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The development of the palate – a brief review

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SUMMARY

Palatogenesis is a complex developmental process that requires two main events: elevation and then fusion of the palatal shelves. There remains controversy concerning the mechanism(s) responsible for palatal shelf elevation, it being proposed that an intrinsic shelf elevation force might be produced either by the generation of a turgor pressure following hydration of the extracellular matrix via its glycoconjugate molecules or by proliferation, migration and/or contraction of the palatal shelf mesenchymal cells. Recent evidence indicates that the shelf elevation force is related to the presence of hyaluronan in the extracellular matrix, to an as yet unknown molecule that is packaged in the mesenchymal cells' Golgi complex, and to CD44 receptor functioning. For fusion of the palatal shelves to occur, the breakdown of the midline epithelial seam relates to apoptosis and redifferentiation of the epithelial cells and this appears to be signalled by the synthesis of type IX collagen just prior to the breakdown of the basement membrane around the midline epithelial seam. The events associated with palatogenesis are controlled by the palatal shelf mesenchyme, under the influence of a variety of homeobox genes and transcription factors and and of several growth factors (particularly TGF-βs).

Key words: Palatogenesis – Palatal shelf elevation – Hyaluronan – CD44 – TGF-β

INTRODUCTION

Palatogenesis is a complex event and is often disturbed to produce the congenital defect known as cleft palate. Consequently, the events and mechanisms responsible for the development of the palate have been much studied, although some controversy remains.

The definitive palate (or secondary palate) develops in the human fetus between the sixth and eighth week of intra-uterine life (e.g. Ferguson, 1978a; Johnston and Sulik, 1990; Sadler, 2000; Berkovitz et al., 2002). By the sixth week of development (Fig. 1), the primitive nasal cavities are separated by a primary nasal septum

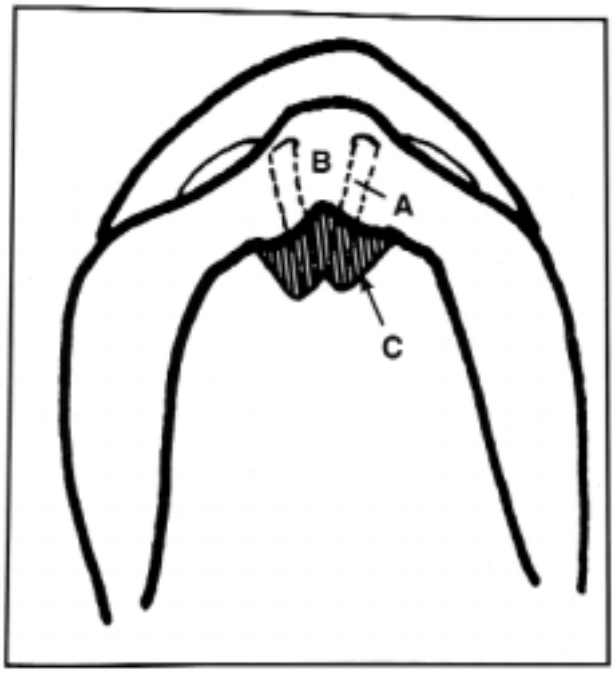

Figure 1. Diagram showing the state of development of the palate by the sixth week of intra-uterine life. A = primitive nasal cavities; B = primary nasal septum; C = primary palate.

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Figure 2. Diagram showing the development of the palate during the sixth week of intra-uterine life. A = lateral palatal shelves; B = primary palate; C = secondary nasal septum.

and are partitioned from the primitive oral cavity by a primary palate. Both the primary nasal septum and primary palate are derived from the frontonasal process of the developing face. The stomodeal chamber is divided at this stage into the small primitive oral cavity beneath the primary palate, and the relatively large oronasal cavity behind the primary palate. As shown in Figure 2, during the sixth week of development, two palatal shelves develop laterally behind the primary palate from the maxillary facial processes. A secondary nasal septum grows down from the roof of the stomodeum behind the primary nasal septum, thus dividing the nasal part of the oronasal cavity into two.

Figure 3 shows the developing head during the seventh week of development. At this stage, the oral part of the oronasal cavity becomes completely filled by the developing tongue. Growth of the palatal shelves continues such that they come to lie vertically. Two peaks of DNA synthesis occur as the palatal shelves are formed: during initial shelf outgrowth and during vertical shelf elongation (Burdett et al., 1988). The reason for mammalian shelves forming with a vertical orientation is unknown. It has been suggested that the potential space in the oronasal cavity is insufficient because of the evolution of a large tongue in mammals (Hayward and Avery, 1957). However, Young et al. (1990) have shown that there is no spatio-temporal relationship between the development of the tongue and the palatal shelves.

During the eighth week of development (Fig. 4), the stomodeum enlarges, the tongue 'drops'

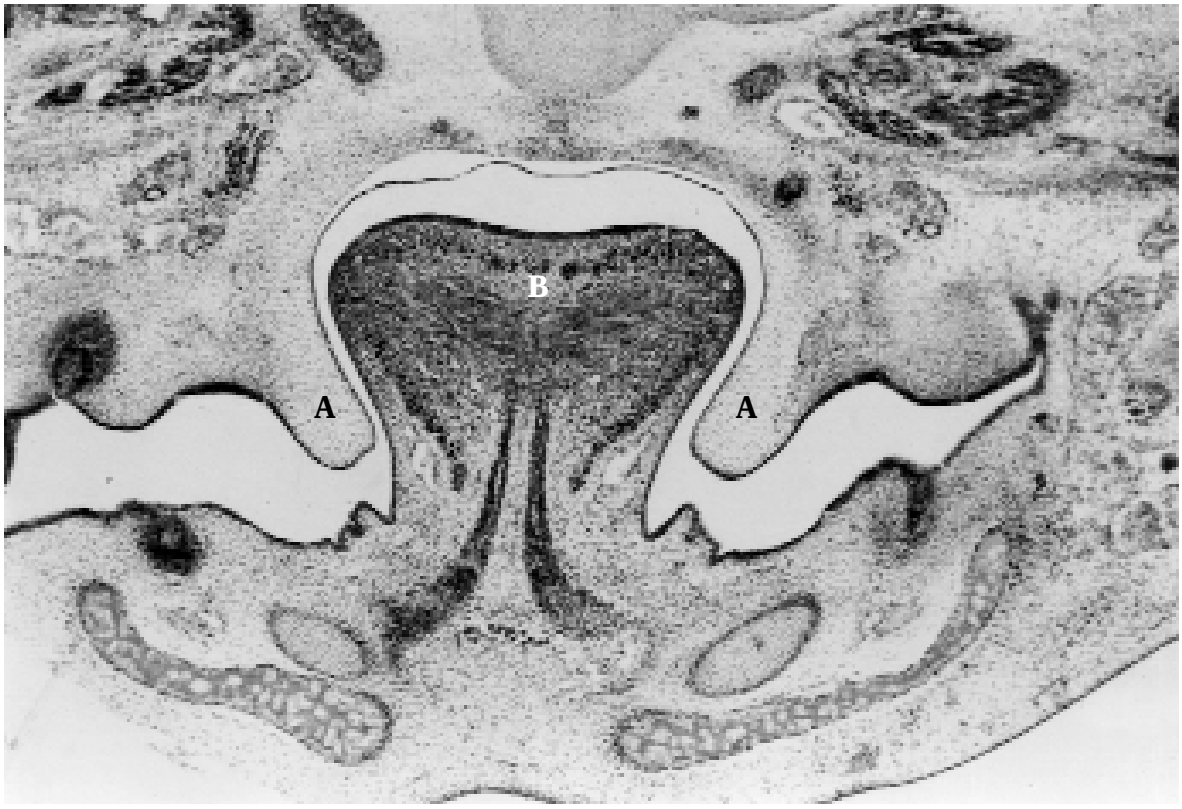


Figure 3. Coronal section through the developing head during the seventh week of development showing the palatal shelves (A). B = developing tongue. (Masson's trichrome). x 55.

and the vertically-inclined palatal shelves become horizontal. It has been suggested that the descent of the tongue is related to mandibular growth and/or a change in the shape of the tongue (e.g. Humphrey, 1971; Diewert, 1974). On becoming horizontal, the palatal shelves contact each other (and the secondary nasal septum) in the midline to form the definitive or secondary palate. The shelves contact the primary palate anteriorly so that the oronasal cavity becomes subdivided into its constituent oral and nasal cavities. Figure 5 shows a coronal section through the developing oronasal regions following contact of the palatal shelves and secondary nasal septum. After contact, the medial edge epithelia of the two shelves fuse to form a "midline epithelial seam" (MES). Subsequently, this degenerates so that mesenchymal continuity is established across the now intact, and horizontal, secondary palate. Fusion of the palatal processes is complete by the twelfth week of development. Behind the secondary nasal septum, the palatal shelves fuse to form the soft palate and uvula.

Concerning the origin of the mesenchyme within the fetal processes contributing to the development of the palate, all of the skeletal and connective tissues that form the face are derived from neural crest (NC) cells that originate along the dorsal margins of the midbrain and rostral hindbrain (Noden, 1978; Couly et al., 1992; Köntges and Lumsden, 1996; Le Douarin and Kalcheim, 1999). Indeed, Been and Song (1978) have shown that localized destruction of midbrain NC inter-

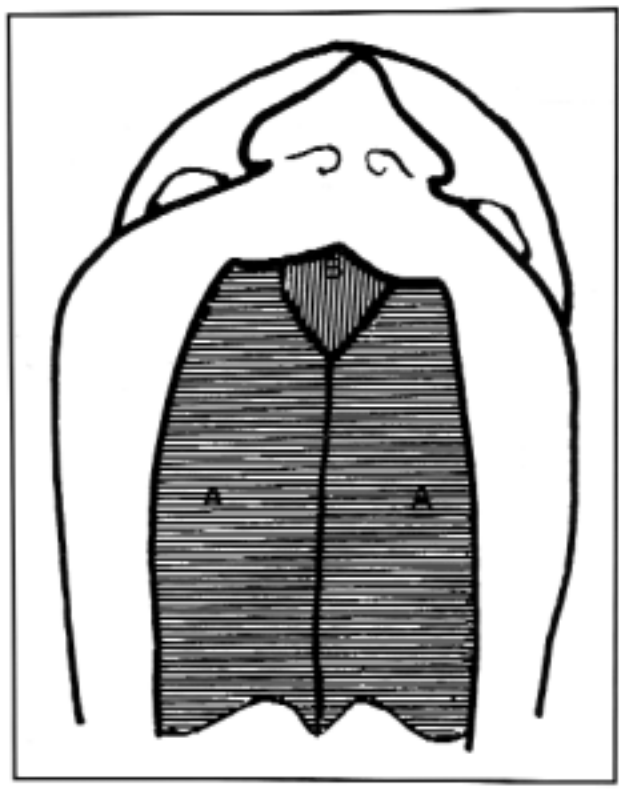


Figure 4. Diagram showing the state of development of the palate during the eighth week of intra-uterine life. A = palatal shelves; B = primary palate.

