos removed 14.5 to 17.5 days post coitum (dpc) to obtain tissue samples prior to, during and immediately following palatal fusion. Immunohistochemistry for FGF8 and SHH was performed on paraffin sections of rat embryo maxillas fixed in paraformaldehyde. Asymmetric FGF8 expression was detectable initially localized to the medial edge epithelium (MEE) of the right palatal shelf after elevation and rotation of the palatal processes above the tongue. Immediately prior to and following contact of the MEE of each of the shelves in the midline, localized regions of bilateral FGF8 and SHH expression were confined to the MEE. FGF8 and SHH were not detected in the epithelium of the medial epithelial seam (MES) formed by the integration of the MEE on each palatal shelf. These results are interpreted as indicating that FGF8 and SHH expression in the MEE play a role in chirality resolution to ensure subsequent merging of the palatal shelves whereas these ligands have a key role as determinants of left-right asymmetry of the embryo.

Introduction

Symmetry around a midline axis underlies the whole of embryonic development which is critically dependent on the successful completion of midline fusion-the resolution of chirality (Shuler 1995). Midline fusion events are critical to normal embryonic development and their disturbance is a major contributor to congenital deformity (Cohen 2001). These fusion events frequently involve trans-differentiation of cells from epithelial to mesenchymal (EMT) or mesenchymal to epithelial (MET) (Hay 1995; Hay *et al.* 1995).

In the rat embryo, morphologically identifiable stages of palatal development begin at 15 dpc with fusion completed by 18 dpc (Gibbins *et al.* 1999; Gibbins *et al.* 2000). The palatal shelves, which initially grow lateral to the developing tongue, arise from the maxillary prominence of the first brachial arch. At this stage, each palatal process is a simple structure comprising a two or three cell layered epithelium overlying mesenchymal cells embedded in a loose extracellular matrix (ECM) with no specialized structures evident. The secondary palate is one of the latest embryonic structures to form and is one of the most frequent sites of malformation (Ferguson 1988).

An important question in the normal development of the faciomaxillary structures is the underlying molecular mechanisms determining left-right (L-R) sidedness (chirality) and the suppression of sidedness (resolution of chirality) that must occur to allow midline fusion of tissues to occur during embryonic growth (Vogel *et al.* 1996; Lewandoski *et al.* 2000). Asymmetrical expression of laterality genes begins just after node formation at the anterior end of the

primitive streak and before development of the notochord. Determination of L-R asymmetry has been linked to the molecular determinates fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) (Riddle *et al.* 1993; Heikinheimo *et al.* 1994; Levin *et al.* 1995; Yost 1995; Levin 1997; Beddington *et al.* 1999; Meyers *et al.* 1999; Capdevila *et al.* 2000; Mercola *et al.* 2001).

The first defined asymmetrical signal in the L-R patterning pathway is SHH, which is initially expressed throughout the node but becomes restricted to the left side (Nusslein-Volhard *et al.* 1980; Echelard *et al.* 1993; Levin *et al.* 1995; Pagan-Westphal *et al.* 1998). SHH is essential for morphogenesis of the frontonasal and maxillary processes, which give rise to the middle and upper face. SHH induces handed gene activities (left specific genes *nodal*, *lefty1*, and *lefty2*) in the left paraxial mesoderm (LPM) (Levin 1997; Logan *et al.* 1998; Meno *et al.* 1998; Monsoro-Burq *et al.* 2001). *Nodal* expression induces expression of *pitx2*, a transcription factor thought to directly specify left identity during organ development (Ryan *et al.* 1998). The stabilization of asymmetry, through the induction of unilateral gene expression patterns and the L-R information, is then transmitted either globally or organ specifically to the organ precursors where effector programs lead to differential morphogenesis.

FGF8 is localized to regions involved in outgrowth, patterning and axis formation during embryogenesis (Heikinheimo *et al.* 1994). Expression patterns show a medial-lateral progression, commencing at the node and/or neural structures and notochord, moving to the intermediate mesoderm and finally reaching a maximum intensity in the LPM. In limb development FGF8 signalling from the apical ectodermal ridge (AER) has been found to be essential

for the initiation, outgrowth and patterning of the limb (Lewandoski *et al.* 2000). FGF8 has opposite roles in the specification of L-R identity in chick and mouse. In the chick, FGF8 is expressed asymmetrically on the right side, posterior to the node (Crossley *et al.* 1995). FGF8 transcription is induced by activin and FGF8 protein inhibits the expression of *nodal* and *pitx2* (Boettger *et al.* 1999; Rodriguez Esteban *et al.* 1999). An activin-like signalling pathway has been shown to act upstream of SHH and *nodal* in the chick node. Activin signalling leads to the restriction of SHH expression to the left of the node and induces FGF8 on the right side of the node. SHH then activates *nodal* lateral to the node, whereas FGF8 acts to inhibit *nodal* expression on the right (Levin *et al.* 1995; Boettger *et al.* 1999). In the mouse, FGF8 is required for the left asymmetric expression of *nodal*, *lefty2*, and *pitx2*. Right-sided inhibition of FGF8 signalling induces bilateral marker gene expression, demonstrating that in rabbits FGF8 acts as a right determinant as in the chick (Fischer *et al.* 2002).

Embryonic midline tissues include the pre-chordal plate mesoderm anteriorly, the notochord and the spinal floor plate in the trunk. The palate is also a midline structure, with both secondary palatal shelves fusing in the midline. While morphogenetic events occurring around the midline require resolution of the chirality initiated at the stage of the primitive streak, fusion of the palatal processes could be considered as the enforced midline union of two processes which have a high degree of handedness. This paper reports the results obtained in a study designed to follow the expression of FGF8 and SHH in the resolution of chirality, during the palatal fusion sequence. The expression profiles for FGF8 and SHH were examined by immunohistochemistry on craniofacial tissues removed from timed rat embryos.

Results

FGF8 and SHH are not detected when the palatal processes are lateral to the tongue

At 15 dpc the developing tongue fills the presumptive nasal and oral cavities. Tissues that will form the secondary palate can be recognized as tapering plates occupying the space between the lateral surface of the tongue and the buccal mucosa, with the epithelia on the respective surfaces in close contact. FGF8 and SHH expression were not detected in the epithelium on the palatal processes, in the regions that would become the medial edges of the palatal shelves following rotation (Fig. 1).

Asymmetrical FGF8 expression in the MEE of the right palatal process, immediately after elevation and rotation to a position above the tongue

During the period from 15.5 to 16.5 dpc the tips of the palatal shelves move from lying lateral to the tongue to occupying a position directly opposite but separate from each other and rostral to the dorsal surface of the tongue. Asymmetrical expression of FGF8 was localized to the medial edge epithelium (MEE) of only the right palatal shelf (Fig. 2), immediately after elevation and rotation to a position above the tongue. FGF8 was evident initially in the simple epithelium on the presumptive tip of the right palatal shelf (MEE) and ceased abruptly in the region where the epithelium became columnar (Fig. 2C). This was detected only in the pharyngeal region. SHH was not detected at this stage (Fig. 3). Controls which included biotin conjugated goat anti-mouse (GAM-b)

polyclonal antibody and pre-incubation with blocking peptides, confirmed the specificity of staining (Fig. 2 D,E,F,G).

FGF8 is expressed bilaterally in the MEE prior to palatal fusion

FGF8 was localized to selected cells in the MEE of both left and right palatal processes prior to the contact of the tips of the palatal shelves in the midline (Fig 4). FGF8 was not detected in the epithelial cells in the oral and nasal regions. Similarly, FGF8 was not detected in the mesenchymal cells of both left and right palatal processes.

Bilateral FGF8 and SHH expression in the pre-integrated MEE, immediately following midline palatal contact

Following contact of the tips of the palatal shelves in the midline, the epithelial cells meld into an uniform medial epithelial seam (MES) by 16.5 dpc which then rapidly disperses leaving occasional islands of epithelial cells isolated in the mesenchyme, by 17.5 dpc. Bilateral expression of FGF8 and SHH was detected, localized to the pre-integrated MEE, immediately prior to midline palatal contact (Fig. 5 & 6). For both ligands the pattern was restricted to scattered cells of the MEE but excluding the periderm. No FGF8 nor SHH was detected in the MES. The columnar nasal epithelium and basal columnar oral epithelial cells contiguous with the MEE and MES did not show evidence of FGF8 or SHH while FGF8 was detected in the mesenchymal cells of both left and right palatal processes. Controls which included GAM-b polyclonal

antibody and blocking peptides, confirmed the specificity of staining (Fig. 5 E & F, 6D).

