Original Article

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

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ABSTRACT   **Secondary palatal fusion is dependent on targeted removal of the epithelium between the palatal shelves. Aseptically delivered rat embryos 15 through 18 days post coitum (dpc) were probed with DIG-labeled antisense and sense ssDNA probes for spliced exon sequences flanking intron E of cytokeratins K5/6 and spliced exon sequences flanking intron F of vimentin. Cytokeratin K5/6 expression was upregulated in the medial edge epithelium (MEE) prior to rotation of the palatal shelves and in the vomerine epithelium in the region of fusion with the palate. K5/6 expression continued in the medial epithelial seam (MES) and in epithelial islands during breakdown of the MES. Vimentin expression was not detected in the MEE prior to rotation but was specifically upregulated in the MEE following rotation and prior to midline contact and continued in the MES and in epithelial cells identifiable during the breakdown of the MES. Initiation of vimentin upregulation in the MEE prior to contact of the palatal shelves was tested by serum-free organ culture of palates from embryos at 15.5 dpc with the shelves separated by a biocompatible membrane. Vimentin upregulation occurred in the epithelium specifically in the region of anticipated contact. These results are interpreted as indicating that i) cytokeratin K5/6 expression may play a critical role in the integration of the epithelial layers of the MES to ensure subsequent merging of the mesenchyme and ii) epithelial cells in the MEE are specifically 'primed' to upregulate expression of mesenchymal genes prior to integration into and breakdown of the MES.**

KEY WORDS: *palatal fusion, in situ hybridization, keratin K5, vimentin, epithelial-mesenchymal transition*

Introduction

Midline fusion events play crucial roles in many aspects of embryological development and are essential for normal formation of maxillo-facial structures during development of the head (Shuler, 1995). An extensively studied midline fusion event is the formation of the secondary palate (Ferguson, 1988; Hay, 1995) where failure of the fusion process contributes to palatal clefting, one of the most common congenital defects in humans (Gorlin *et al.*, 1990). In the developing maxilla in the rat, fusion of the secondary palate depends on the successful completion of a complex sequence of events occurring over a period from 15.5 to 17.5 days post coitum (dpc). The sequence of events in the rat is similar to that in the human except that approximately 10 days are required to progress through the sequence in the human (Greene and Pratt, 1976). Within a period of approximately 12 h in the rat, the tips of the two palatal shelves align in the midline, the medial edge epithelium (MEE) covering the tips comes into precise apposition and integrates to form the medial epithelial seam (MES). The MES disappears over the next 12 h as the mesenchyme from each side merges to form the fused palatal shelf.

The regional specificity of integration and dispersion of the MEE and sparing of the contiguous nasal and oral epithelium indicates

that the epithelium of the MEE must develop distinguishing characteristics immediately prior to or during the fusion process. Despite the close approximation of the epithelial surfaces of the MEE with the epithelium of the buccal and lingual mucosa prior to fusion, integration and dispersion only occurs in the MEE in the midline during palatal fusion in normal animals. Misdirected specificity of MEE integration has been described in mice homozygous for deletion of Jag2 which codes for the ligand for the Notch family of transmembrane receptors. These animals show a most unusual phenotype of fusion of the tips of the unrotated palatal shelves with the lateral surfaces of the tongue and die perinatally due to palatal clefting (Jiang *et al.*, 1998). The unique nature of the MEE prior to fusion is also indicated by the critical and possibly controlling role played by TGF-β3 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) where the requirement for TGF-β3 appears to be exclusive as midline fusion of the primary palate and the mandible occurs even if TGF-β3 is not present (Chai *et al.*, 1997; Kaartinen *et al.*, 1997). This paper reports the results obtained in a study designed to follow the expression of genes characteristic of epithelial or mesenchymal cellular differentiation during the palatal fusion sequence. The expression profiles of the genes for the intermediate filaments keratin K5/6 and vimentin were examined in the rat by *in situ* hybridization (ISH) on cranio-facial tissues removed from timed embryos and also in palatal explants in organ culture using probes for spliced exon sequences flanking specific introns in these genes. The results obtained indicate that expression of K5/6 and vimentin mRNAs are upregulated in the palatal epithelium in the region of presumptive fusion and that the pattern of vimentin upregulation anticipates the fusion process.

Results

Early expression of K5/6 and absence of vimentin expression in oral epithelium prior to palatal shelf elevation

At 15.5 dpc, the developing tongue fills both the presumptive nasal cavity and the oral cavity. The tissues that will form the secondary palate can be recognized as tapered 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 (Fig. 1A,C).

K5/6 expression was detected initially in the epithelium on the palatal processes in the regions that would become the edges of the palatal shelves following rotation (Fig. 1A,B). In frozen sections, expression of K5/6 was evident initially in the simple epithelium on the presumptive tips of the palatal shelves and ceased abruptly in the region where the epithelium became columnar (Fig. 1B, arrowhead). In the adjacent oral epithelium, expression of K5/6 was most intense in the thin, periderm-like layer on the surface and extended into the presumptive stellate reticulum of the dental anlages (Fig. 1A). The dorsal surface of the tongue was not removed in the preparation of embryonic heads at 15.5 dpc and it showed intense staining for K5/6 expression in the superficial layers. Expression of K5/6 was evident also in the ectoderm in the head region at 15.5-16 dpc, consistent with the early expression of K5 as described in the mouse ectoderm at equivalent developmental stages (Byrne *et al.*, 1994).