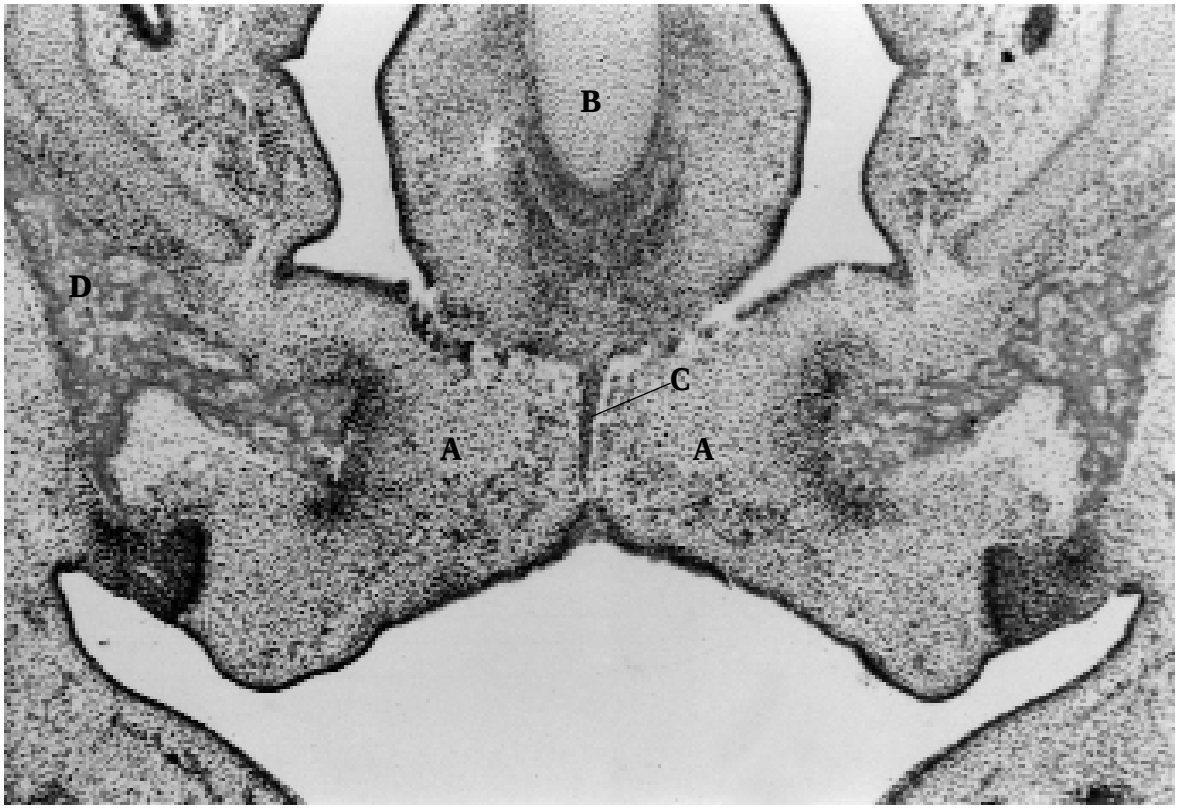


Figure 5. Coronal section through developing oronasal regions following contact of the palatal shelves (A) and secondary nasal septum (B); C = midline epithelial seam; D = developing bone of maxilla. (Masson's trichrome). x 55.

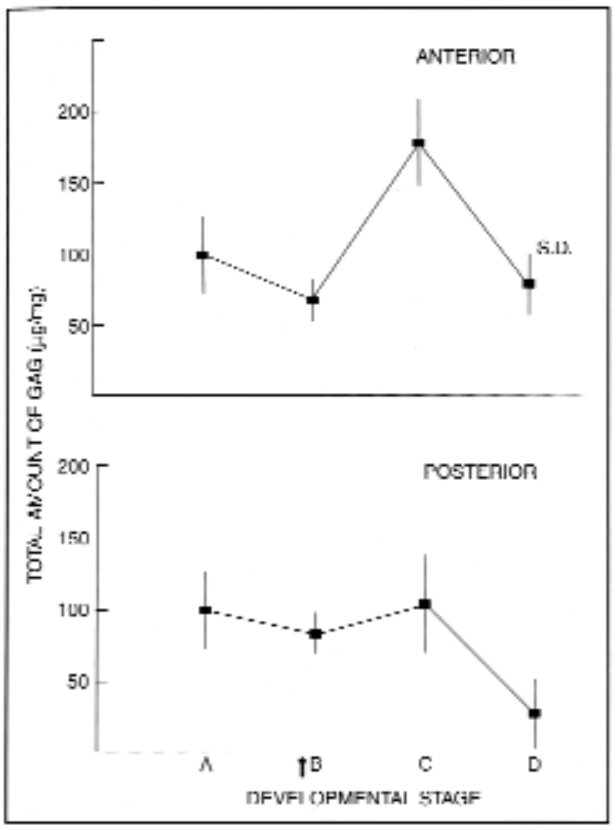


Figure 6. Graphs illustrating the changing amounts of glycosaminoglycans (GAG) during development of the anterior (presumptive hard) and posterior (presumptive soft) palates. Stage A – prior to shelf elevation; Stage B – after shelf elevation; Stage C – during shelf fusion and early histogenesis; Stage D – a stage of marked histogenesis after fusion. Courtesy of Dr G.D. Singh and B.J. Moxham and the editor of *Archives of Oral Biology*.

feres with palatal closure. In mammalian fetuses, cranial NC cells do not always migrate before the neural tube closes (e.g. Tan and Morriss-Kay, 1986). Furthermore, recent work suggests that craniofacial development does not depend on NC pre-programming but is controlled by a complex combination of cell and tissue interactions involving NC plasticity (Trainor and Krumlauf, 2001). Evidence is also available that suggests that NC cells can be reprogrammed and that their fate and identity depends upon the cellular signals they receive as they migrate to their target tissues (e.g. Schilling et al., 2001). This seems to occur as a result of alteration in *Hox* gene identity. However, there is some work indicating that NC cells have some identity at their place of origin near the neural tube; although elaboration of their development is reached via integration and interaction with signals from surrounding tissue environments through which they migrate (Grammatopoulos et al., 2000; Pasqualetti et al., 2000).

Recent research on palatogenesis has concentrated on two main events: palatal shelf elevation and the initial stage of fusion of the shelves.

Palatal shelf elevation

Several mechanisms have been proposed to account for the rapid movement (Ferguson, 1978; Brinkley, 1980) of the palatal shelves from the vertical to the horizontal position and the source of the force(s) responsible for palatal shelf reorientation/elevation is a matter of controversy. Two categories of explanations have been provided: either the forces are extrinsic to the shelves or they are generated intrinsically by the shelf mesenchyme.

Those extrinsic forces that have been proposed often relate to movement of the tongue. For example, there have been hypotheses that include downward movement of the tongue due to a mandibular growth spurt clearing a path for palatal shelf elevation (e.g. Asling et al., 1960; Diewert, 1974), a downward displacement of the tongue by the nasal septum again clearing a path for shelf elevation (e.g. Zeiler et al., 1964), and a lowering of the tongue due to a fetal mouth opening reflex (e.g. Humphrey, 1969, 1971). It has also been suggested that the tongue physically pushes the palatal shelves upwards (e.g. Walker, 1971). However, it is now generally thought that the palatal shelf elevation force is not extrinsic in origin. Ferguson (1978a) reviewed the literature relating to extrinsic forces and concluded that the chronology of events extrinsically did not necessarily synchronise with shelf elevation. Furthermore, following tongue excision during palatogenesis, no spatio-temporal relationship exists and aglossia and microglossia in humans does not prevent palatal closure (Young et al., 1990). In addition, palatal shelves are seen to elevate in organ culture in the absence of a tongue or a lower jaw.

It has been proposed that the intrinsic shelf elevation force might develop as a result of hydration of extracellular matrix (ECM) components (principally hyaluronan) in the shelf mesenchyme (e.g. Larsson et al., 1959; Pratt et al., 1973; Ferguson, 1978a; Brinkley and Morris-Wiman, 1984, 1987; Singh et al., 1994, 1997), or as a result of mesenchymal cell activity (e.g. Shah, 1979, 1980; Innes, 1978; Wee et al., 1979; Zimmerman, 1979; Babiarz et al., 1979; Luke, 1984; Bulliet and Zimmerman, 1985; Brinkley and Bookstein, 1986; Shah et al., 1989). Of course, the intrinsic shelf elevating force might be multifactorial, although there is as yet no experimental evidence to support what otherwise might be considered a “commonsense” view.

Much recent work has focussed on the changes occurring in the ECM of the palatal shelf’s mesenchyme during shelf elevation. The changing amounts of glycosaminoglycans (GAG) during development of the anterior (presumptive hard) and posterior (presumptive soft) palates have been reported by Singh et al. (1994) and are illustrated in Fig. 6. The findings show that the most significant changes occur after eleva-

tion and that, during the time of elevation, there are no differences between the anterior and posterior regions of the shelves even though, in the species studied here (the rat), the posterior region of the shelf does not elevate but grows initially with a horizontal disposition (Coleman, 1965; Cleaton-Jones, 1976a; Singh et al., 1994). Singh et al. (1997) have also reported that, when palatal clefts are induced in the rat by 5-fluoro-2-deoxyuridine (FUDR), GAG biosynthesis is suppressed.