MES remnants express FGF8 at the completion of palatal fusion

On completion of palatal fusion at approximately 18 dpc, the MES dispersed, leaving remnants on the oral and nasal borders. FGF8 was detected in both the oral and nasal epithelial cells in regions of remnant MES (Fig. 7) prior to complete dispersal of the MES at both the nasal and oral surfaces. Control sections confirmed the specificity of staining (Fig. 7E).

Discussion

It is noteworthy that the midline fusion of the palatal processes, which are apparently products of chiral morphogenesis, is related to the expression of specific ligands strongly implicated in the generation of chirality. Paradoxically, both the generation and resolution of chirality appear to employ overlapping mechanisms, at least for this developmental sequence.

The epithelial cells of the MEE in the pharyngeal region asymmetrically expressed FGF8 immediately after elevation and rotation to a position above the tongue. Interestingly, during pharyngeal arch patterning, FGF8 was also found to be expressed in the anterior endoderm and the overlying ectoderm (Wall *et al.* 1995; Graham *et al.* 2001).

FGF8 was detected in isolated epithelial cells of both the left and right MEE immediately prior to contact of the tips of the palatal shelves in the midline. Previously, vimentin mRNA expression was found to be specifically and uniformly up-regulated in the MEE, prior to midline contact (Gibbins *et al.* 1999). Uniform temporally and spatially restricted expression of *R-twist* mRNA was also observed in the MEE. *R-twist* transcripts were detected in a localised epithelial area at the tip of the shelves prior to fusion (Bloch-Zupan *et al.* 2001). It could be postulated that the epithelial cells in the MEE are specifically primed for midline contact. The observed restriction of FGF8 expression to isolated cells implies programming at the level of the cell rather than a tissue domain.

The MES is formed by the integration of the opposing epithelia covering

the tips of the palatal shelves (MEE) (Farbman 1969; Hayward 1969). At midline palatal contact, MES regions (which had integrated) did not show any expression of FGF8 or SHH. Marked reduction in staining possibly related to down-regulation of expression was noted in zones of cell contact between opposing MES indicating that contact could trigger this response. However, in the pre-integrated epithelia (MEE), there was bilateral, cell specific detection of both FGF8 and SHH. In confirmation of the site-specificity of expression, nasal and oral epithelial cells contiguous with the MEE and MES did not stain for FGF8 or SHH. The bilateral expression of FGF8 and SHH in specific relationship to regions of anticipated MEE integration is characteristic and should be considered in relation to the rapidity with which this process must necessarily occur. It could be postulated that both FGF8 and SHH expression at the protein level play a role in MEE and MES epithelial integration.

During MES dispersion, epithelial cells accumulate at the oral and nasal aspects to form epithelial triangles (Carette *et al.* 1992). FGF8 was detected in isolated cells of the epithelial triangles (remnants of the MES) at both the oral and nasal regions. Fusion of the tips of palatal shelves with the lateral surface of the tongue has been reported during mis-directed MEE integration in mice homozygous for *Jag2* deletion (Jiang *et al.* 1998). The unique priming of the MEE prior to fusion is also indicated by the critical and possibly controlling role played by TGF β 3 in the fusion of the murine secondary palate both *in vitro* (Brunet *et al.* 1995; Kaartinen *et al.* 1997) and *in vivo* (Proetzel *et al.* 1995; Kaartinen *et al.* 1997).

Studies in *xenopus* and mice have demonstrated that midline structures,

including the notochord and floor palate, are required for normal L-R differentiation. Apoptosis along the primitive streak midline has been proposed as a mechanism to prevent L-R communication (Kelly *et al.* 2002). Also, the early response gene *sil* has been found to block midline signalling (Izraeli *et al.* 1999). FGF8 knockout embryos die during gastrulation, preventing the analysis of laterality defects in FGF8 null embryos (Sun *et al.* 1999). A conditional mouse knockout FGF8 displays a loss of *nodal* and *pitx2* expression, implicating FGF8 as a left determinant (Meyers *et al.* 1999). Interestingly, SHH mutant mice have also been found to have midline defects (Chiang *et al.* 1996).

Transient loss of SHH signalling in the embryonic face results in defects, analogous to hypotelorism and cleft lip/palate, and which are characteristic of the mild forms of holoprosencephaly. In contrast, excess SHH leads to hypertelorism, a medio-lateral widening of the frontonasal process (Wall *et al.* 1995; Hu *et al.* 1999). Perturbations associated with midline and laterality identities lead to numerous congenital defects, including cyclopia, situs inversus, and heteritaxia. L-R axis defects in humans occur at a frequency of 1 in 8500 live births, but account for many more deaths *in utero* (Brown *et al.* 1990; Casey 2001; Cohen 2001). Mutational analysis of the SHH gene in newborns with oral clefts indicated silent polymorphisms (Orioli *et al.* 2002). Chromosomal 22 micro-deletion (del22q11) syndrome presents with congenital cardiovascular defects and craniofacial anomalies, which include hypertelorism and cleft palate. It is the most common micro-deletion syndrome occurring in an estimated 1 in 4000 live births and encompasses both DiGeorge and Velocardiofacial syndromes (Greenberg *et al.* 1984; Scambler *et al.* 1992; Lindsay 2001).

The results reported in this paper provide evidence that the fusion of the rat secondary palate exhibits a pattern of FGF8 and SHH expression that supports an interpretation of L-R chirality resolution. Epithelial cells in the MEE are specifically primed to up-regulated expression of both FGF8 and SHH genes prior to MEE contact and MES integration. Expression of FGF8 and SHH during secondary palatal fusion of the rat embryo may be analogous to their role played in the determination of handedness in the chick, mouse and rabbit primitive streak. However, in the secondary palate, resolution of chirality is also needed for midline fusion to take place. Further studies will be required to determine the laterality genes which control these changes during palatal development.

Materials and Methods

Animal Preparation

Time mated Sprague-Dawley female rats were confirmed by vaginal mucus plug after a twelve hour mating period. Gestation times were determined from the morning following removal of the male rat as being half a dpc. The uterine horns of pregnant dams timed to be from 14.5 to 17.5 dpc were surgically removed under anesthesia and the embryos removed under sterile conditions. The embryos were decapitated and the stage in the fusion process determined by inspection of the palates under a dissecting microscope (Gibbins *et al.* 1999; Gibbins *et al.* 2000).

Fixation and Tissue Processing

Timed rodent embryos prior to, during and immediately following palatal fusion (14.5 to 17.5 dpc) were removed, fixed in 4% paraformaldehyde in PBS for a minimum of 6 hours and washed three times in 70% ethanol. Tissues were placed in tissue processing cassettes and processed, going through the following changes: 40 minutes each in 50% ethanol, 70% ethanol, 80% ethanol, then 2x 60 minutes each in 95% ethanol, and 100% ethanol, followed by 2x 90 minutes in xylene then taken through 2x 60 minutes of paraffin followed by a further 90 minutes in paraffin before mounting and orientating the specimens in cassettes. Paraffin embedded tissues were sectioned at 5 μ m then sections were dewaxed and rehydrated (Gibbins *et al.* 1999; Gibbins *et al.* 2000).

Immunohistochemistry

Twelve embryos, consisting of 3 embryos from each time sequence (14.5, 15.5, 16.5 and 17.5 dpc) were utilized in this study. Sections at different timed stages were blocked in 20% normal horse serum in PBS for 1 hour at RT, then reacted for 1 hour at room temperature with either a 1:50 dilution of goat polyclonal anti-FGF8 or anti-SHH antibody (Santa Cruz Biotechnology SC-6958 & SC-1194) in 10% FCS in PBS. After three 10 minutes washes in PBS, the sections were reacted with 1:100 dilution of rabbit anti-goat horseradish peroxidase antibody in 10% FCS in PBS at room temperature for one hour. The slides were then washed with three 10 minutes washes in PBS. The specific binding of the antibodies was visualized with 3' dimaminobenzidine tetrahydrochloride (DAB). Controls were run in parallel with goat anti-mouse (GAM-b) polyclonal antibody and for both FGF8 and SHH, pre-incubation of the primary antibody with blocking peptides (Santa Cruz Biotechnology SC-6958P and SC-1194P).