In embryonic heads removed at this stage, individual mesenchymal cells throughout the orofacial apparatus stained for vimentin

expression, but the intensity of staining was not equivalent in all cells. Clusters of mesenchymal cells exhibiting intense expression were evident (bands of strong staining in the mandibular mesenchyme Fig. 1C), with other regions showing less intense staining. Regions of intense activity were distributed in patterns bilaterally symmetrical around the midline axis and the patterns of distribution were dependent on the stage of development and consistent in adjacent sections of the same specimen. The epithelial cells covering the palatal shelves at this stage showed no evidence of vimentin expression while individual cells in the mesenchyme of the palatal shelf show strong vimentin expression (Fig. 1D).

Expression of vimentin initiated in palatal shelf epithelium prior to shelf contact

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 (Fig. 1E,F). The signal for expression of K5/6 was intense in the epithelium in the thinned regions on the tips of the shelves (Fig. 1F). Expression of vimentin in the mesenchyme was evident as localized regions showing a very strong signal (Fig. 1G). At this stage, the epithelium on the opposing palatal shelves also showed a strong signal for vimentin expression. Vimentin expression in the epithelium on the horizontally aligned palatal shelves was confined to the tips of the shelves in the regions of potential contact (Fig. 1H). The adjacent oral and nasal epithelium was free of staining for hybridized vimentin probe. The epithelium in the tip regions of the palatal shelves was uniformly thinner than the adjacent epithelium on both the palatal and nasal surfaces (Fig. 1G) and the vimentin expression was evident in the majority of the cells of this thinned region (Fig. 1H). The expression of K5/6 was more extensive than vimentin and continued into the periderm-like superficial layer on the oral surface. The patterns of expression for each of the genes were symmetrical across the midline.

Expression patterns of K5/6 and vimentin retained during epithelial fusion prior to breakdown of the seam

Following contact of the tips of the palatal shelves in the midline, the epithelial cells meld into a uniform epithelial seam (the MES) by 16.5 dpc (Fig. 1I,J,K) which then rapidly disperses leaving occasional islands of epithelial cells isolated in the mesenchyme (Figs. 1L,M,N and 2A to E) that persist for a short time and then disappear by 17.5 dpc . The expression patterns for K5/6 evident in the epithelial cells prior to fusion were also evident in epithelial cells identifiable during the integration (Fig. 1I) and dispersion (Fig. 1L,M,N) of the MES. Strong staining for expression of K5/6 occurred consistently in the epithelium during integration to form the MES (Fig. 1I). K5/6 expression persisted in the epithelium on both the nasal (Fig. 1N) and oral surfaces of the palatal shelves (Fig. 1M) immediately adjacent to the region of fusion and in morphologically identifiable islands (Fig. 1M,N, arrowheads). In the whole-mount preparations – viewed from the direction of the large arrowhead in (Fig. 1J) – expression of vimentin was apparent in the epithelium along the line of fusion (Fig. 1K).

Unlike the strong expression of vimentin in the majority of epithelial cells of the MEE prior to integration (Fig. 1H), not all cells in the remaining MES (Fig. 2C) and islands (Fig. 2D,E) showed strong expression for vimentin. Strong staining for vimentin expression was evident in the oral epithelium still continuous with the MES at the time

of removing and fixing the tissue (Fig. 2C). Expression of vimentin was also evident in cells in the region of oral epithelium immediately adjacent to the medial seam (Fig. 2C) and in the epithelium that will form the nasal lining as a consequence of the fusion process (Fig. 2E). Fragments of cytoplasm showing weak expression of vimentin (Fig. 2E) relative to the sense control (Fig. 2F) were associated with morphologically identifiable epithelial islands containing cells showing strong expression (Fig. 2E, arrowheads). The patterns of staining were consistent in sequential sections of the same specimens.

Expression of K5/6 in region of fusion between nasal vomer and palate

In the anterior region of the developing maxilla the downgrowth of the vomerine nasal process in the midline is coordinated with the development of the secondary palate, ending with fusion to the nasal surface of the palate to form the nasal septum. A striking finding in this study was the up-regulation of K5/6 expression evident in the nasal epithelium covering the vomer specifically in the region of potential fusion with the nasal surface of the recently fused palate (Fig. 1O, arrowhead). The induction of expression of K5/6 was confined entirely to the region of flattened nasal epithelium directly opposite the epithelium on the newly-formed nasal palatine surface also expressing K5/6 following the midline fusion of the palatal shelves (Fig. 1O).

At the same time as the fusion of the secondary palate is taking place, the ectoderm in the head region undergoes stratification and begins formation of hair follicles. The pattern of expression detected in the ectoderm in the head region (Fig. 1P) corresponded exactly with the pattern of K5 and K14 described by Byrne *et al.* (1994) in their study of early development of mouse ectoderm.

K5/6 and vimentin gene expression profile retained in palatal shelves separated by a biocompatible barrier in organ culture

The expression profiles for the genes for both K5/6 and vimentin were examined in palates explanted from 15.5 to 16 dpc embryos and cultured for 24 to 48 h in serum-free medium with a biocompatible barrier (nuclepore membrane) preventing direct contact between the palatal shelves (arrowheads in Fig. 2G,H,J). The patterns of expression detected in the organ cultures matched closely the profiles found *in vivo*. Strong K5/6 expression was concentrated in the thinned epithelium in the region of potential contact and in the epithelium extending onto the oral surface (Fig. 2H). In presumptive nasal epithelium retained in the explant (near the edge in contact with the Millipore substrate), K5/6 was not expressed. The epithelium on the tips of the palatal shelves in the region of presumptive fusion showed strong expression for vimentin (Fig. 2I). Vimentin expression was confined to the epithelium at the tips of the shelves and was not evident in contiguous epithelium on the presumptive oral and nasal surfaces. Strong vimentin expression in the organ cultures was also evident in mesenchymal cells in the body of the explants confirming the biosynthetic capability of the mesenchymal cells under the culture conditions. The sense controls for cytokeratin K5/6 and vimentin in the organ cultures were uniformly negative (Fig. 2J).