Three types of GAG are found in the developing palatal shelves *in vivo* (Singh et al., 1994, 1997): hyaluronan, heparan sulphate and chondroitin-4-sulphate (Fig. 7). If palatal shelves are cultured *in vitro*, dermatan sulphate is also present (e.g. Burkitt, 1990), highlighting the problem of extrapolating from the findings of tissue culture to the *in vivo* situation. Furthermore, there may be species differences since, using early histochemical techniques, it has been claimed that chondroitin-6-sulphate may be present in mouse palatal shelves (Larsson, 1962).

Much attention has been paid to the role of hyaluronan in shelf elevation. It has been proposed that hyaluronan is a GAG involved in shelf elevation because it is highly electrostatically charged, it displays non-ideal osmolarity, and its open coil molecule is capable of binding up to 10 times its own weight in water (e.g. Pratt et al., 1973; Brinkley and Morris-Wiman, 1987). This view has the support of the work of Foreman et al. (1991), where an increase in water content of the palatal shelves was observed up until shelf fusion.

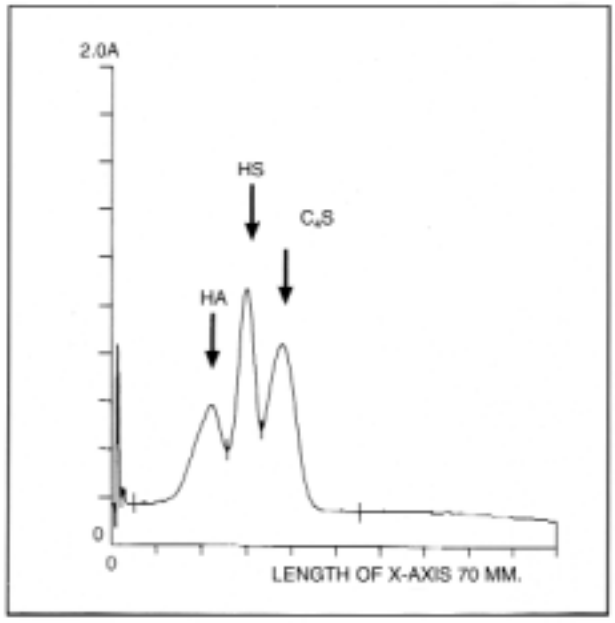


Figure 7. Densiometric scan of electrophoretograms showing the GAGs within the palatal shelves. HA – hyaluronan; HS – heparan sulphate and C_4S – chondroitin 4-sulphate. Courtesy of Dr G.D. Singh and B.J. Moxham and the editor of *Archives of Oral Biology*.

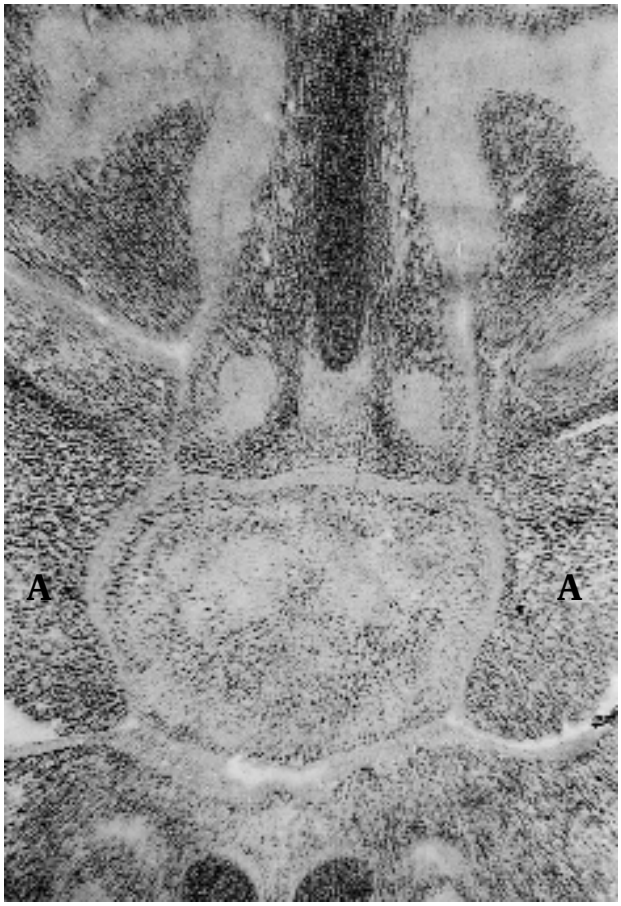


Figure 8. Section through a vertical (pre-elevation) palatal shelf (A) stained using the hyaluronectin/anti-hyaluronectin technique to demonstrate the presence of hyaluronan. x 40.

Figure 8 shows a section through a vertical (pre-elevation) palatal shelf stained using the hyaluronectin/anti-hyaluronectin technique (Girard et al., 1986) and shows intense staining for hyaluronan within the palatal shelf mesenchyme. Singh et al. (1994) have investigated the changing concentrations of hyaluronan within the anterior and posterior regions of palatal shelves (Fig. 9). Statistically, there is significantly more hyaluronan in the shelves immediately before elevation than immediately after elevation. However, the data do not agree with some reports that there is less hyaluronan posteriorly than anteriorly (Knudsen et al., 1985), the pattern of change in hyaluronan again being similar both anteriorly and posteriorly even though the posterior region does not undergo elevation to reach the horizontal (Coleman, 1965; Cleaton-Jones, 1976a; Singh et al., 1994).

Singh et al. (1994) also reported that, although heparan sulphate and chondroitin-4-sulphate are present within the palatal shelves throughout palatogenesis, these GAGs do not show detectable changes at the time of palatal shelf elevation.

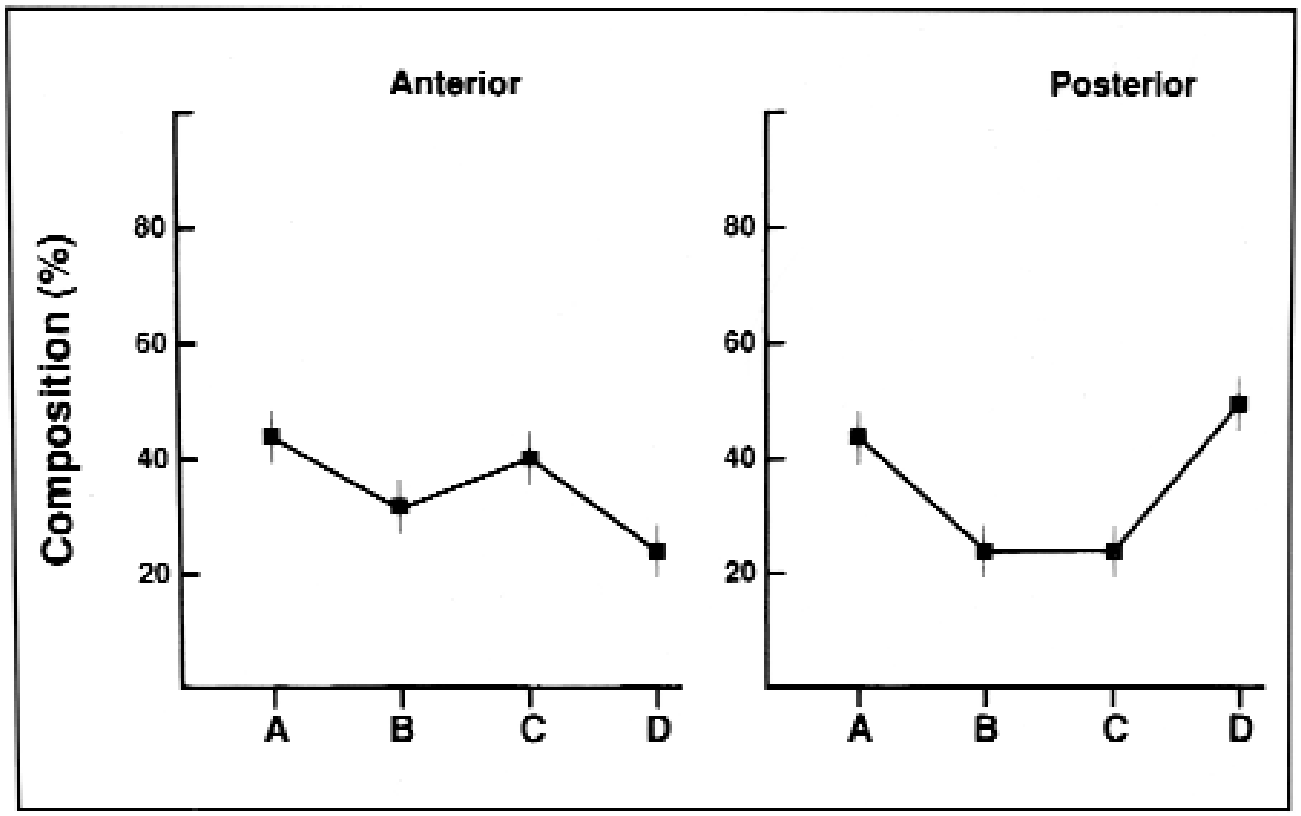
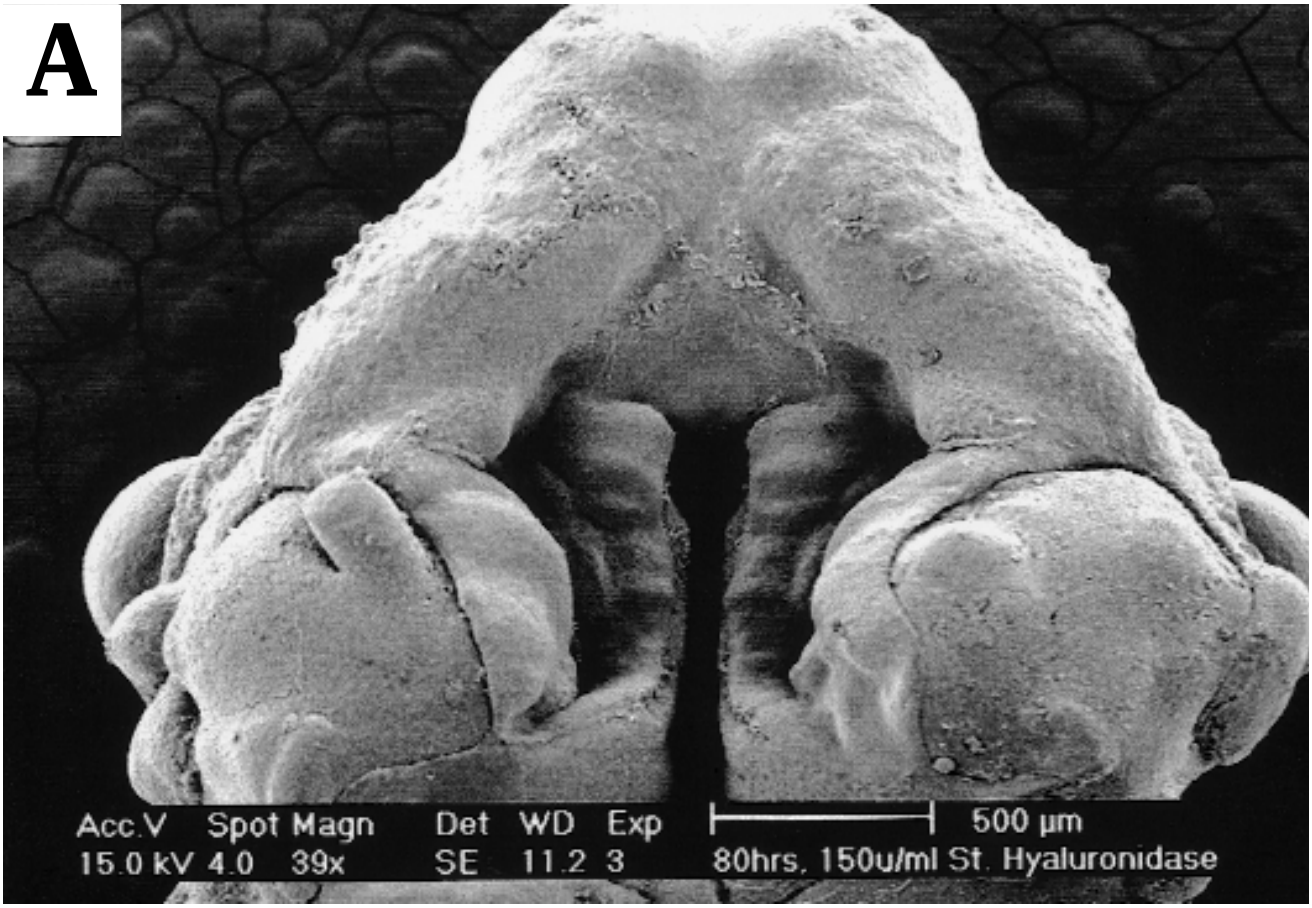