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References

- BEDDINGTON, R. S. and ROBERTSON, E. J. 1999. Axis development and early asymmetry in mammals. *Cell* **96**(2): 195-209.
- BLOCH-ZUPAN, A., HUNTER, N., MANTHEY, A. and GIBBINS, J. 2001. R-twist gene expression during rat palatogenesis. *Int J Dev Biol* **45**(2): 397-404.
- BOETTGER, T., WITTLER, L. and KESSEL, M. 1999. FGF8 functions in the specification of the right body side of the chick. *Curr Biol* **9**(5): 277-80.
- BROWN, N. A. and WOLPERT, L. 1990. The development of handedness in left/right asymmetry. *Development* **109**(1): 1-9.
- BRUNET, C. L., SHARPE, P. M. and FERGUSON, M. W. 1995. Inhibition of TGF-beta 3 (but not TGF-beta 1 or TGF-beta 2) activity prevents normal mouse embryonic palate fusion. *Int J Dev Biol* **39**(2): 345-55.
- CAPDEVILA, J., VOGAN, K. J., TABIN, C. J. and IZPISUA BELMONTE, J. C. 2000. Mechanisms of left-right determination in vertebrates. *Cell* **101**(1): 9-21.
- CARETTE, M. J. and FERGUSON, M. W. 1992. The fate of medial edge epithelial cells during palatal fusion in vitro: an analysis by DiI labelling and confocal microscopy. *Development* **114**(2): 379-88.
- CASEY, B. 2001. Genetics of human situs abnormalities. *Am J Med Genet* **101**(4): 356-8.
- CHIANG, C., LITINGTUNG, Y., LEE, E., YOUNG, K. E., CORDEN, J. L., WESTPHAL, H. and BEACHY, P. A. 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**(6599): 407-13.

-
- COHEN, M. M., JR. 2001. Asymmetry: molecular, biologic, embryopathic, and clinical perspectives. *Am J Med Genet* **101**(4): 292-314.
- CROSSLEY, P. H. and MARTIN, G. R. 1995. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**(2): 439-51.
- ECHELARD, Y., EPSTEIN, D. J., ST-JACQUES, B., SHEN, L., MOHLER, J., MCMAHON, J. A. and MCMAHON, A. P. 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**(7): 1417-30.
- FARBMAN, A. I. 1969. The epithelium-connective tissue interface during closure of the secondary palate in rodent embryos. *J Dent Res* **48**(5): 617-24.
- FERGUSON, M. W. 1988. Palate development. *Development* **103** Suppl: 41-60.
- FISCHER, A., VIEBAHN, C. and BLUM, M. 2002. FGF8 acts as a right determinant during establishment of the left-right axis in the rabbit. *Curr Biol* **12**(21): 1807-16.
- GIBBINS, J. R., BRENT, S., SRIVASTAVA, M., GARIBOTTO, N., TAZAWA, Y. M., CAMERON, A. and HUNTER, N. 2000. Rapid disappearance of the medial epithelial seam during palatal fusion occurs by multifocal breakdown that is preceded by expression of alpha smooth muscle actin in the epithelium. *Int J Dev Biol* **44**(2): 223-31.

-
- GIBBINS, J. R., MANTHEY, A., TAZAWA, Y. M., SCOTT, B., BLOCH-ZUPAN, A. and HUNTER, N. 1999. Midline fusion in the formation of the secondary palate anticipated by upregulation of keratin K5/6 and localized expression of vimentin mRNA in medial edge epithelium. *Int J Dev Biol* **43**(3): 237-44.
- GRAHAM, A. and SMITH, A. 2001. Patterning the pharyngeal arches. *Bioessays* **23**(1): 54-61.
- GREENBERG, F., CROWDER, W. E., PASCHALL, V., COLON-LINARES, J., LUBIANSKI, B. and LEDBETTER, D. H. 1984. Familial DiGeorge syndrome and associated partial monosomy of chromosome 22. *Hum Genet* **65**(4): 317-9.
- HAY, E. D. 1995. An overview of epithelio-mesenchymal transformation. *Acta Anat* **154**(1): 8-20.
- HAY, E. D. and ZUK, A. 1995. Transformations between epithelium and mesenchyme - Normal, pathological and experimentally induced. *American Journal of Kidney Diseases* **26**(4): 678-690.
- HAYWARD, A. F. 1969. Ultrastructural changes in the epithelium during fusion of the palatal processes in rats. *Arch Oral Biol* **14**(6): 661-78.
- HEIKINHEIMO, M., LAWSHE, A., SHACKLEFORD, G. M., WILSON, D. B. and MACARTHUR, C. A. 1994. Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mech Dev* **48**(2): 129-38.
- HU, D. and HELMS, J. A. 1999. The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development* **126**(21): 4873-84.

-
- IZRAELI, S., LOWE, L. A., BERTNESS, V. L., GOOD, D. J., DORWARD, D. W., KIRSCH, I. R. and KUEHN, M. R. 1999. The SIL gene is required for mouse embryonic axial development and left-right specification. *Nature* **399**(6737): 691-4.
- JIANG, R., LAN, Y., CHAPMAN, H. D., SHAWBER, C., NORTON, C. R., SERREZE, D. V., WEINMASTER, G. and GRIDLEY, T. 1998. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**(7): 1046-57.
- KAARTINEN, V., CUI, X. M., HEISTERKAMP, N., GROFFEN, J. and SHULER, C. F. 1997. Transforming growth factor-beta3 regulates transdifferentiation of medial edge epithelium during palatal fusion and associated degradation of the basement membrane. *Dev Dyn* **209**(3): 255-60.
- KELLY, K. A., WEI, Y. and MIKAWA, T. 2002. Cell death along the embryo midline regulates left-right sidedness. *Dev Dyn* **224**(2): 238-44.
- LEVIN, M. 1997. Left-right asymmetry in vertebrate embryogenesis. *Bioessays* **19**(4): 287-96.
- LEVIN, M., JOHNSON, R. L., STERN, C. D., KUEHN, M. and TABIN, C. 1995. A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**(5): 803-14.
- LEWANDOSKI, M., SUN, X. and MARTIN, G. R. 2000. Fgf8 signalling from the AER is essential for normal limb development. *Nat Genet* **26**(4): 460-3.
- LINDSAY, E. A. 2001. Chromosomal microdeletions: dissecting del22q11 syndrome. *Nat Rev Genet* **2**(11): 858-68.

-
- LOGAN, M., PAGAN-WESTPHAL, S. M., SMITH, D. M., PAGANESSI, L. and TABIN, C. J. 1998. The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* **94**(3): 307-17.
- MENO, C., SHIMONO, A., SAIJOH, Y., YASHIRO, K., MOCHIDA, K., OHISHI, S., NOJI, S., KONDOH, H. and HAMADA, H. 1998. lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. *Cell* **94**(3): 287-97.
- MERCOLA, M. and LEVIN, M. 2001. Left-right asymmetry determination in vertebrates. *Annu Rev Cell Dev Biol* **17**: 779-805.
- MEYERS, E. N. and MARTIN, G. R. 1999. Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. *Science* **285**(5426): 403-6.
- MONSORO-BURQ, A. and LE DOUARIN, N. M. 2001. BMP4 plays a key role in left-right patterning in chick embryos by maintaining Sonic Hedgehog asymmetry. *Mol Cell* **7**(4): 789-99.
- NUSSLEIN-VOLHARD, C. and WIESCHAUS, E. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**(5785): 795-801.
- ORIOLI, I. M., VIEIRA, A. R., CASTILLA, E. E., MING, J. E. and MUENKE, M. 2002. Mutational analysis of the Sonic Hedgehog gene in 220 newborns with oral clefts in a South American (ECLAMC) population. *Am J Med Genet* **108**(1): 12-5.
- PAGAN-WESTPHAL, S. M. and TABIN, C. J. 1998. The transfer of left-right positional information during chick embryogenesis. *Cell* **93**(1): 25-35.