Discussion

Specificity of expression

Expression of intermediate filament genes was studied at the level of messenger RNA as a sensitive indicator of changes in gene expression occurring in the epithelial cells of the embryonic palate during the fusion process. High specificity was achieved with the ssDNA probes that were designed to span specific intron splice sites. The high homology between the relevant exon sequences of K5 and K6 meant that the probe used could detect both keratin sub-types (Paine *et al.*, 1992) and for this reason the probe is referred to as K5/6. The extremely good correspondence between the patterns of K5 detected with this probe in the ectoderm with the patterns reported in the development of the mouse skin (Byrne *et al.*, 1994) indicate that the probe is reliably indicating K5 expression.

Upregulation of K5/6 prior to fusion

The cells of the MEE expressed K5/6 prior to fusion and the cells that remain on both the nasal and oral surfaces immediately adjacent to the line of fusion following the breakdown of the MES continued to express K5/6 until at least 18 dpc when tissues ceased to be collected. The columnar nasal epithelium and the basal columnar oral epithelial cells contiguous with the MEE and the MES did not show evidence of K5/6 expression throughout the fusion sequence from 15.5 dpc to 17.5 dpc. The upregulation of K5/6 in specific relationship to regions of anticipated fusion is most dramatic in the nasal epithelium at the base of the vomer which fuses with the palate to form the nasal septum. K5/6 expression in the vomerine epithelium stops abruptly at the junction of the simple epithelium with the columnar epithelium on the lateral surfaces of the vomer (Fig. 1O). K5 mRNA expression has been shown to be upregulated in the ectoderm in the developing mouse concomitantly with the onset of stratification (Byrne *et al.*, 1994) and could be demonstrated to follow a similar pattern in the ectoderm in the rat in this study (Fig. 1P). Upregulation of K5/6 mRNA in strictly localized regions of the developing nasal and oral cavities in a simple epithelium that will disappear before stratification is even initiated was an unexpected finding. Formation of the MES is known to involve melding of the epithelia covering the opposing shelves to form an integrated structure complete with fully-formed desmosomal attachments prior to dispersion of the epithelium (Fitchett and Hay, 1989; Shuler *et al.*, 1991). One possible role of K5 expression in an epithelium already expressing K8 (unpublished results) could be a necessity to form a physical bond between the opposing shelves of sufficient strength to resist separation for a critical period of time to allow preparation for mesenchymal links to form across the midline. Keratin filaments containing K5 and the associated desmosomal structures normally formed in a stratified epithelium rather than those formed with K8/K18 in a simple epithelium may be required to resist increased shear forces generated by a muscular tongue that is becoming active at this stage (Luke, 1976) together with an increase in tensile force due to lateral expansion of the head. Defective palatal fusion has not been reported in K14 null mice which develop severe blistering disorders in the skin similar to human Epidermolysis bullosa simplex (Lloyd *et al.*, 1995). The residual keratin network formed by co-polymerization of K15 with K5 (Lloyd *et al.*, 1995) may be functionally competent in early development in the K14 null animals allowing normal development of the palate. Even K18 which normally copolymerizes with K8 is able to partially compensate for the absence of K14 when overexpressed as a transgene in K14 null animals (Hutton *et al.*, 1998). Ablation of the keratin K5 gene itself would be necessary to determine if K5 expression is required for palatal fusion.