Figure 9. Graphs showing the changing concentrations of hyaluronan within the anterior and posterior regions of palatal shelves. A-D stages of palatogenesis described in Figure 6. Courtesy of Dr G.D. Singh and B.J. Moxham and the editor of *Archives of Oral Biology*.

That FUDR is associated with the production of cleft palates (e.g. Singh et al., 1997) has also been implicated in supporting the notion that hyaluronan is important in palatogenesis. Accordingly, Ferguson (1978b) has reported that FUDR's interference in DNA synthesis fits with its reported effects on glycoconjugate production *in vitro* (e.g. Dorfman et al., 1975).

More recent studies (Thomas, 1999; Thomas, Hall and Moxham, unpublished data) have revealed the presence during palatogenesis of enzymes associated with hyaluronan synthesis, of a cell surface receptor associated with hyaluronan, of the hyaluronan binding ECM components versican and hyaluronectin, and of hyaluronan binding sites. Furthermore, using an organ culture system (Figs. 10-13), agents that alter hyaluronan content or size, that disrupt GAG substitution on proteoglycans, or that alter the balance of matrix molecules secreted via the Golgi complex and hyaluronan produced at the cell surface all affect palatogenesis. Figure 10 illustrates the effects of streptomyces hyaluronidase, an enzyme that specifically degrades hyaluronan. Streptomyces hyaluronidase treated shelves produced clefts. In addition, link protein is absent whilst versican was evident in the mesenchyme and CD44 in the ectoderm. The results suggest that palate development is disrupted in the absence of hyaluronan. Figure 11 shows the effects of chlorcyclixine, a substance that enhances degradation of hyaluronan and chondroitin sulphate to lower the molecular weight products, with little effect on their synthesis and no appreciable effect on DNA synthesis. Chlorcyclixine treated shelves exhibit a cleft. CD44 and link protein are absent from the shelves but versican is evident throughout the mesenchyme. Therefore, the size of the GAG chain may influence palatogenesis. The effects of UDP-xylose are illustrated in Fig. 12. UDP-xylose is a natural inhibitor of UDPGD, the enzyme responsible for the conversion of UDP-glucose to UDP-glucuronic acid. UDP-xylose treated shelves exhibited normal palate development. Link protein was again absent, but versican and CD44 exhibited the same distribution as seen in control cultures. Therefore, inhibition of UDPGD has no effect on palate development (assuming that it was able to enter the tissue). Figure 13 shows the effects of Brefeldin A. This inhibits vesicular transport through the Golgi complex. Hyaluronan synthesis is not affected as this GAG undergoes a different synthetic pathway to the other GAGs, being formed at, or near, the plasma membrane by the hyaluronan synthase/enzyme complex. Brefeldin A produced a cleft. While versican was evident throughout the mesenchyme, link protein and CD44 were absent. Exposure to BFA at sequential 10h window periods suggests that BFA only causes a cleft in the initial 30h. The results indicates



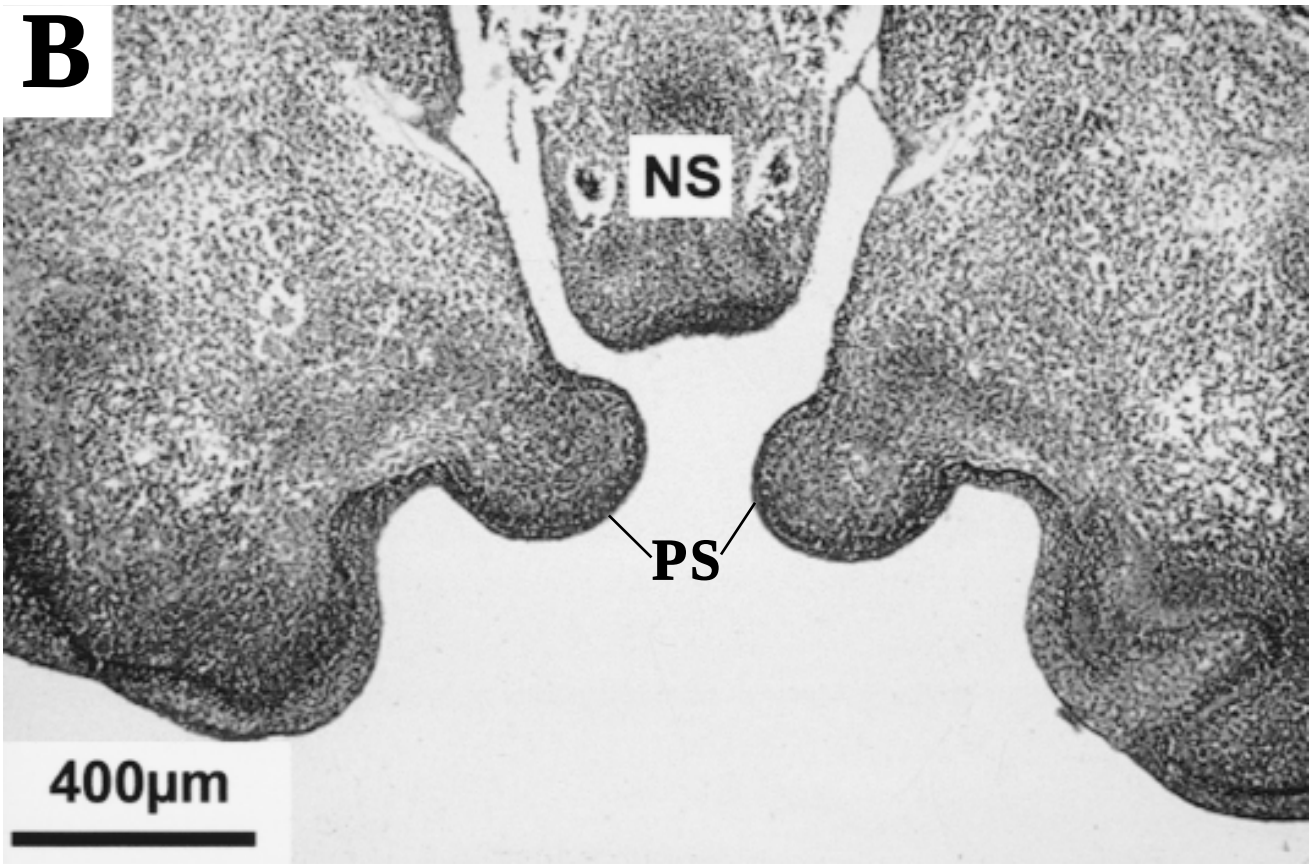


Figure 10. The production of clefts produced during organ culture of rat developing palates following the introduction of streptomyces hyaluronidase to the culture medium and shown by scanning electronmicroscopy (**A**) and light microscopy (**B**). NS = nasal septum; PS = unfused palatal shelves. Scale bars: 10A = 500 µm; 10B = 400 µm. Courtesy of S.Thomas, R. Hall and B.J. Moxham.