-
- PROETZEL, G., PAWLOWSKI, S. A., WILES, M. V., YIN, M., BOIVIN, G. P., HOWLES, P. N., DING, J., FERGUSON, M. W. and DOETSCHMAN, T. 1995. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* **11**(4): 409-14.
- RIDDLE, R. D., JOHNSON, R. L., LAUFER, E. and TABIN, C. 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**(7): 1401-16.
- RODRIGUEZ ESTEBAN, C., CAPDEVILA, J., ECONOMIDES, A. N., PASCUAL, J., ORTIZ, A. and IZPISUA BELMONTE, J. C. 1999. The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. *Nature* **401**(6750): 243-51.
- RYAN, A. K., BLUMBERG, B., RODRIGUEZ-ESTEBAN, C., YONEI-TAMURA, S., TAMURA, K., TSUKUI, T., DE LA PENA, J., SABBAGH, W., GREENWALD, J., CHOE, S., NORRIS, D. P., ROBERTSON, E. J., EVANS, R. M., ROSENFELD, M. G. and IZPISUA BELMONTE, J. C. 1998. Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* **394**(6693): 545-51.
- SCAMBLER, P. J., KELLY, D., LINDSAY, E., WILLIAMSON, R., GOLDBERG, R., SHPRINTZEN, R., WILSON, D. I., GOODSHIP, J. A., CROSS, I. E. and BURN, J. 1992. Velo-cardio-facial syndrome associated with chromosome 22 deletions encompassing the DiGeorge locus. *Lancet* **339**(8802): 1138-9.
- SHULER, C. F. 1995. Programmed cell death and cell transformation in craniofacial development. *Crit Rev Oral Biol Med* **6**(3): 202-17.

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- SUN, X., MEYERS, E. N., LEWANDOSKI, M. and MARTIN, G. R. 1999. Targeted disruption of *Fgf8* causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* **13**(14): 1834-46.
- VOGEL, A., RODRIGUEZ, C. and IZPISUA-BELMONTE, J. C. 1996. Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**(6): 1737-50.
- WALL, N. A. and HOGAN, B. L. 1995. Expression of bone morphogenetic protein-4 (BMP-4), bone morphogenetic protein-7 (BMP-7), fibroblast growth factor-8 (FGF-8) and sonic hedgehog (SHH) during branchial arch development in the chick. *Mech Dev* **53**(3): 383-92.
- YOST, H. J. 1995. Vertebrate left-right development. *Cell* **82**(5): 689-92.

**FIGURE
LEGENDS**

FIGURE LEGENDS

Figure 1. FGF8 and SHH were not detected in the palatal processes at 14.5 to 15.5 dpc.

A) A section of the whole embryo revealing no expression of FGF8 in the palatal processes. B) At low magnification, FGF8 was not detected in the palatal shelves when the palatal processes were lateral to the tongue. C) Similarly, SHH was not detected in the palatal shelves at this stage.

Figure 2. FGF8 detected in the MEE of the right palatal shelf at 16.0 dpc.

A) Section at low magnification, revealing asymmetrical FGF8 expression. B) At higher magnification, FGF8 was not detected in the MEE of the left palatal process. C) At higher magnification, FGF8 was detected in the MEE of the right palatal process, immediately after elevation and rotation to a position above the tongue. D&E) Control of primary antibody pre-incubated with FGF8 blocking peptide. E&F) Similarly, control GAM-b did not reveal any FGF8.

Figure 3. SHH was not detected in the MEE at 16.0 dpc.

A) Section at low magnification, revealing no SHH expression in the MEE of both the left and right palatal processes, immediately after elevation and rotation to a position above the tongue. B) At higher magnification, SHH is not detected in the MEE of the left palatal process. C) Similarly, at higher magnification SHH is not detected in the MEE of the right palatal process.

Figure 4. FGF8 was detected bilaterally in MEE prior to palatal contact.

A) Section at low magnification, revealing bilateral FGF8 expression in the MEE of both left and right palatal processes. B) Higher magnification reveals specific bilateral FGF8 detection in MEE cells. FGF8 was only detected in the cells of the medial edge of the palatal processes. FGF8 was not detected in epithelial cells of the oral and nasal regions.

Figure 5. FGF8 was detected bilaterally in the pre-integrated MEE at palatal contact (16.5 to 17.5 dpc).

A&B) Sections at low magnification, revealing FGF8 expression in the embryo and fusing palate. C) Higher magnification of the nasal region, reveals specific bilateral FGF8 detection in the pre-integrated MEE. FGF8 was not detected in the central MEE which is integrated and proceeding to MES. D) Similarly, in the oral region, FGF8 was detected in specific bilateral pre-integrated MEE. E) Control with FGF8 blocking peptide. F) Control GAM-b only.

Figure 6. SHH was detected bilaterally in the pre-integrated MEE at palatal contact (16.5 to 17.5 dpc).

A) Section at low magnification, revealing bilateral SHH expression. B) Higher magnification of the nasal region, reveals specific bilateral SHH detection in the pre-integrated MEE. SHH was not detected in the central MEE which is in contact and proceeding to MES. C) Similarly, in the oral region, specific bilateral detection of SHH occurs only in the pre-integrated MEE. D) Control GAM-b only.

Figure 7. FGF8 was detected in the MEE remnants at completion of palatal fusion.

A&B) Sections at low magnification, revealing FGF8 expression at the completion of palatal fusion. C) Higher magnification of the nasal region, reveals specific FGF8 detection in the remnant MEE cells, prior to integration. The central MES is integrated and FGF8 is not detected. D) Similarly, in the oral region, specific FGF8 expression is detected only in the remnant MEE cells, prior to integration. E) Control GAM-b only.