Upregulation of vimentin indicates initiation of epithelial transdifferentiation

Formation and disappearance of identifiable epithelial structures appears obligatory for midline fusion events in the developing head region in mammals (Ferguson, 1988) and will also occur in birds *in vitro* if the developing beak is removed and TGF-β3 is added (Sun *et al.*, 1998b). In the rodent secondary palate, the medial epithelial seam (MES) forms and disperses rapidly, within a period of less than 24 h. Transdifferentiation of the epithelial cells into mesenchymal cells (EMT) along the line of fusion as the mechanism for the dispersion of the MES has the support of a substantial body of accumulated evidence (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler *et al.*, 1992; Shuler, 1995; Sun *et al.*, 1998b). EMT as a mechanism for dispersion of the MES is supported by the localized upregulation of vimentin expresion in cells prior to contact in the midline demonstrated in this study and interpreted as indicating initiation of mesenchymal gene expression in cells to undergo dispersion as fusion progresses. EMT involves a switch in the pattern of gene expression from epithelial to mesenchymal and an associated change in phenotype from stationary and integrated to motile and independent (Hay, 1995). Loss of expression of the cell surface molecules syndecan-1 and E-cadherin associated with adhesion of epithelial cells has been observed and interpreted as indicating cells switching from an epithelial to a mesenchymal phenotype in anticipation of the dispersion of the seam during palatal fusion (Sun *et al.*, 1998a). In the case of the cells of the MES in the rat, these changes must be achieved within a time constraint of the order of 24 h observed for the process as whole and DNA synthesis has been shown to cease 24-36 h before epithelial contact in the midline (Greene and Pratt, 1976). This means that the cells of the MEE would have to be committed to differentiate along a mesenchymal pathway prior to contact so as to be able to disperse into the mesenchyme of the palate as mesenchymal cells within the time available. Aspects of EMT have been studied *in vitro* in culture systems based on tumor-derived cell lines (Boyer *et al.*, 1989; Gibbins *et al.*, 1991) where the changes in gene expression following malignant change included down-regulation of keratin and upregulation of vimentin and collagenase (Boyer *et al.*, 1989; Paine *et al.*, 1992, 1993). Similar changes in gene expression could be evoked in a tumor-derived cell line by activation of an inducible transfected fos gene (Reichmann *et al.*, 1992) but upregulation of expression of these genes by *fos* required several days. It was shown subsequently that the upregulation of the mesenchymal-type genes was preceded by a switch in Fibroblast Growth Factor Receptor Type 2 (FGFR2) from an epithelial type (KGFR) to a mesenchymal type (BEK) that occurred within 4 h of stimulation of the transfected *fos* gene (Scotet *et al.*, 1995). The results reported here indicate that the MEE is a specific target population primed to initiate changes in expression in anticipation of subsequent signal or signals presumably required to complete the transition process. The observation that not all the cells in the MES and in the residual seam and islands show strong vimentin expression (Fig. 2C,D,E) suggests that not all the cells of the MES undergo EMT during dispersion and this corresponds with the demonstration of alternative fates such as apoptosis (Mori *et al.*, 1994) or migration (Carette and Ferguson, 1992) for some of the cells of the MES. It is considered unlikely that vimentin gene expression itself in the cells of the MES plays a critical role in the fusion process as mice homozygous for a null gene for vimentin develop and function normally and have not been reported to suffer from defects in midline fusion events during development, including palatal clefting (Colucci-Guyon *et al.*, 1994). The localized upregulation in vimentin expression in the cells of the MEE does indicate that these epithelial cells are functionally distinct from nearby oral and nasal epithelium. Also, upregulation of vimentin in the MEE in organ culture where physical contact between the opposing palatal shelves was prevented indicates that the putative 'priming' signal does not require contact between the epithelia of the shelves. This result suggests that the signal was either present in the tissue before explanting or was generated locally in the explant during the period of culture.

Control of expression of K5/6 and vimentin in anticipation of fusion

The apparently coordinated expression of K5/6 and vimentin in the epithelium that will be involved in midline fusion in the secondary palate indicates that this epithelium is responding to an inductive signal. A similar regionally restricted activation of an epithelial-to-mesenchymal cell transition has been demonstrated in the developing heart where the endothelial cells generate the cushion mesenchyme prior to cardiac septation and valvulogenesis (Nakajima *et al.*, 1994). Studies *in vitro* have shown that the regional specificity in endocardial cells in the developing heart appears to require both a paracrine factor produced by the myocardium and expression of TGF-β3 in the endocardial cells for EMT of the endocardial cells to occur (Nakajima *et al.*, 1998). The requirement for TGF-β3 in the development of the heart may be less strict than for the fusion of the secondary palate as developmental defects in the heart have not been reported in TGF-β3 null homozygous mice (Proetzel *et al.*, 1995; Kaartinen *et al.*, 1997). Another interesting correspondence between the mechanisms underlying the process of EMT in fusion of the secondary palate and development of the cardiac outflow tract is the recognition that the neural crest is known to be a major contributor to both craniofacial mesenchyme (Johnston and Bronsky, 1995) and early cardiac development (Creazzo *et al.*, 1998). The upregulation of vimentin expression in palatal explants in organ culture indicates that the epithelial cells in the MEE of the secondary palate must have received an inductive signal prior to the excision of the palates. Fusion of the secondary palate appears to rely on a different mechanism from fusion of the primary palate and mandibular prominences as these are not affected in mice lacking the gene for TGF-β3 (Chai *et al.*, 1997). The particular requirement for TGF-β3 for fusion of the secondary palate appears to be involved in the mechanisms underlying the dispersion rather than the formation of the MES as the MEE from the apposing palatal shelves from TGF-β3 -/- mice will adhere but will not form a mesenchymal bridge in organ culture without exogenous TGF-β3 (Kaartinen *et al.*, 1997).

The results reported in this paper provide initial evidence that the epithelial cells in the regions of presumptive fusion in the secondary palate exhibit a pattern of intermediate filament gene expression at the level of messenger RNA that supports an interpretation of priming of the cells in anticipation of integration into a common epithelial structure followed by dispersion of the cells into the mesenchyme bridging across the midline. Further studies will be required to determine the factors controlling these changes in gene expression in the development of the palate.