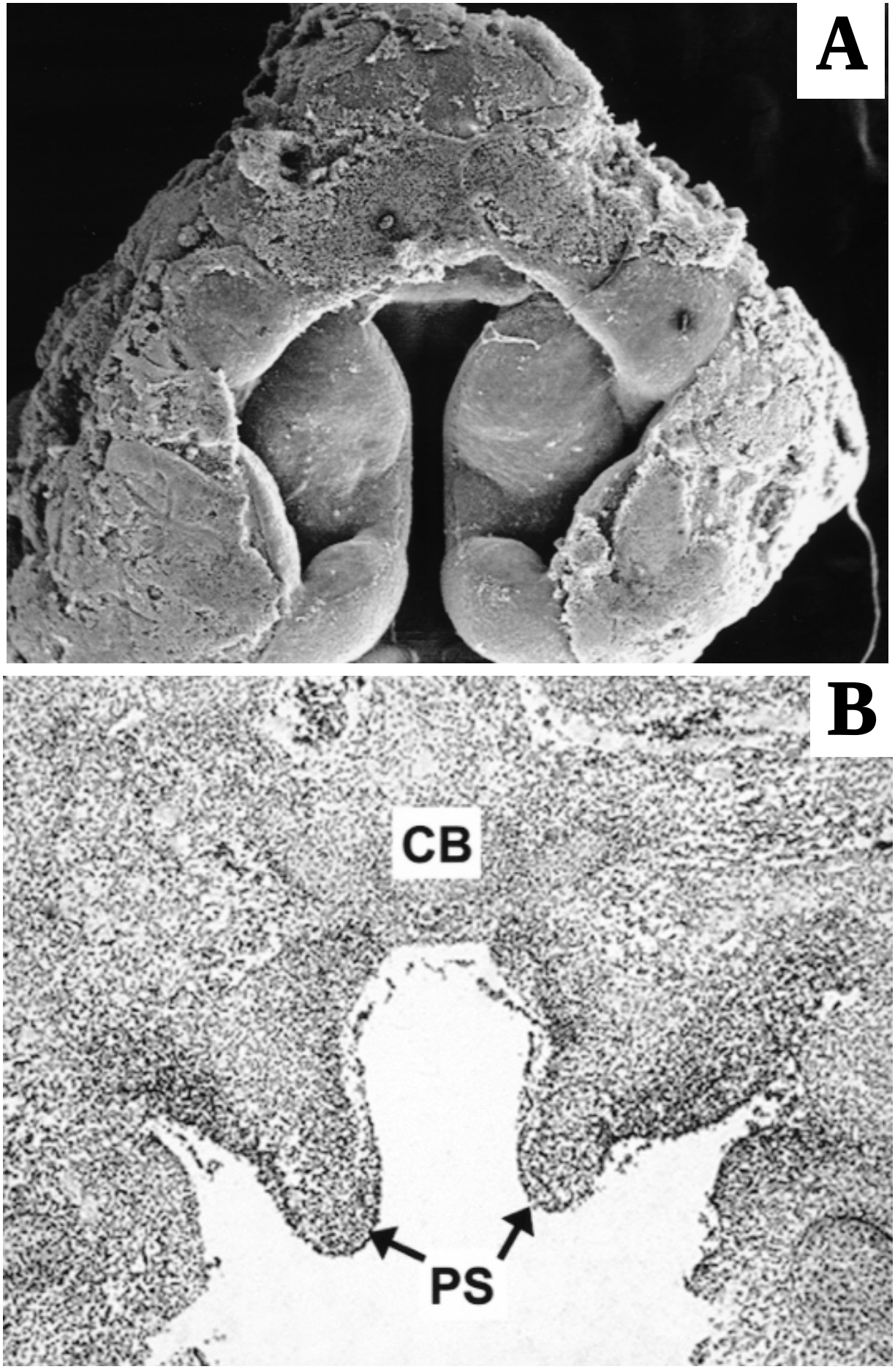


Figure 11. The production of clefts produced during organ culture of rat developing palates following the introduction of chlorcyclixine to the culture medium and shown by scanning electronmicroscopy (**A**) and light microscopy (**B**). CB = cranial base; PS = unfused palatal shelves. (11A = x 50; 11B = x 100). Courtesy of S.Thomas, R. Hall and B.J. Moxham.

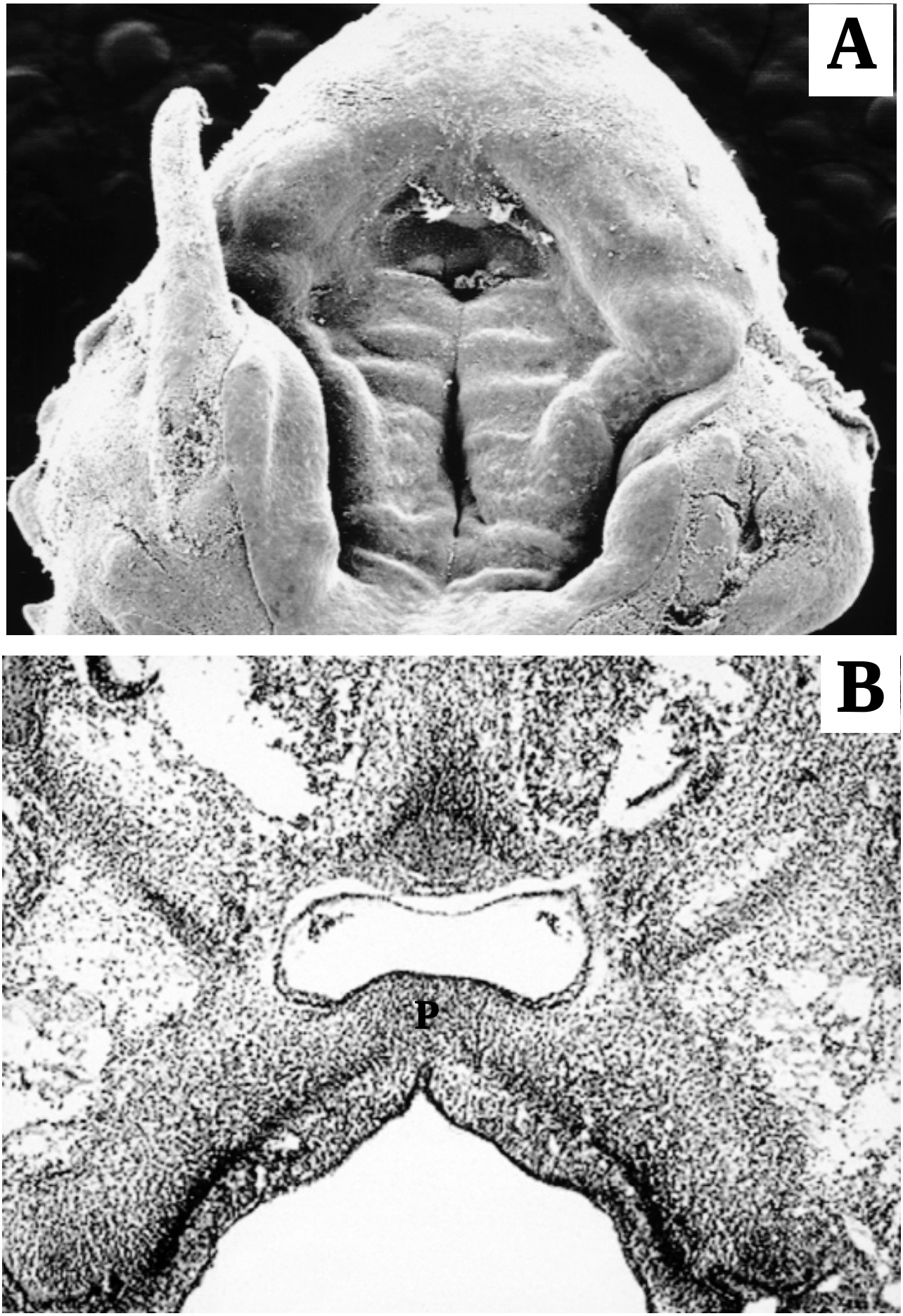
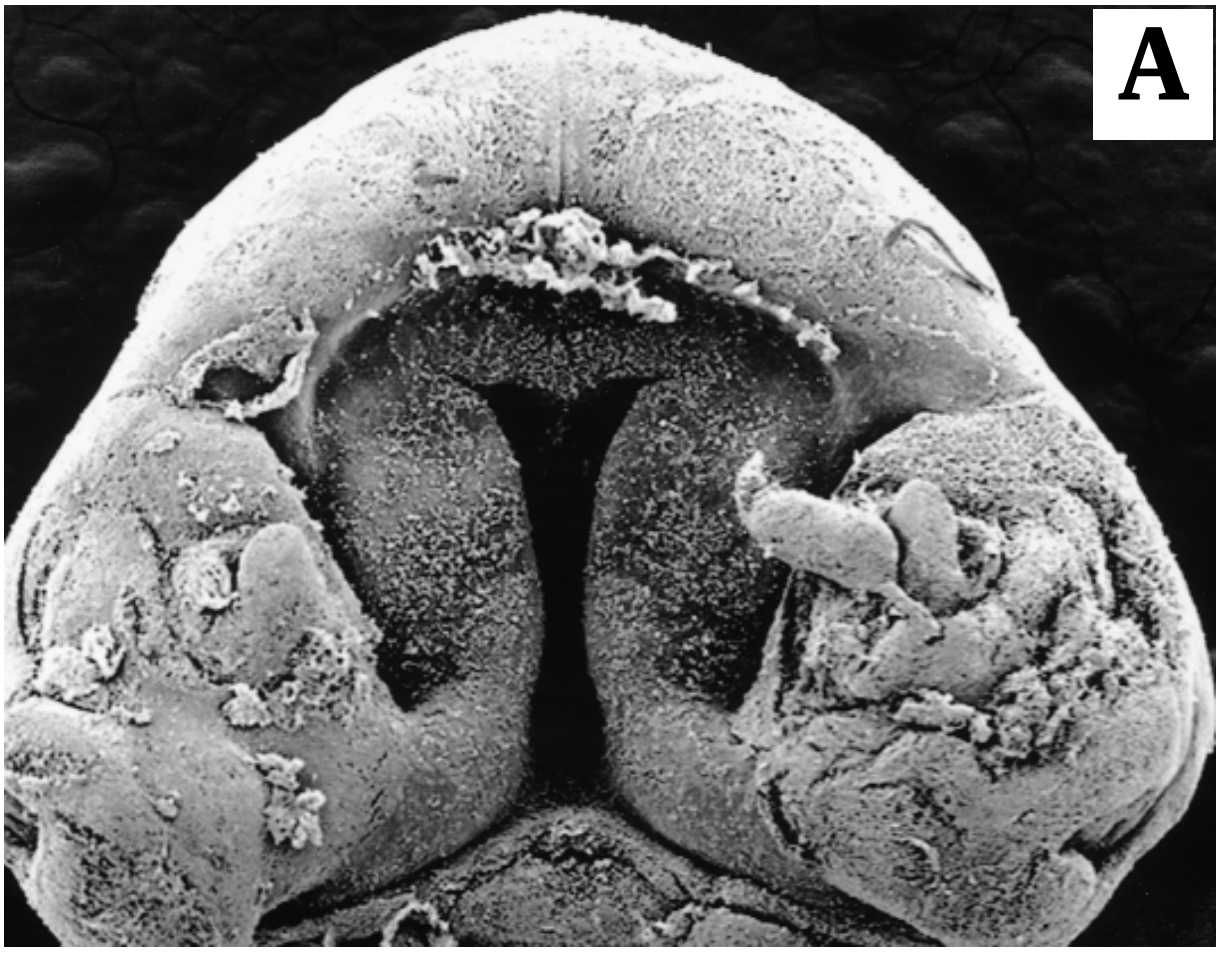


Figure 12. Normal palatogenesis during organ culture of rat palates following the introduction of UPD-xylose to the culture medium and shown by scanning electronmicroscopy (**A**) and light microscopy (**B**). P = fused palatal shelves. (12A = x 60; 12B = x 95). Courtesy of S.Thomas, R. Hall and B.J. Moxham.