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FIGURES

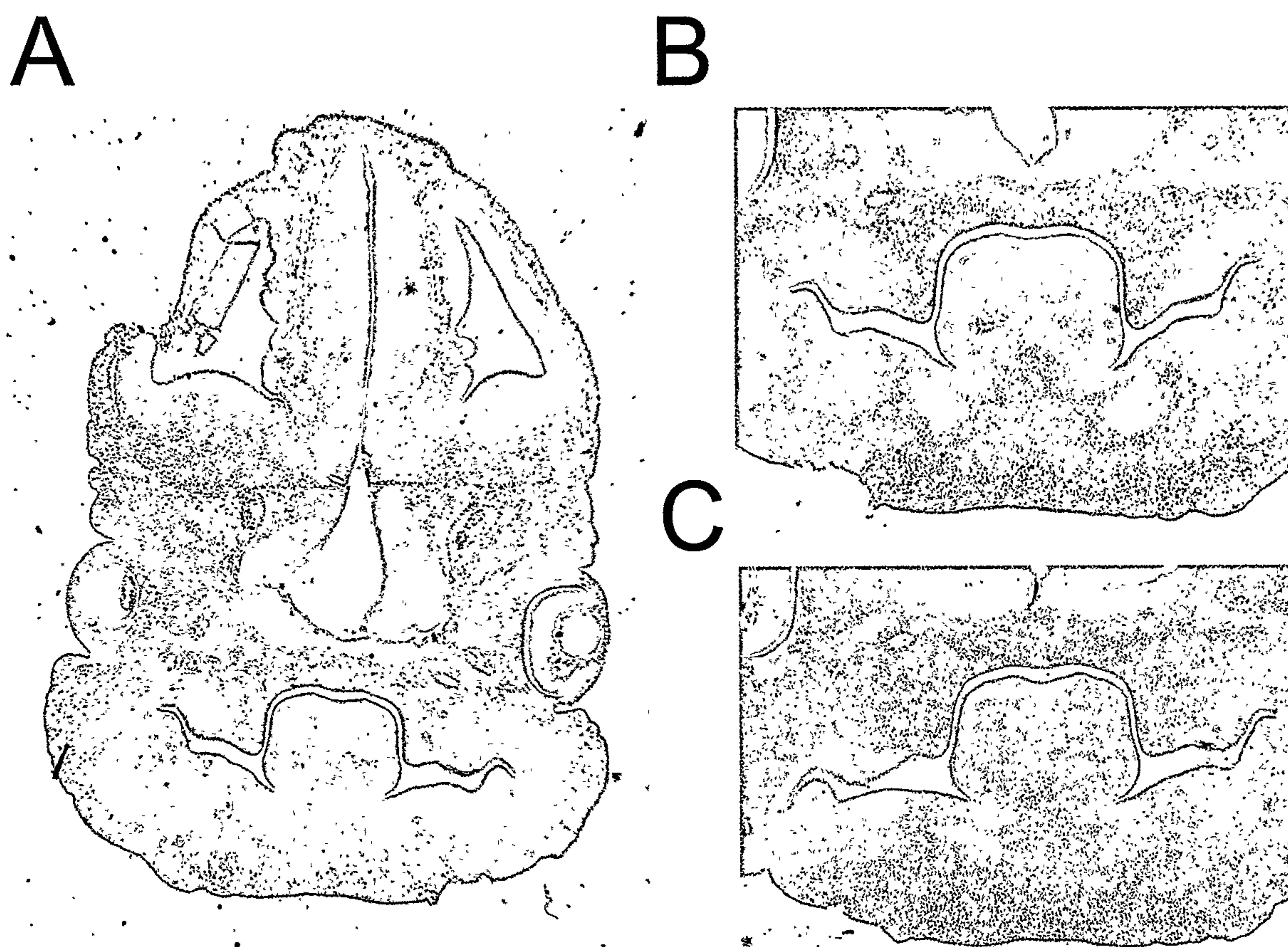


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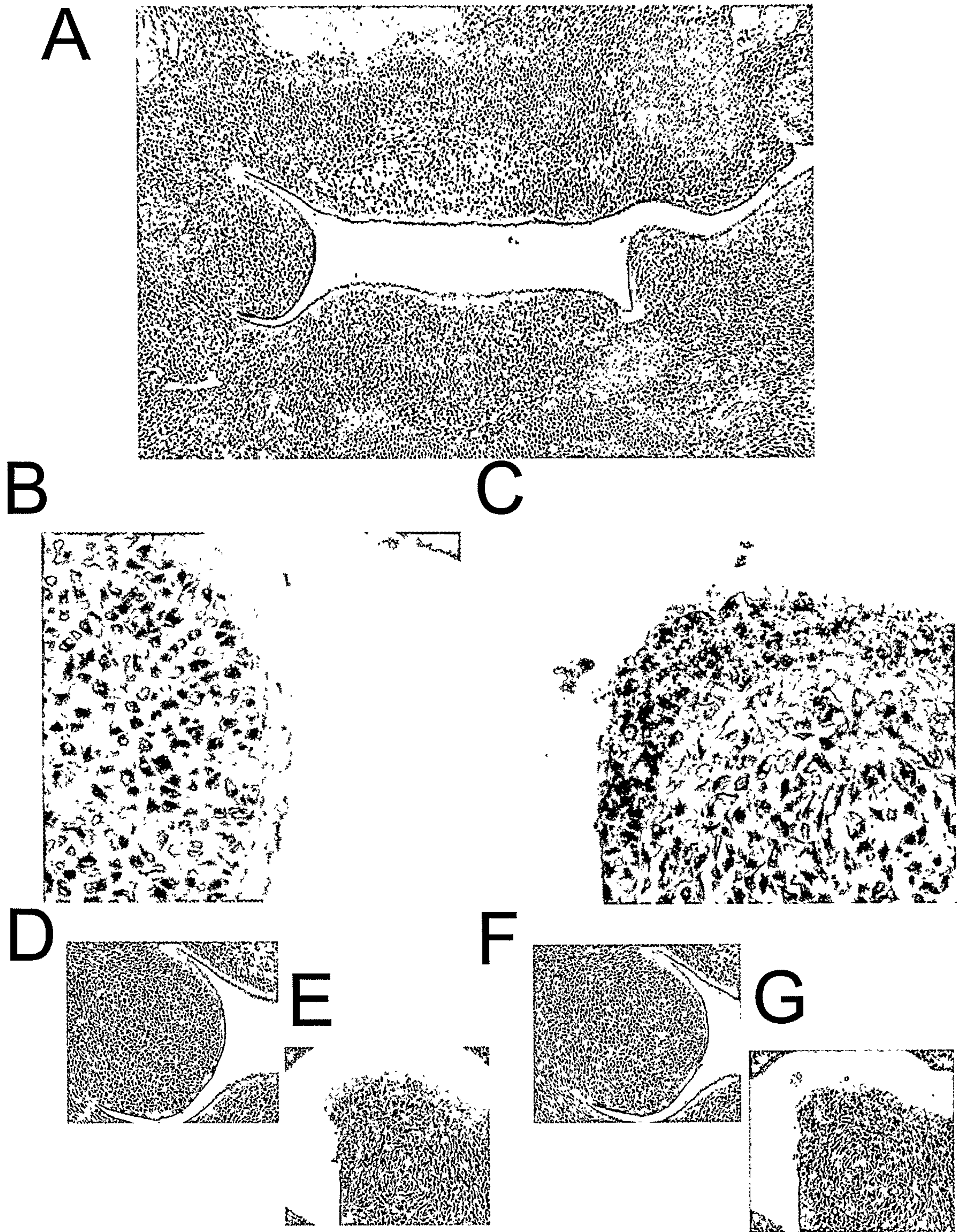


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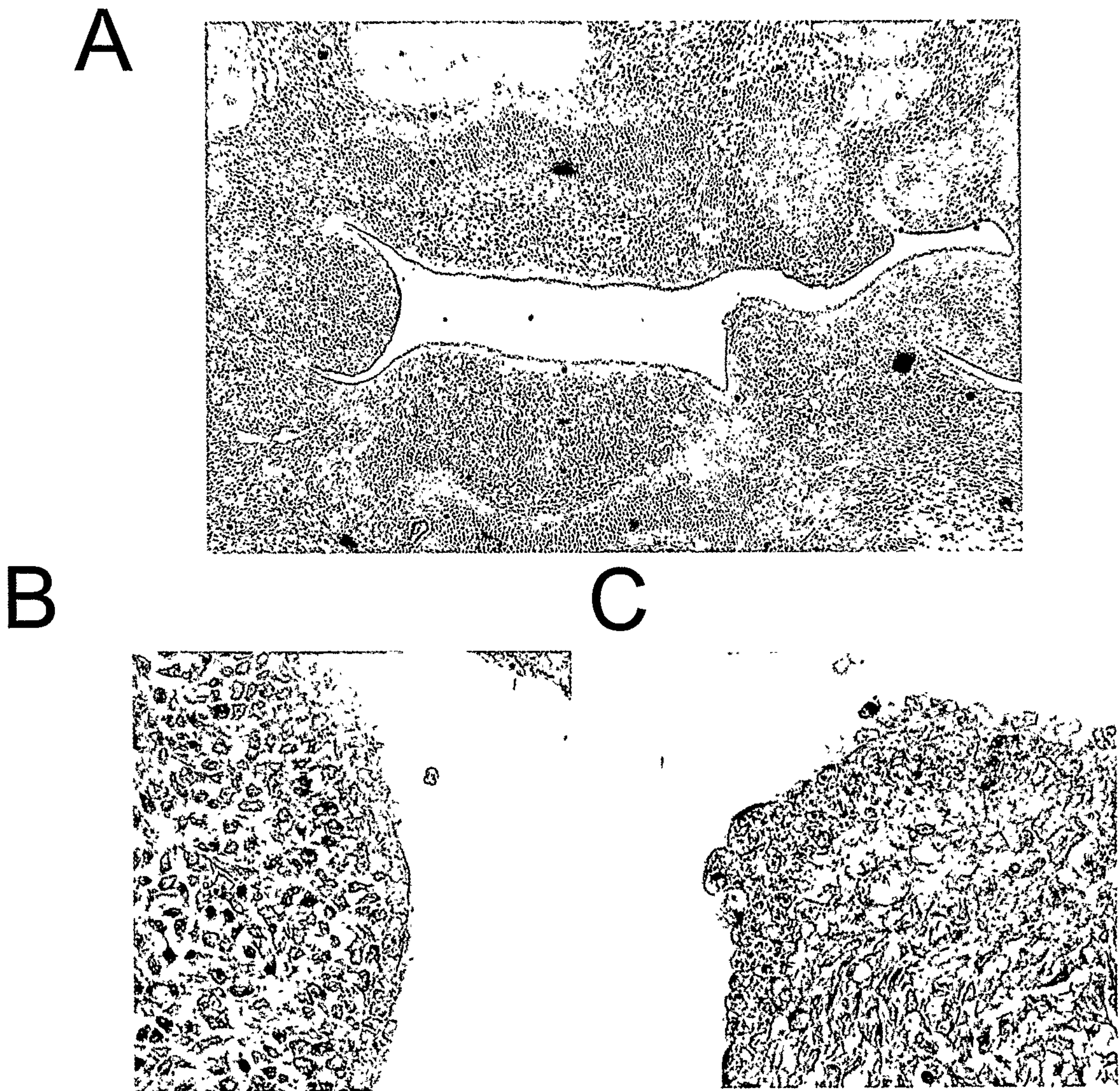