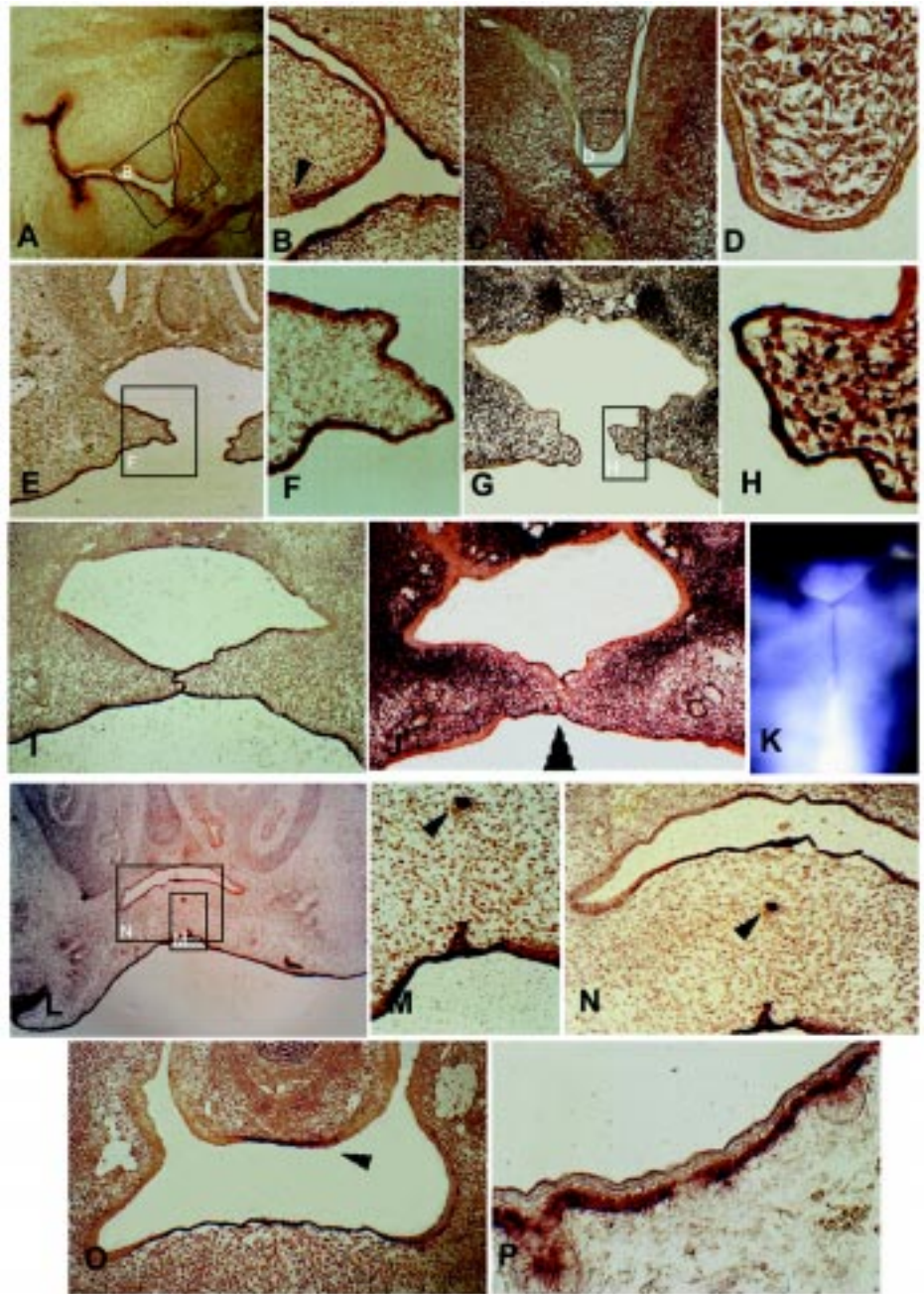**Fig. 1. Keratin K5/6 and vimentin differentially expressed during palatal fusion *in vivo*. (A-D)** *15.5 dpc. The tissue that will form the palate lies lateral to the tongue, which occupies the developing nasal cavity. The tongue is to the right of the palatal shelves in the illustrations.* **(A,B)** *Cytokeratin K5/6. At low magnification (A) expression is evident in the maxillary and mandibular tooth germs and the dorsal surface of the tongue. The palatal shelf adjacent to the tongue in the outlined box at higher magnification (B) shows strong expression of K5 in regions of the oral mucosa and lateral surface of the tongue. Expression of K5 in the developing palatal shelf ends abruptly in the region of low columnar epithelium (arrowhead in B).* **(C,D)** *Vimentin. Localized areas of intense staining for vimentin expression in the mesenchyme at low magnification (C) contrast with the absence of vimentin expression in the oral epithelium. Epithelium at the tip of the palatal shelf does not show evidence of vimentin expression at higher magnification (D).* **(E-H)** *Sixteen to 16.5 dpc. The tongue has moved down and out of the nasal cavity and the palatal shelves have rotated to lie opposite each other but have not come into contact. (The tongue has been removed in the preparation of these specimens).* **(E,F)** *Cytokeratin K5/6. Expression in the epithelium on the ends of the opposed palatal shelves and extending onto the presumptive nasal and oral surfaces of the palatal shelves with no expression in the epithelium on the lateral and upper surfaces of the nasal cavity (E). The simple epithelium (F) in the region of presumptive contact (the MEE) shows high expression of K5/6.* **(G,H)** *Vimentin. Regions of high expression in the mesenchyme form patterns symmetrical about the midline evident at low magnification (G). The epithelium on the ends of the palatal shelves at higher magnification (H) shows staining indicative of strong expression confined to the region of the MEE. The intensity of staining in the epithelial cells is equivalent to individual cells in the underlying mesenchyme.* **(I-K)** *16.5 to 17 dpc. The palatal shelves have come into contact in the midline and the epithelium from each shelf has intercalated to form the midline epithelial seam (MES).* **(I)** *Cytokeratin K5/6. The epithelium of the MES shows strong staining for K5/6 expression as does the surface of the epithelium on the oral and nasal side of the palate. The epithelium lining the sides of the developing nasal cavity does not show expression.* **(J,K)** *Vimentin. (J) Intense expression in the mesenchyme in a pattern symmetrical about the midline. Strong expression in regions of the MES and also in the epithelium on the nasal and oral surfaces immediately adjacent to the MES. (K) Whole-mount preparation of the region of midline contact and generation of the MES (viewed from the direction indicated by the arrowhead in panel J). The dark regions at the top and sides are due to the intensity of staining of the large bulk of mesenchymal tissues deeper in the specimen.* **(L-N)** *Seventeen to 17.5 dpc. The thickness of the palatal shelves has increased and the MES is in the process of disintegration allowing the mesenchyme to merge across the midline.* **(L,M** *and* **N)** *Cytokeratin K5/6. Expression of K5/6 in the midline is confined to the remnants of the MES evident as either islands of cells (arrowheads in M and N) or segments still continuous with the oral epithelium. On the oral surface, K5/6 expression occurs across the entire surface but is confined to the epithelium near the midline on the nasal side of the palate (N). Localized expression is also evident in the simple nasal epithelium on the roof of the nasal cavity in the midline directly opposite the region of palatal fusion (N). The intensity of staining in regions of the remnants of the MES is reduced relative to adjacent epithelial cells continuous with the surface (M).