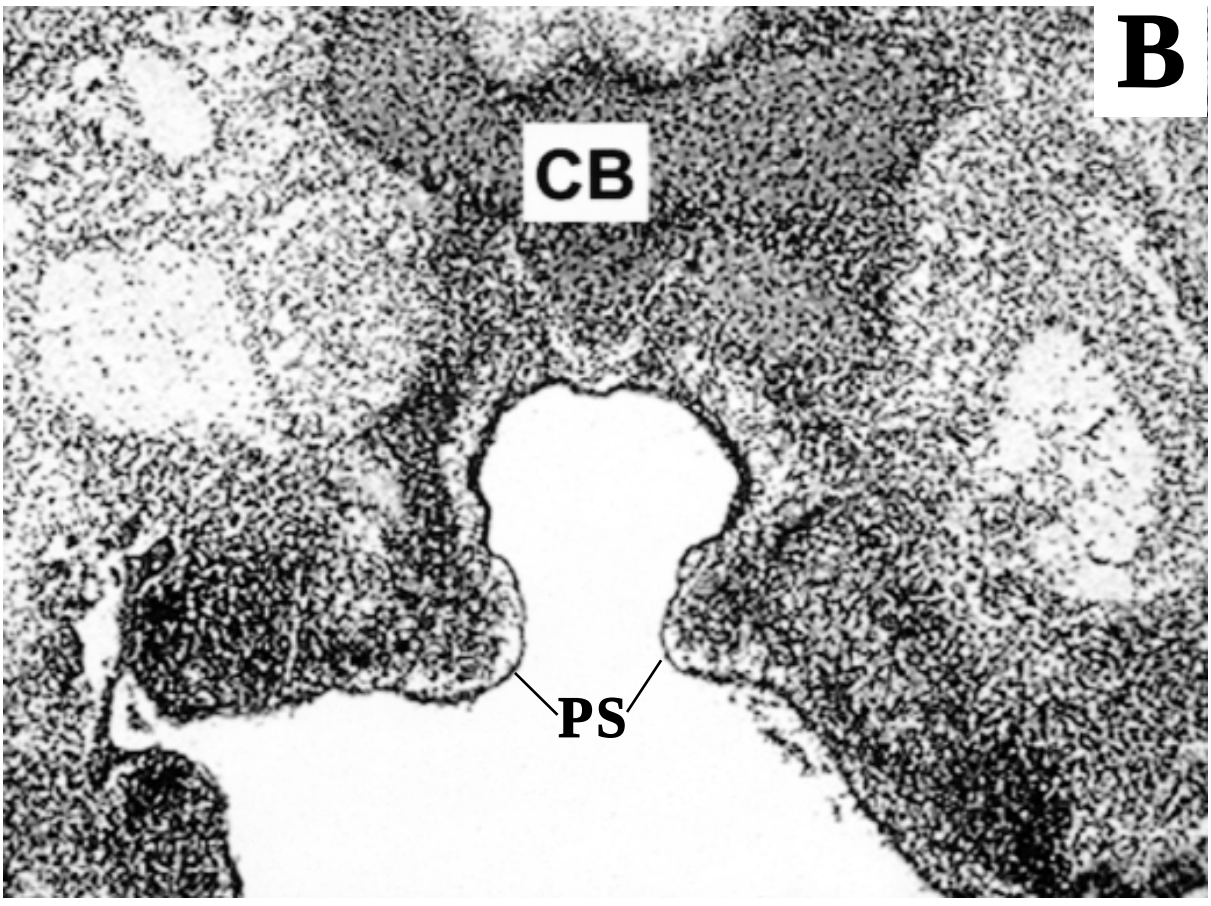


Figure 13. The production of clefts produced during organ culture of rat developing palates following the introduction of brefeldin A to the culture medium and shown by scanning electronmicroscopy (**A**) and light microscopy (**B**). CB = cranial base; PS = unfused palatal shelves. (13A = x 50; 13B = x 100). Courtesy of S.Thomas, R. Hall and B.J. Moxham.

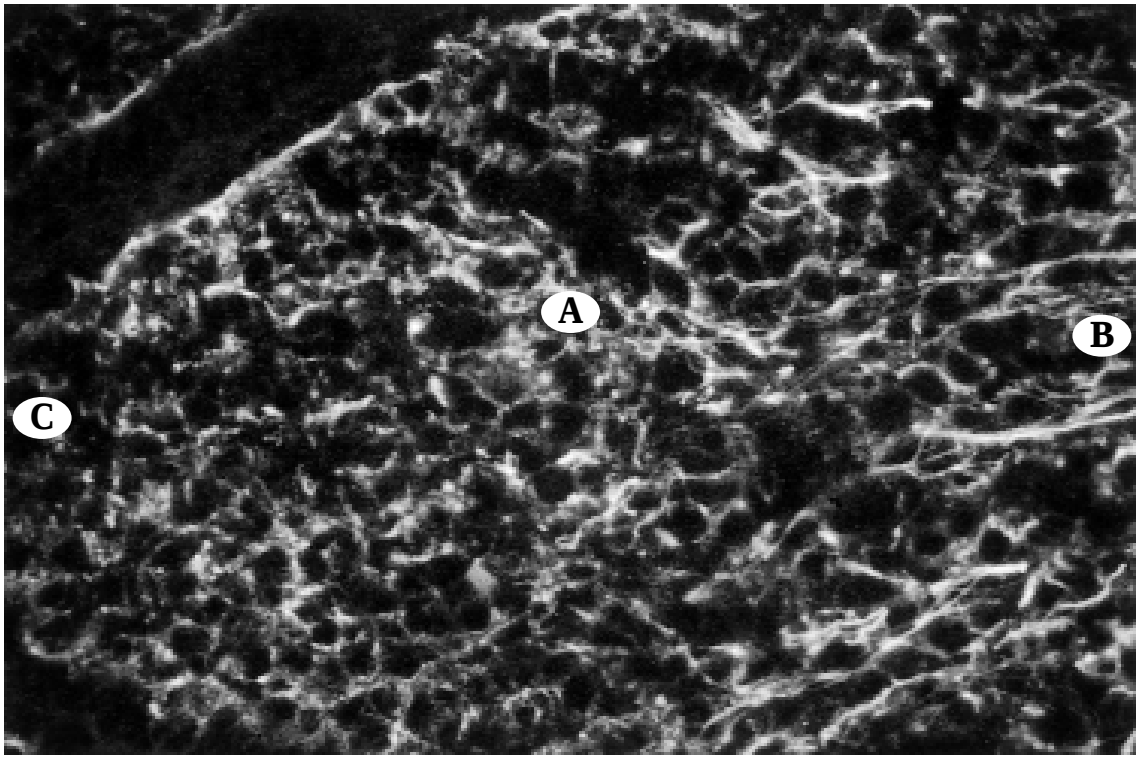


Figure 14. Section of a palatal shelf labelled immunocytochemically with antibodies against type I collagen. A = collagen bundles; B = base of palatal shelf; C = tip of palatal shelf. x 300. Courtesy of Professor M.W.J. Ferguson.

that a set of macromolecules other than hyaluronan, and synthesised in the Golgi complex, plays an important role in normal palate development.

Other recent studies at our laboratories at Cardiff (Hudson and Hall, unpublished data) have been concerned with the expression of hyaluronan binding protein splice variants of CD44, versican and RHAMM and isoforms of hyaluronan synthases (*Has*) and hyaluronidases (*Hyal*) in the developing rat palate. Expression of CD44 (the major hyaluronan binding protein) was found to be both transient and dynamic during shelf elevation with differential expression of CD44 transcripts containing variant exons v1, v2, v8 and v9. It was also noted that larger transcripts (containing more variant exons) were present after shelf elevation. It can be argued that expression of distinct *Has* and *Hyal* splice variants is necessary during palatogenesis in order for correct tissue formation to occur because both enzymes produce different sizes of hyaluronan, thus promoting distinct cellular responses depending on cell type (Itano et al., 1999). Small hyaluronan chains can induce gene expression (McKee et al., 1996), cell signalling responses and cell differentiation (Termeer *et al.*, 2000), and cell proliferation and growth (Mohapatra et al., 1996, Bourguignon et al., 1997), whereas large hyaluronan chains at high concentrations inhibit cell growth and induce cell adhesion and migration (Noble et al., 1998). Versican splice variants differ in size and GAG chain number and are thought to form bridges, helping to stabilize the ECM and create the necessary turgor pressure to enable shelf elevation.

Other ECM components, including proteoglycans, are probably of importance to shelf elevation. Versican and decorin (but not biglycan) have been identified at a range of molecular weights corresponding to various processed forms. The extent to which aggregation and disaggregation of proteoglycans occurs at different locations of the palatal shelf and at different stages of palatogenesis is unknown; although this could have significant functional implications associated with shelf elevation. The role of collagen within the palatal shelves is disputed. Pratt and King (1972) showed that cleft palates can result from the administration of lathyrogens that have specific effects on collagen crosslink formation. Hassell and Orkin (1976) described collagen bundles with defined orientation next to the basement membrane of the palatal shelves and reported that the rate of collagen synthesis was greatest just prior to shelf elevation. Indeed, it has been suggested that these collagen fibres "direct" the shelf elevation force (e.g. Bulleit and Zimmerman, 1985) and/or contribute to a critical volume of the shelves necessary for their re-orientation (Ben-Khaial and Sha, 1994). Immunohistochemically, type 1 collagen can easily be identified (Fig. 14) (Ferguson, 1988). Indeed, stout bundles of collagen can be seen running down the centre of the palatal shelf and these are orientated from the base towards the tip of the shelf.