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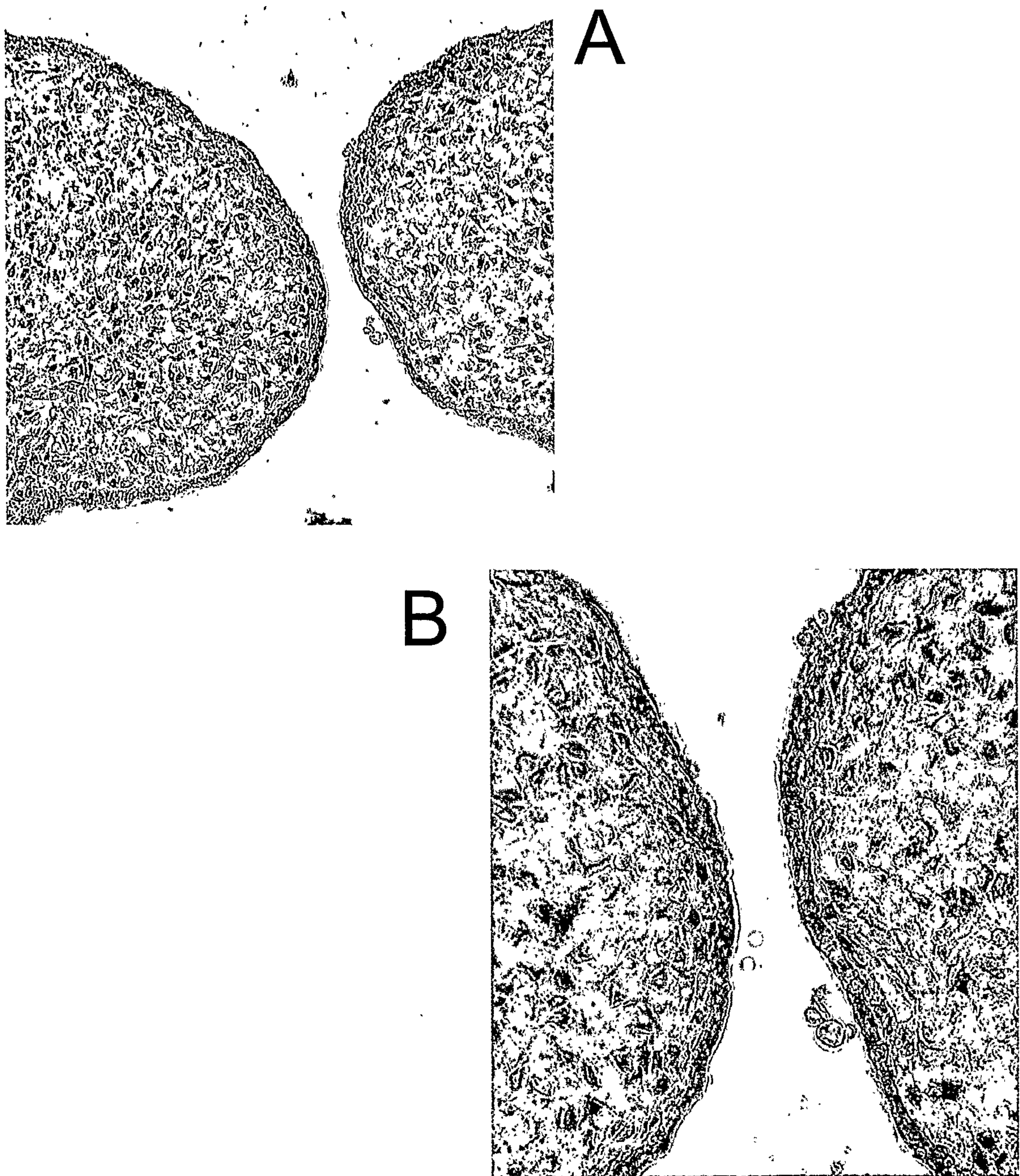


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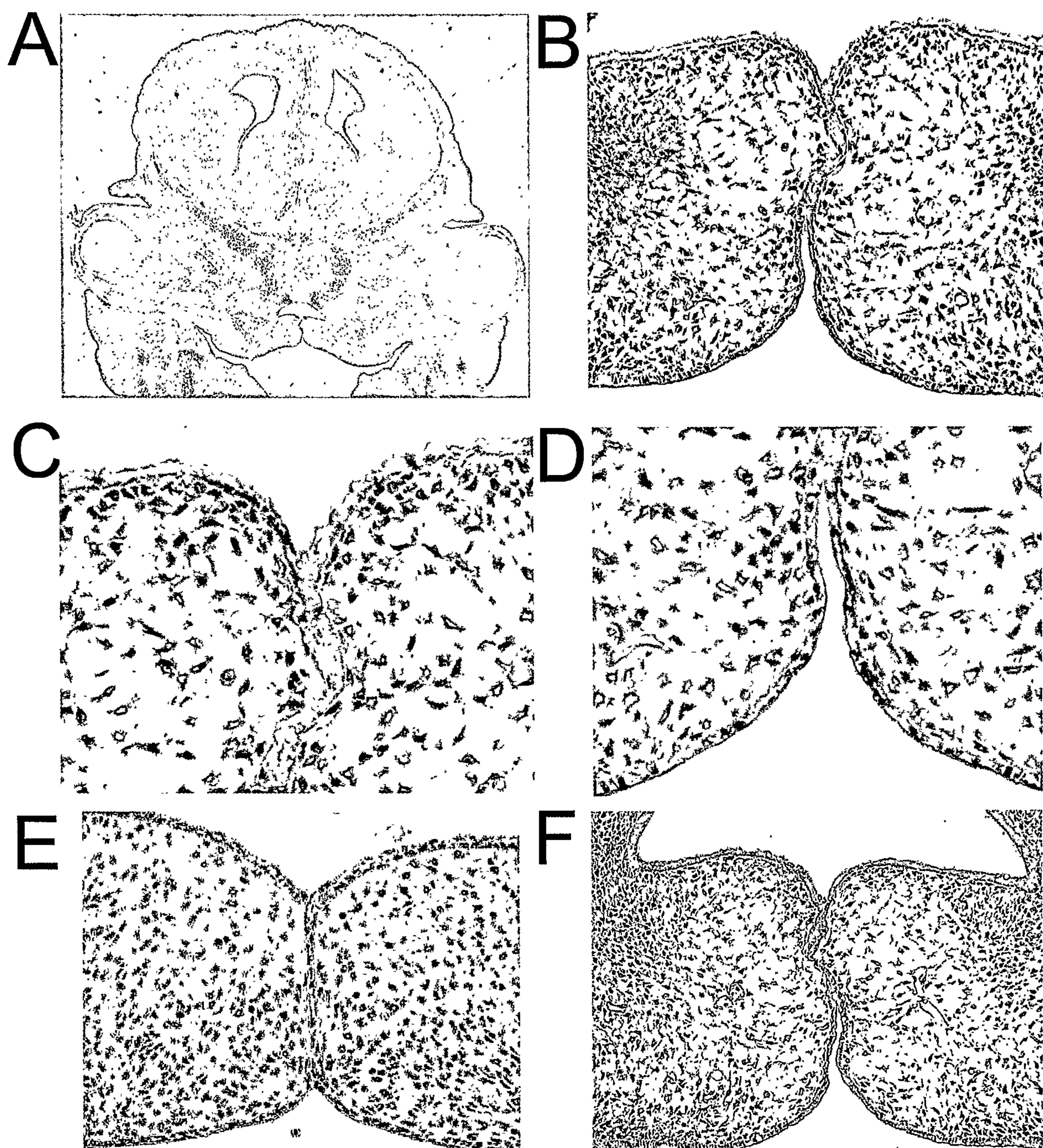


Figure 5. FGF8 was detected bilaterally in the pre-integrated MEE at palatal contact (16.5 to 17.5 dpc).