* **(O)** *Cytokeratin K5/6. In the anterior region of the secondary palate the nasal vomerine process also fuses with the dorsal surface of the developing palate by a similar mechanism to form the nasal septum. The epithelium on the vomer in the region of presumptive fusion shows a highly localized upregulation of K5/6 that does not extend to adjacent and contiguous epithelium on the lateral surfaces of the vomer.* **(P)** *Ectoderm - Cytokeratin K5/6. Expression of K5 and K14 in the developing ectoderm in the mouse is confined to the basal layers when the ectoderm undergoes stratification and begins formation of hair follicles (Byrne et al., 1994). The pattern of staining with the probe used in this study of K5/6 expression in the rat shows expression in the basal layers of the stratifying ectoderm and absence of expression in the downgrowths of the initial hair follicles; a pattern consistent with the pattern of expression defined in the developing mouse ectoderm.*
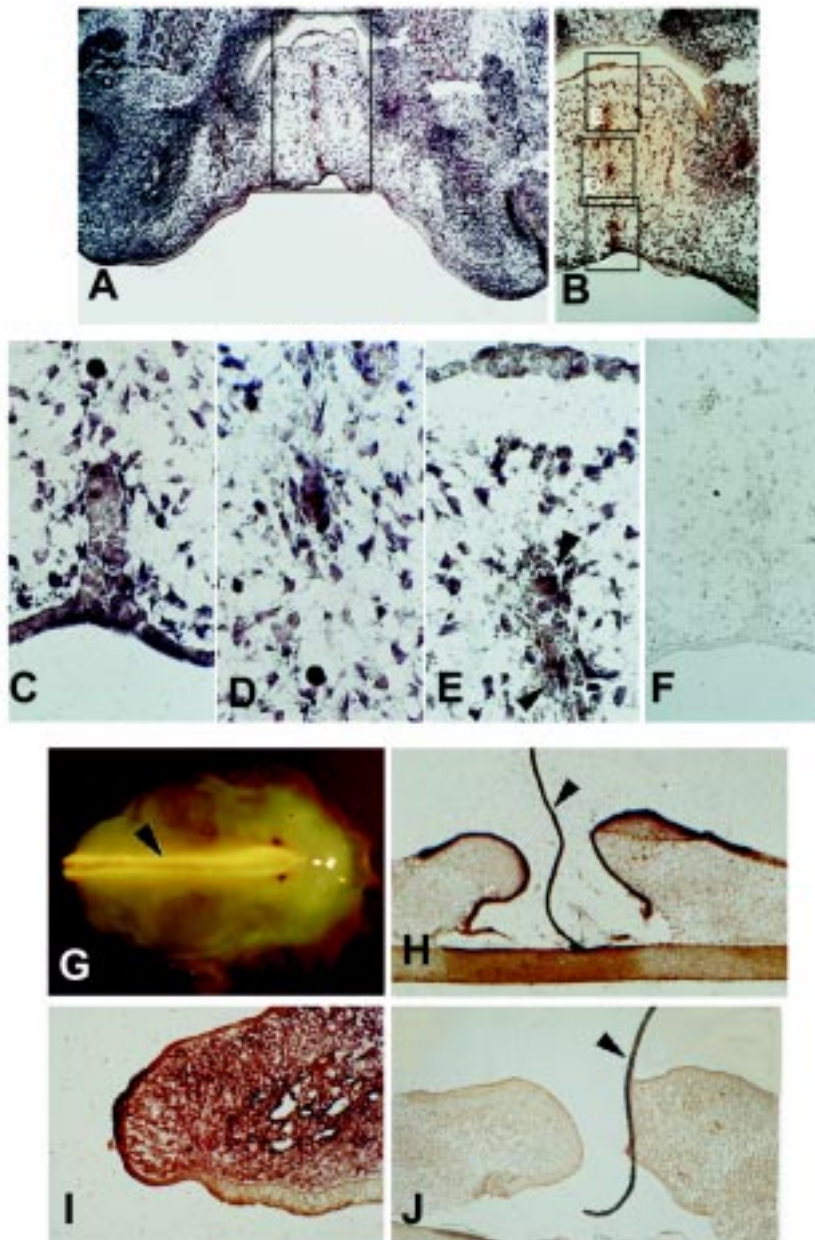
Fig. 2. Vimentin expression in epithelial cells during seam dispersal *in vivo* and prior to palatal shelf contact in organ culture. (A-F) *Seventeen dpc - Vimentin. Expression of vimentin during disappearance of the MES when fragments of the MES can still be identified.* **(A)** *The region of fusion shown in greater detail in panels B through F. The separation of the epithelium on the nasal side of the palate producing a vesicular space evident at the top of the boxed region is a consistent observation at this stage of development irrespective of the method of specimen preparation.* **(B)** *The regions presented individually in panels C, D and E.* **(C)** *Residual MES continuous with the epithelium on the oral side of the palate. Individual cells still identifiable as part of the disintegrating MES show strong expression of vimentin. Strong staining is also evident in the intact epithelium on the oral surface.* **(D)** *Apparently isolated island near the middle of the region of palatal fusion morphologically recognizable as epithelial showing regions of strong vimentin expression.* **(E)** *Remnants in the zone of the MES with regions showing strong expression of vimentin (arrowheads) surrounded by profiles consistent with cytoplasmic debris.* **(F)** *A section from the same region processed identically following hybridization with the sense ssDNA probe for vimentin as a control for the antisense ssDNA probe shows no reaction product in the epithelium or mesenchyme.* **(G-J)** *Organ culture with biocompatible physical separator.* **(G)** *Plan view of an organ culture of a palatal explant cultured for 48h with a biocompatible barrier (arrowhead) preventing direct contact between the palatal shelves. The anterior end of the explant is on the right and the barrier extends beyond the posterior edge of the explant. The Millipore membrane substrate for the organ culture has been stained prior to placing of the explants to facilitate observation and handling of the tissues.* **(H)** *Cytokeratin K5/6. Frozen section of organ culture with palatal shelves prevented from coming into physical contact by a Nuclepore membrane (arrowhead) shows strong expression of K5/6 in the epithelium in the region of the MEE and the oral surface. (The palatal explants were placed on the Millipore support with the oral surface uppermost after microdissection from the embryonic maxilla).* **(I)** *Vimentin. Strong expression of vimentin localized in the epithelium in the region of the MEE in organ culture with a membrane barrier. Adjacent epithelium on both the oral and the nasal surfaces does not show expression. Strong staining for hybridized probe is also evident in the mesenchyme in the central region of the palatal shelf.* **(J)** *Control sense ssDNA. K5/6 sense probe control for organ culture in panel H. The biocompatible separator has been displaced during preparation of the frozen section. (Sense ssDNA probes for K5/6 or vimentin run as controls for the antisense ssDNA probes and processed identically following hybridization showed no reaction product in the epithelium or mesenchyme).*