The role of the mesenchymal cells within the palatal shelves has also been controversial. There is evidence that a critical number of cells are required for palatal shelf elevation to occur (e.g. Shah et al., 1989) but there is no reliable evidence as yet that these cells, by their rapid division and proliferation or by their migration or contraction, can generate a palatal shelf elevation force (particularly in view of the rapidity of shelf elevation). The density of palatal shelf mesenchymal cells appears to change during palatogenesis (e.g. Brinkley and Bookstein, 1986). This could be the result of variations in cell number and/or of cell redistribution. It was once believed that differential rates of cell mitoses/proliferation might produce the shelf elevation force (Luke, 1984; Bulliet and Zimmermann, 1985). ^{3}H-thymidine studies have shown that there are differential rates of mesenchymal cell proliferation (e.g. Cleaton-Jones, 1976b). However, the differential rates are probably related to histogenic changes and do not necessarily account for the generation of the shelf elevation force. Brinkley and Bookstein (1986) showed that shelf re-orientation is accompanied by changes in mesenchymal cell density and distribution. They suggested that high local cell densities were enhanced by cell division but that decreased cell density (which cannot be accounted for by an increase in cell size) was probably related to displacement of cells by expansion of the ECM. Ferguson (1978a) also noted the closely packed nature of mesenchymal cells before elevation and commented upon the greater cell density within the posterior region of the developing palate (a region which is the last to fuse).

In addition to mesenchymal cell proliferation, the production of a shelf elevation force might also be related to changes, at the critical time, in cellular morphology (e.g. Brinkley and Bookstein, 1986) and in particular to changes in the intracellular microfilamentous and microfibrillar systems (e.g. Kuhn et al., 1980). Babiarz et al. (1979) reported that palatal shelf mesenchymal cells before elevation were elongated and polarized, the cells nearest the basement membrane being perpendicularly aligned to the membrane. After shelf elevation, the cells became more rounded with short cellular projections. Babiarz et al. (1979) considered that these changes were indicative of cell contraction and that this could be the means of generating the shelf elevation force. Innes (1978) and Shah (1979, 1980) reported that shelf mesenchymal cells possess contractile, microfilaments. In addition, contractile proteins have been isolated from palatal shelf mesenchymal cells, leading to the claim that “actin- and myosin-like systems” may be involved in shelf elevation (Babiarz, Allenspach and Zimmerman, 1975). Indeed, Babiarz et al. (1979) reported on the presence of microfilaments containing actinomyosin and suggested that these were associated with cell migration that could be responsible for shelf elevation. Wee and Zimmermann (1980) reported that cytochalasin B inhibits palate shelf elevation by disrupting actin crosslinking in the cytoskeleton. However, they also found that curare (a microfilament antagonist) enhanced shelf elevation *in vitro*, thus providing evidence against the notion that microfilamentous systems are primarily responsible for shelf re-orientation. Furthermore, it is not clear whether changes in the palatal shelf mesenchymal cells are primarily associated with the re-orientation mechanism or whether they are the effect of cell displacements/cell activities caused by changes in the ECM during the period of shelf re-orientation (e.g. Pratt et al., 1973).

There have been many qualitative electronmicroscopic investigations of the palatal shelf mesenchymal cells (e.g. De Angelis and Nalbandian, 1968; Babiarz et al., 1975; Innes, 1978, 1981, 1985; Ferguson, 1981a). Essentially, these studies show that the mesenchymal cells appear to be very active, possessing many mitochondria, abundant cisternae of endoplasmic reticulum, a well-developed Golgi complex, and large numbers of glycogen particles (organelles appropriate for cells actively synthesising and secreting ECM proteins and entirely consistent with the view that the gradual accumulation of GAG is correlated with the synthesizing organelles of the mesenchymal cells). Shah (1979) described ultrastructural changes occurring during normal palatogenesis, noting that the cells elongated after shelf elevation. Lieb and De Paola (1981) found that the mesenchyme was tightly packed with polygonal cells possessing centrally placed ovoid nuclei with prominent nucleoli. They also reported that there was a large complement of free ribosomes and polysomes and very little intercellular space. Recently, it has been reported that filopodia-like structures appear on the surface of palatal shelf cells at the time of fusion (Taya et al., 1999). Similar events occur during development of the intermaxillary segment when the facial processes fuse (Symons and Moxham, 2002). Despite these many studies, to date there have been remarkably few quantitative electronmicroscopic studies. Brinkley and Bookstein (1986) have undertaken some quantitative studies on the development of the mouse secondary palate. The purpose of their investigation was to determine differences in cell density at various stages of palatogenesis *in vitro* and consequently, with the exception of the nuclei, they did not measure the organelles within the mesenchymal cells.

It is obvious that, whether or not the palatal shelf mesenchymal cells are involved in the

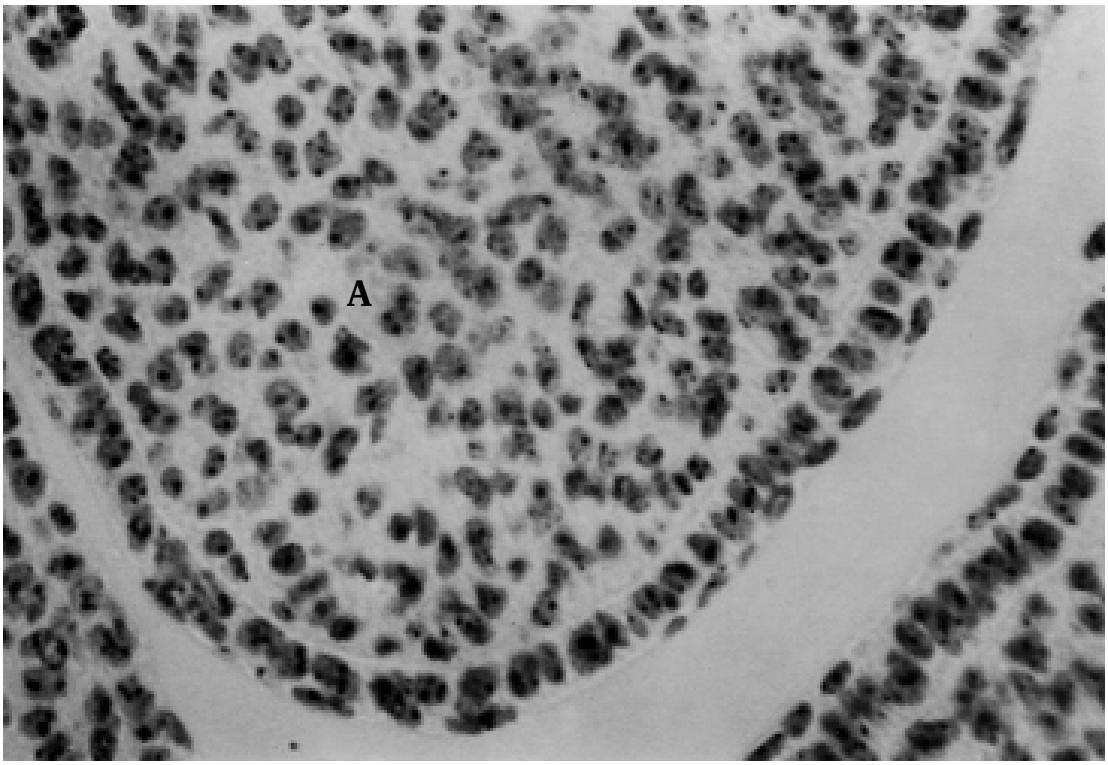


Figure 15. Silver staining of the palatal shelves (A) to assess the degree of activity of the mesenchymal cells. The black silver grains in the mesenchymal cell nuclei highlight Nucleolar Organiser Regions (NORs). Silver stain. x 500. Courtesy of Dr. G.D. Singh and B.J. Moxham.

generation of the shelf elevation force, the cells have to maintain (and control) events taking place in the shelf ECM. Indeed, using special silver staining techniques to highlight nucleolar organiser regions (NORs), the degree of protein synthesising activity of the mesenchymal cells in the palatal shelf at different stages of palatogenesis has been assessed (Singh and Moxham, 1993) (Fig. 15). The number and configuration of "grains" within the NORs reflect the overall degree of protein synthesis by the cells. This staining procedure confirmed that the rate of protein synthesis during palatogenesis is high, is higher before elevation than after elevation, and is higher still during later stages of histogenesis. These results accord with the changes occurring in GAG synthesis at various stages of palatogenesis. The AG-NOR staining technique further shows that protein synthesis is severely depressed during cleft formation, but the technique is unable to demonstrate major differences between anterior and posterior regions.

Although hyaluronan in the palatal shelves is most often associated with the development of a turgor pressure for shelf elevation via attraction of water molecules, this GAG also influences cellular activity. For example, hyaluronan produces large intercellular spaces during early palatogenesis to prevent cell-cell and cell-matrix interactions, allowing assembly of ECM constituents and presentation of growth factors that in turn influence cell growth and differentiation by altering the local concentration of intercellular signals (Toole, 2000). Following shelf elevation, there is a decline in hyaluronan shelf content (Singh et al., 1994), probably via CD44 receptor-mediated endocytosis of hyaluronan and hyaluronidases that produce shorter hyaluronan chains. This enables the onset of palatal tissue differentiation. Hyaluronan that is taken up into cells can bind to intracellular hyaluronan binding proteins, including some RHAMM splice variants. Such binding induces cell signalling pathways that can, in turn, induce changes in the cytoskeleton. During differentiation, the intercellular matrix becomes more dense where hyaluronan is replaced by proteoglycans, but the remaining hyaluronan binds to such proteoglycans (including hyaluronan binding proteins such as versican, cell surface RHAMM and CD44) to form a stable ECM.