A&B) Sections at low magnification, revealing FGF8 expression in the embryo and fusing palate. C) Higher magnification of the nasal region, reveals specific bilateral FGF8 detection in the pre-integrated MEE. FGF8 was not detected in the central MEE which is integrated and proceeding to MES. D) Similarly, in the oral region, FGF8 was detected in specific bilateral pre-integrated MEE. E) Control with FGF8 blocking peptide. F) Control GAM-b only.

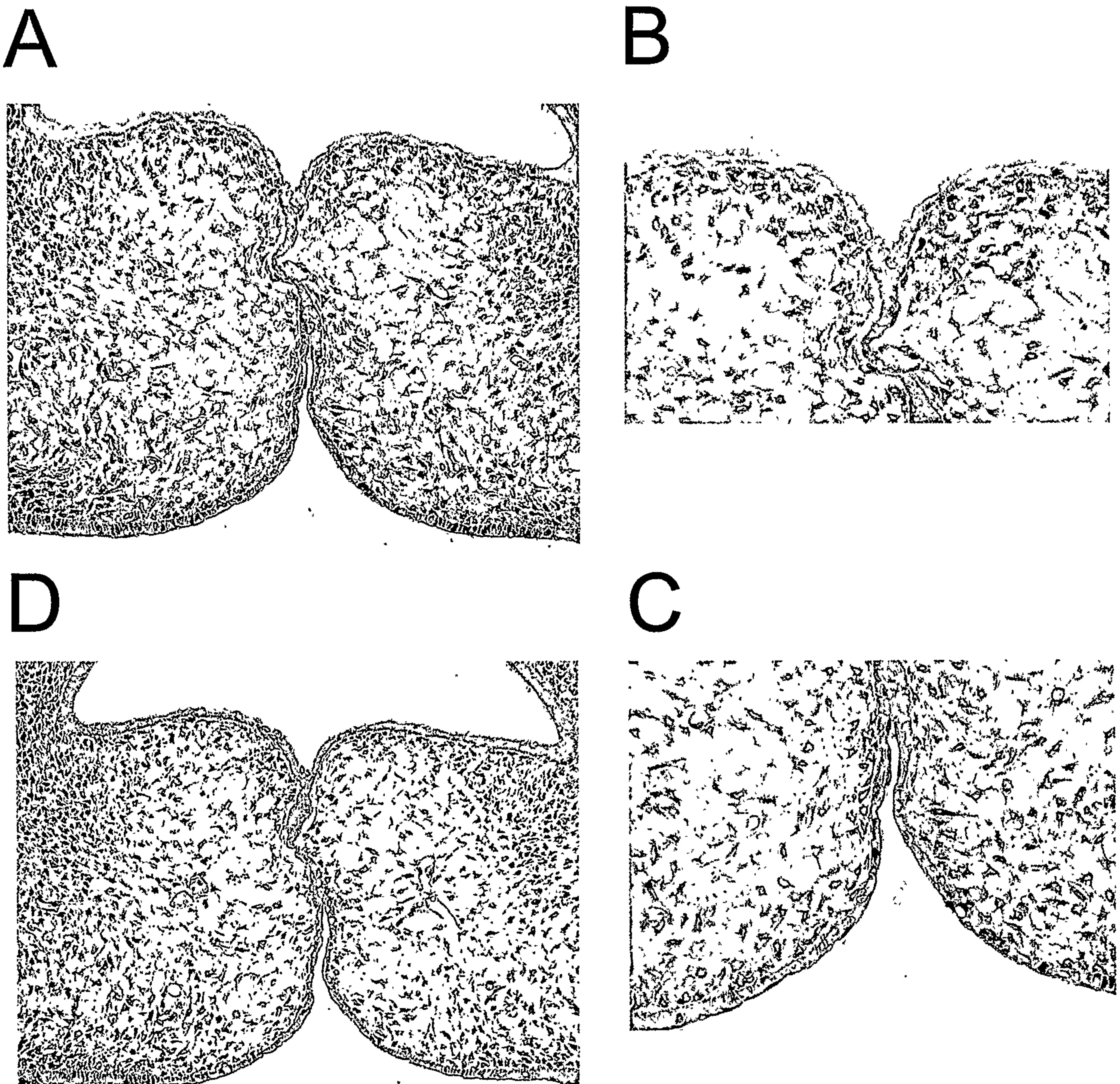


Figure 6. SHH was detected bilaterally in the pre-integrated MEE at palatal contact (16.5 to 17.5 dpc).

A) Section at low magnification, revealing bilateral SHH expression. B) Higher magnification of the nasal region, reveals specific bilateral SHH detection in the pre-integrated MEE. SHH was not detected in the central MEE which is in contact and proceeding to MES. C) Similarly, in the oral region, specific bilateral detection of SHH occurs only in the pre-integrated MEE. D) Control GAM-b only.

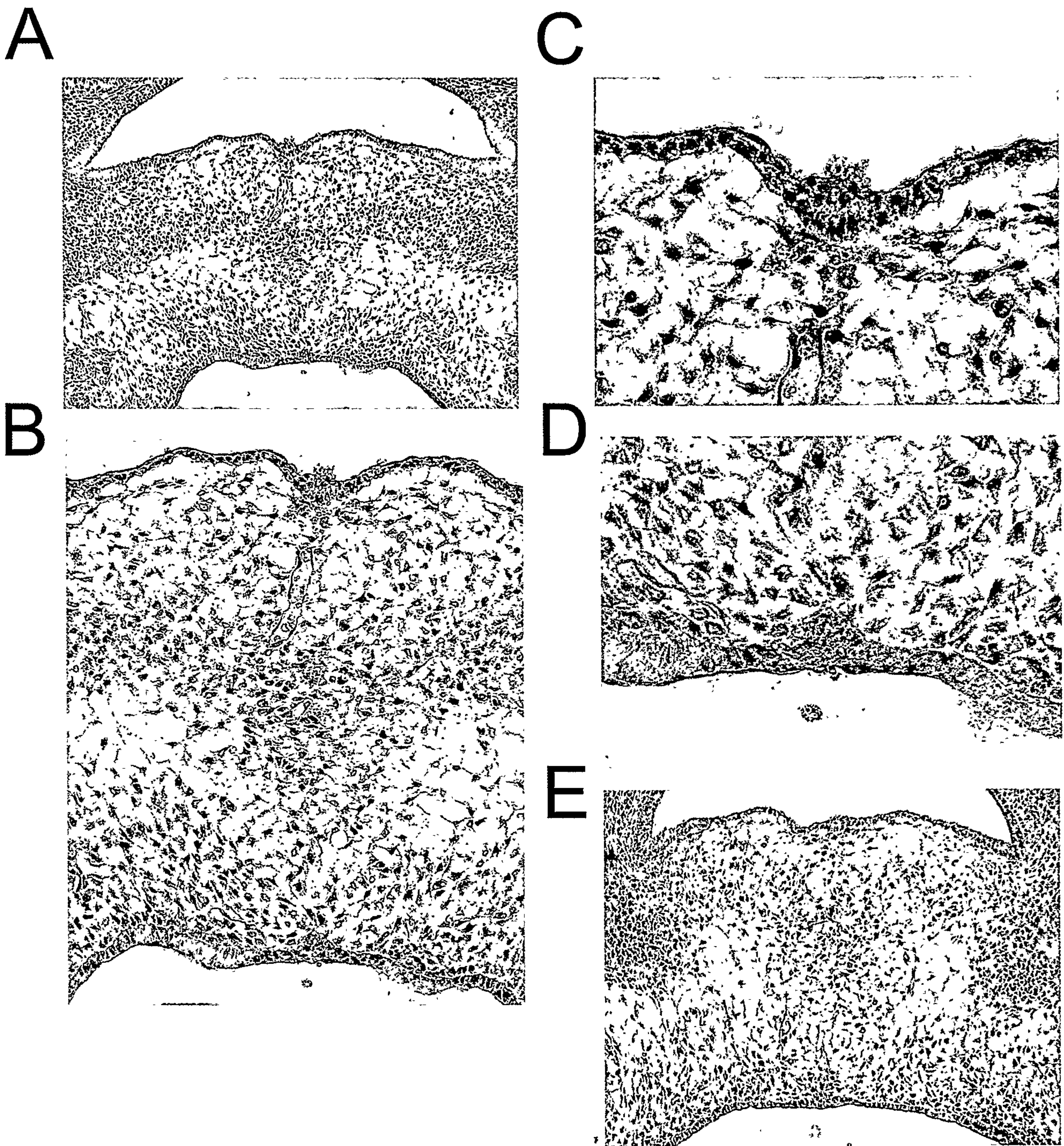


Figure 7. FGF8 was detected in the MEE remnants at completion of palatal fusion.