Materials and Methods

Care was taken to maximize the preservation and retention of RNA by preventing RNAse exposure in all preparatory and experimental steps. Gloves were worn at all times and all glassware and instruments were washed and rinsed in MilliQ H2O and baked in a dry air oven at 160-180°C for 6 h or overnight (O/N). All solutions used for the detection of mRNA in tissues were prepared using autoclaved MilliQ-H2O.

Tissue collection

Individual male rats were placed O/N in cages containing Sprague-Dawley female rats in prime breeding condition. Gestation times were determined from the morning following removal of the male rat as being half a day post coitum (dpc). The uterine horns of pregnant dams timed to be from 15 to 18 dpc were surgically removed under anaesthesia and the embryos dissected under sterile conditions. The embryos were decapitated, the mandible removed from the heads of embryos 16.5 dpc or older,

and the stage in the fusion process determined by inspection of the palates under a dissecting microscope.

Organ culture of palatal explants

Palates were micro-dissected under sterile conditions from the heads of embryos obtained before the palatal shelves had come into contact (15.5-16 dpc) and placed on the Millipore rafts so that the two palatal shelves were correctly orientated but separated by the Nuclepore membrane. Care was taken not to damage the epithelial surfaces of the palatal shelves during dissection and positioning of the explants. The Millipore rafts were then placed in Falcon organ culture dishes containing sufficient serum-free culture medium to wet the Millipore raft from the underside and cultured in a humidified incubator in 95% air/ 5% CO_2 at 37°C for 24 or 48 h. The culture medium was based on the medium used by Brunet *et al.* (1995) with the addition of non-essential amino acids. At the end of the culture period, the rafts were removed, fixed immediately and subsequently processed identically to tissues removed directly from animals.

Tissue processing for frozen sections

The dissected embryonic heads and organ culture rafts were fixed for a minimum of 2 h in ice-cold Karnovsky's fixative – 2% paraformaldehyde+2.5% glutaraldehyde in phosphate-buffered saline (PBS) –, washed in PBS, soaked in 30% sucrose in PBS+2 mM $MgCl_2$ overnight, then embedded in O.C.T. compound and stored in the -70°C freezer. Cryostat sections at 7-8 μm were mounted on Silane-coated slides, left to dry for 30 min and stored desiccated at -70°C prior to hybridization.

Preparation of probes

Digoxygenin (DIG)-labeled ssDNA probes were generated using the method described in Paine *et al.* (1995). Rat cytokeratin K5 probes 197bp in length were generated using a plasmid containing cDNA for exons 5 and 6 as template with primers TGGACCTGGACAGCATCATCG (rk51J) and TCTCAGCCTCTGGATCATCCG (rk53) (Paine *et al.*, 1992).