A&B) Sections at low magnification, revealing FGF8 expression at the completion of palatal fusion. C) Higher magnification of the nasal region, reveals specific FGF8 detection in the remnant MEE cells, prior to integration. The central MES is integrated and FGF8 is not detected. D) Similarly, in the oral region, specific FGF8 expression is detected only in the remnant MEE cells, prior to integration. E) Control GAM-b only.

APPENDIX

FGF-8 (N-19): sc-6958**BACKGROUND**

Fibroblast growth factor-1 (FGF-1), also designated acidic FGF, and fibroblast growth factor-2 (FGF-2), also referred to as basic FGF, are members of a family of growth factors that stimulate proliferation of cells of mesenchymal, epithelial and neuroectodermal origin (1). Additional members of the FGF family include the oncogenes FGF-3 (Int2) and FGF-4 (hst/Kaposi), FGF-5, FGF-6, FGF-7 (KGF), FGF-8 (AIGF), FGF-9 (GAF) and FGF-10 (2-8). Members of the FGF family share 30-55% amino acid sequence identity, similar gene structure, and are capable of transforming cultured cells when overexpressed in transfected cells. Cellular receptors for FGFs are members of a second multigene family including four tyrosine kinases, designated Flg (FGFR-1), Bek (FGFR-L), TKF and FGFR-3 (9).

SOURCE

FGF-8 (N-19) is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the amino terminus of FGF-8 α of mouse origin (identical to corresponding human sequence).

PRODUCT

Each vial contains 200 μ g IgG in 1.0 ml of PBS containing 0.1% sodium azide and 0.2% gelatin.

Blocking peptide is available for competition studies (sc-6958 P) (100 μ g peptide in 0.5 ml PBS with 0.1% sodium azide and 100 μ g BSA).

SPECIFICITY

FGF-8 (N-19) reacts with each of the FGF-8 alternative splice forms of mouse, rat and human origin by Western blotting and immunohistochemistry.

Recommended dilution range for Western blot analysis: 1:100–1:1000. Recommended starting dilution: 1:100.

Molecular Weight of FGF-8: 26 kDa.

Western blotting positive controls: A-431 WCL, sc-2201; NIH/3T3 WCL, sc-2210; IMR-32 WCL, sc-2409.

STORAGE

Store at 4° C, do not freeze; stable for one year from the date of shipment.

RESEARCH USE

For research use only, not for use in diagnostic procedures.

REFERENCES

1. Rifkin, D.B. and Moscatelli, D. 1989. Recent developments in the cell biology of fibroblast growth factor. *J. Cell Biol.* **109**: 1-6.
2. Moore, R., Casey, G., Brookes, S., Dixon, M., Peters, G., and Dickson, C. 1986. Sequence, topography and protein coding potential of mouse *int-2*: a putative oncogene activated by mouse mammary tumor virus. *EMBO J.* **5**: 919-924.
3. Delli Bovi, P., Curatola, A.M., Kern, F.G., Greco, A., Ittmann, M., and Basilico, C. 1987. An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. *Cell* **50**: 729-737.
4. Zhan, X., Bates, B., Hu, X., and Goldfarb, M. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* **8**: 3487-3495.
5. Marics, I., Adelaide, J., Raybaud, F., Mattei, M., Coulier, F., Planche, J., Lapeyriere, O., and Birnbaum, D. 1989. *Oncogene* **4**: 335-340.
6. Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H., and Matsumoto, K. 1992. Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. *Proc. Natl. Acad. Sci. USA* **89**: 8928-8932.
7. Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. 1993. Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Mol. Cell. Biol.* **13**: 4251-4259.
8. Beer, H.D., Florence, C., Dammeier, J., McGuire, L., Werner, S., and Duan, D.R. 1997. Mouse fibroblast growth factor 10: cDNA cloning, protein characterization, and regulation of mRNA expression. *Oncogene* **15**: 2211-2218.
9. Dionne, C.A., Crumley, G., Bellot, F., Kaplow, J.M., Searfoss, G., Ruta, M., Burgess, W.H., Jaye, M., and Schlessinger, J. 1990. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J.* **9**: 2685-2692.

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Shh (N-19): sc-1194

BACKGROUND

The *Drosophila* segment polarity gene hedgehog (hh) encodes a precursor protein which undergoes autocleavage to generate amino and carboxy terminal peptides of 19 kDa and 25 kDa molecular weight respectively (1-3). Both proteins are secreted and appear to function in embryonic and imaginal disc patterning (1-5). Several vertebrate homologs of *Drosophila* hh have been identified. These include Sonic hedgehog (Shh) (alternatively designated Vhh-1), Desert hedgehog (Dhh) and Indian hedgehog (Ihh) (1,6). Each contain amino terminal signal peptides and apparently function as secreted proteins involved in the mediation of various cell-cell interactions (1,6). Shh resembles *Drosophila* hh in that it is processed to generate an amino terminal 19 kDa secreted peptide that is retained at or near the cell surface and a carboxy terminal 27 kDa glycosylated more diffusible peptide (2,3,7).

SOURCE

Shh (N-19) is an affinity purified goat polyclonal antibody raised against a peptide mapping at the amino terminus of Sonic hedgehog protein (Shh) of human origin (identical to corresponding mouse sequence).

PRODUCT

Each vial contains 200 µg IgG in 1.0 ml of PBS containing 0.1% sodium azide and 0.2% gelatin.

Blocking peptide is available for competition studies (sc-1194 P) (100 µg peptide in 0.5 ml PBS with 0.1% sodium azide and 100 µg BSA).

SPECIFICITY

Shh (N-19) reacts with the amino terminal subunit of Sonic hedgehog (Shh) of mouse, rat and human origin by Western blotting, immunoprecipitation, immunohistochemistry and ELISA; non cross-reactive with Dhh or Ihh.

Recommended dilution range for Western blot analysis: 1:100-1:1000. Recommended starting dilution: 1:100.

STORAGE

Store at 4° C, do not freeze; stable for one year from the date of shipment.

RESEARCH USE

For research use only, not for use in diagnostic procedures.

REFERENCES

1. Roelink, H., Porter, J.A., Chiang, C., Tanabe, Y., Chang, D.T., Beachy, P.A., and Jessell, T.M. 1995. Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**: 445-455.
2. Fan, C.M., Porter, J.A., Chiang, C., Chang, D.T., Beachy, P.A., and Tessier-Lavigne, M. 1995. Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81**: 457-465.
3. Marti, E., Bumcrot, D.A., Takada, R., and McMahon, A.P. 1995. Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**: 322-325.
4. Li, W., Ohlmeyer, J.T., Lane, M.E., and Kalderon, D. 1995. Function of protein kinase A in hedgehog signal transduction and *Drosophila* imaginal disc development. *Cell* **80**: 553-562.
5. Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T.M., and Edlund, T. 1995. Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**: 747-756.
6. Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., and McMahon, A.P. 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**: 1417-1430.
7. Johnson, R.L. and Tabin, C. 1995. The long and short of hedgehog signaling. *Cell* **81**: 313-316.