Rat vimentin probes 189bp in length were generated with primers for conserved regions of exons 6 and 7 flanking intron F of the R. norvegicus mRNA sequence X62952 designed using CPrimer and Amplify 1.2. Forward primer (fV6) TACCAGGACACTATTGGCCG and reverse primer (rV6) GGAAGAAAAGTTTGGAAGAGGC were used with DNA from a pGEM-T plasmid (Promega) containing the vimentin exon 6 and 7 sequences flanking intron F as the primary template to generate the relevant antisense and sense probes on a Corbett Research Thermal Sequencer (FTS-320). This probe was designed specifically to span the sixth intron of the gene for the intermediate filament vimentin in rat and to discriminate from sequences other than vimentin in the Genbank database. The spliced product was cloned from cDNA and the intron and flanking exon regions were cloned from genomic DNA. The cloned DNA was sequenced, the identity of the exon probe confirmed and the intron compared with the same intron from other rodent species (mouse and hamster) and human. The exon clone was used to generate ssDNA probes with the specific primers. Template sequences were confirmed with bi-directional automatic sequencing before being used to generate labeled probes and the labeled probes were checked by electrophoresis prior to each labeling run. Primers were synthesised by Macromolecular Resources DNA Express (Department of Biochemistry, Colorado State University).

In situ hybridization (ISH)

Permeabilization and pre-hybridization treatment

Slides were allowed to come to room temperature, washed in PBS for 5 min, 0.3% triton-x-100 in PBS for 20 min, PBS, 50 mM TRIS pH 7.4, then digested in proteinase K solution (50 mM TRIS+4 μg/ml proteinase K) at 37°C for 20 min. Digestion was stopped with 0.1 M Glycine in PBS, the slides post-fixed in 0.4% paraformaldehyde for 5 min on ice, rinsed in H_2O and transferred to 2xSSC for 5 min incubation. Three hundred μl of pre-hybridization solution was applied to each slide and then incubated at 55°C in a moist incubation chamber for at least 30 min. The pre hybridization solution was composed of 50% deionised formamide, 6xSSC, 5xDenhart's solution (1xDenhart's solution consists of 0.1g polyvinylpyrrolidone, 0.1g ovine serum albumin, 0.1g Ficoll 400, DEPC-H20 to 500 ml), 0.1 mg/ml sperm DNA, 10% dextran sulfate, 5 mg/ml sodium pyrophosphate, 0.5% SDS and 1 mM levamisole).

Hybridization, post-hybridization stringency washes

One hundred-300 μl of probe/pre-hybridization mixture was applied to each slide at a probe concentration of at least 200 ng/ml and allowed to hybridize overnight at 55°C.

Each hybridization experiment comprised an antisense probe and both a sense probe and no-probe controls. The slides were then washed at high stringency in 2xSSC for 15 min at RT, followed by washes at 55°C in 2xSSC for 15 min, three times in 0.2xSSC for 15 min and twice in 0.1xSSC for 15 min then returned to RT in a 0.1xSSC wash for 15 min followed by buffer I (100 mM Tris HCl pH 7.5, 150 mM NaCl) for 5 min at RT.

Whole-mount preparation and processing

The maxilla containing the developing palates was dissected *in toto* from the heads of 15.5 and 16.5 dpc embryos and placed immediately in ice-cold 4% paraformaldehyde in PBS for 2 h, washed 3x5 min PBT (0.1% Tween 20 in PBS) then dehydrated through graded methanol/PBT to 100% methanol for storage at -20°C. For subsequent processing, the tissue was re-hydrated through graded methanol/PBT, incubated for 1 h in 6% H_2O_2 in PBT, washed 3x5 min PBT then incubated in 10 μg/ml Proteinase K for 5 min. Following a 5 min wash in 2 mg/ml glycine/PBT the tissues were post-fixed in 0.2% glutaradehyde/ 4% paraformaldehyde in PBT for 20 min, washed 3x5 min PBT then in 0.1% Sodium Borohydride in PBT for 20 min before prehybridization at 55°C for 1 h and hybridization with DIG-labeled probe at 1 μg/ml overnight at 55°C. The tissues were then washed 50% Formamide/ 2xSSC for 20 min at 55°C, then in NTET (NaCl 500 mM, 10 mM Tris pH 7.5, 5 mM EDTA, 0.1% Tween20) at 37°C for 2x10 min before treatment with 100 μg/ml RNAase in NTET at 37°C for 30 min. After stringency washing in 50% Formamide/2xSSC for 15 min at 55°C, 15 min in 2SSC/0.1% Tween20, then 0.2xSSC/0.1% Tween20 for 15 min at 55°C the tissues were transferred to Buffer I for detection of hybridized probe. All steps were performed with gentle agitation on a mechanical rocker.

Detection of hybridized probe

Detection of hybridized probe was preceded by blocking for 30 min to 1 h at RT with 2% sheep or goat serum in buffer I containing 0.3% Triton-X100 followed by 30 min to 1 h in 2% blocking solution (Boehringer Mannheim) in Buffer I at RT. Two hundred μl-500 μl anti-DIG primary antibody (Boehringer Mannheim) diluted 1:250 in 2% blocking solution was added to each slide and incubated at RT for 2 h. After washing in buffer I then Buffer III (100 mM Tris HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2) at RT, 500 μl of freshly prepared color substrate [200 μl of BCIP/NBT stock solution (Boehringer Mannheim) in 10 ml of Buffer III containing 2 mM levamisole] was placed on each slide and incubated O/N in the dark at RT. After a brief rinse in tap water the slides were coverslipped using aqueous mounting medium and photomicrographs recorded on print color film with a Carl Zeiss photomicroscope. The whole-mount preparations were processed similarly with the major differences being longer time periods for each step, inclusion of 0.1% Tween 20 in Buffer I, overnight incubation at 4°C in antibody blocking solution and shorter colour development incubation under visual inspection at 15 min intervals. The whole-mounts were photographed against a dark field with a Wild-Leitz dissecting microscope.

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