Finally, although the production of cleft palates following the administration of FUDR is thought to be related to interference in ECM glycoconjugate production (e.g. Dorfman et al., 1975; Ferguson, 1978b; Singh et al., 1997), alternative explanations are possible in terms of cell activity within the palatal shelves. Indeed, Amwayi and Luke (1990) reported that FUDR produces a decrease in mesenchymal cell proliferation.

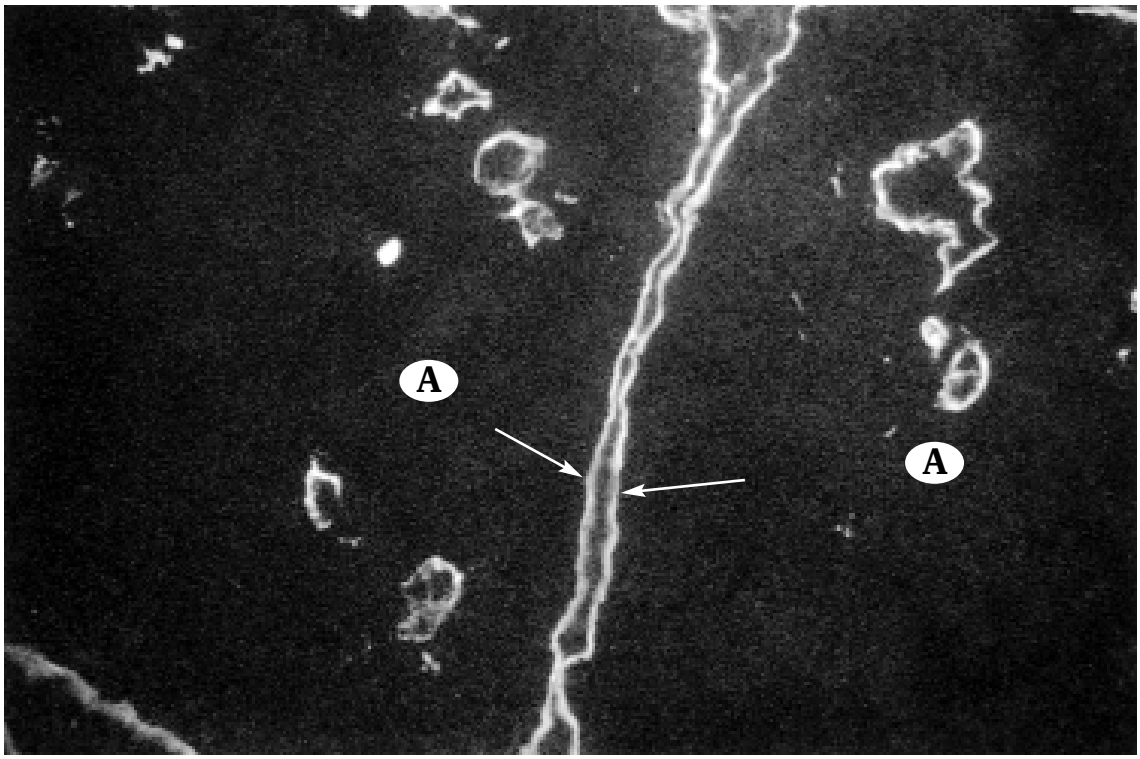


Figure 16. Fusing palatal shelves (A) immunocytochemically labelled with antibodies against type IV collagen found in basal lamina (arrowed). x 120. Courtesy of Professor M.W.J. Ferguson.

Fusion of the palatal shelves

Once the palatal shelves have elevated, they contact each other (initially in the middle third of the palate; Ferguson 1988) and adhere by means of an "adhesive" glycoprotein that coats the surface of the medial edge epithelia of the shelves (Greene and Kochhar, 1974; Pratt and Hassell, 1975; Souchon, 1975; Greene and Pratt, 1977). Additionally, the epithelial cells develop desmosomes (De Angelis and Nalbandian, 1968; Morgan and Pratt, 1977) and consequently an epithelial seam is formed (Morgan and Pratt, 1977; Ferguson, 1988) (see Fig. 5). The adherence of the medial edge epithelia is specific as palatal epithelia will not fuse with epithelia from other sites (e.g. the tongue) (Ferguson et al., 1984). This may be related to the fact that the proteins associated with the formation of desmosomes (i.e. desmoplakin) appear specifically on the cell membranes of the medial edge epithelia just prior to shelf contact (Ferguson, 1988). An intact basal lamina lies on either side of the epithelial seam.

The signals that are responsible for the breakdown of the midline epithelial seam (MES) are not yet fully understood. Nevertheless, the breakdown of the basal lamina is likely to be a significant event (e.g. Ferguson, 1988). Figure 16 demonstrates the fusing palatal shelves immunocytochemically stained with antibodies against type IV collagen found in basal lamina (Ferguson, 1988; Fyfe and Ferguson, 1988). At this early stage of fusion, the basal lamina remains intact. At a later stage of fusion (Fig. 17), with migration of the epithelial cells into the mesenchyme, the MES is disrupted and the migrating cells initially carry with them fragments of the disrupted basal lamina (Fyfe and Ferguson, 1988). Fibrils comprising tenascin and type III collagen have been shown to run at right angles to the basal lamina and may provide guiding pathways for the migrating epithelial cells (Fyfe and Ferguson, 1988; Fyfe et al., 1988). Evidence indicates that the events leading to the breakdown of the MES occur in single isolated palatal shelves and therefore do not depend upon shelf contact (Ferguson et al., 1984; Ferguson, 1988).

Almost as soon as the MES is formed, it thins to a layer two or three cells thick (Mato et al., 1966; Ferguson, 1988). This thinning may be the result of three processes. First, the MES is thinned by growth of the palate (in terms of oronasal height) and by epithelial cell migration from the region of the MES onto the oral and nasal aspects of the palate (e.g. Fyfe and Ferguson, 1988). Second, there is programmed cell death (apoptosis) in the MES. For example, by using the TUNEL technique, and by assessing the presence of macrophages, Martinez-Alvarez et al. (2000) have shown that MES cells die in the developing mouse palate at the time of fusion. Programmed cell death is also suggested by the finding that DNA synthesis ceases in the medial

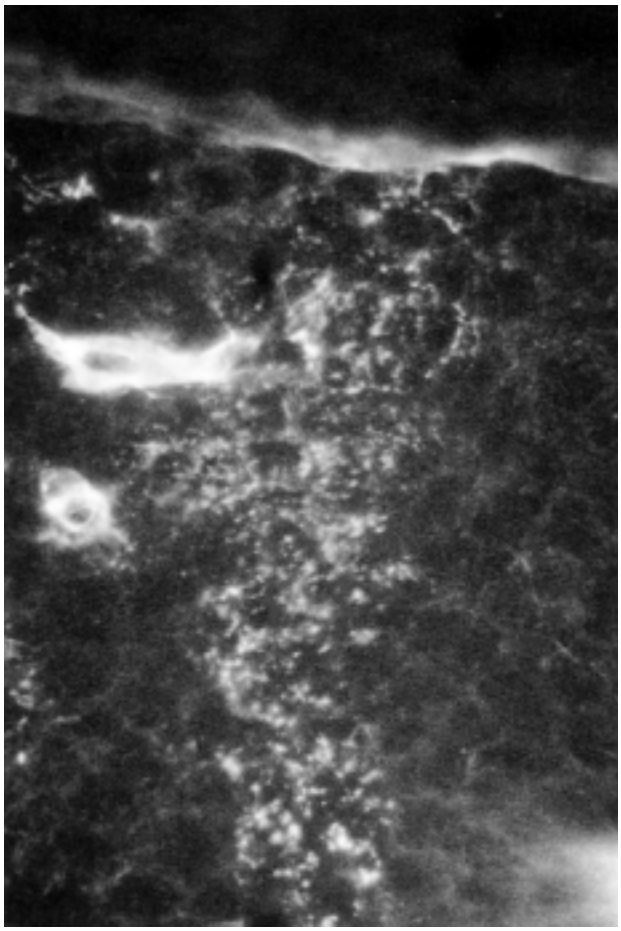


Figure 17. Late stage of fusion of the palatal shelves immunocytochemically labelled for type IV collagen and showing disruption of the midline epithelial seam. x 250. Courtesy of Professor M.W.J. Ferguson.

edge epithelial cells one day prior to shelf contact (Hudson and Shapiro, 1973). Furthermore, cyclic AMP increases just before shelf fusion (e.g. Ferguson, 1987) and exogenous cyclic AMP is associated with precocious cell death in the medial edge epithelia (Pratt and Martin, 1975). It has also been shown that epidermal growth factor (EGF) inhibits medial edge cell death (Hassell, 1975; Pratt et al., 1984; Pratt, 1984) and that this inhibition is blocked by exogenous cyclic AMP (Hassell and Pratt, 1977). Care must be taken, however, when interpreting the effects of cyclic AMP since physiologically it is an intracellular messenger and may therefore be mediating differential gene expression triggered by other events occurring at the cell surface. Martinez-Alvarez et al. (2000) also suggested that TGF-β_3 is an inducer of apoptosis during palatal fusion. Third, there is good evidence that some of the epithelial cells migrate from the MES into the palatal shelf mesenchyme and differentiate into cells indistinguishable from the mesenchymal cells (e.g. Ferguson, 1988). Indeed, it is well known that epithelial cells can migrate and differentiate into mesenchymal-like cells in other circumstances during development. Although labelling of MES cells with vital lipophilic markers has not clarified whether such cells migrate and/or transform into mesenchyme, *in vitro* studies that involve infecting the cells with the replication-defective helper-free retroviral vector CXL carrying the *Escherichia coli* lacZ gene (thus enabling analysis of β-galactosidase activity in the cells and the determination of cell fate) indicate that the cells of the MES transform into mesenchyme during palatal fusion (Martinez-Alvarez et al., 2000).

There have been many experiments to help clarify the nature of the epithelial-mesenchymal interactions during fusion of the palatal shelves. In the main, these experiments have involved the separation and then the recombination in culture of the epithelial and mesenchymal components of the shelves. Overall, these experiments have shown that, as with epithelial-mesenchymal interactions for tooth development, it is the mesenchyme that signals epithelial differentiation and behaviour (e.g. Ferguson and Honig, 1984). The nature of this signal is controversial. Figure 18 shows the medial edge

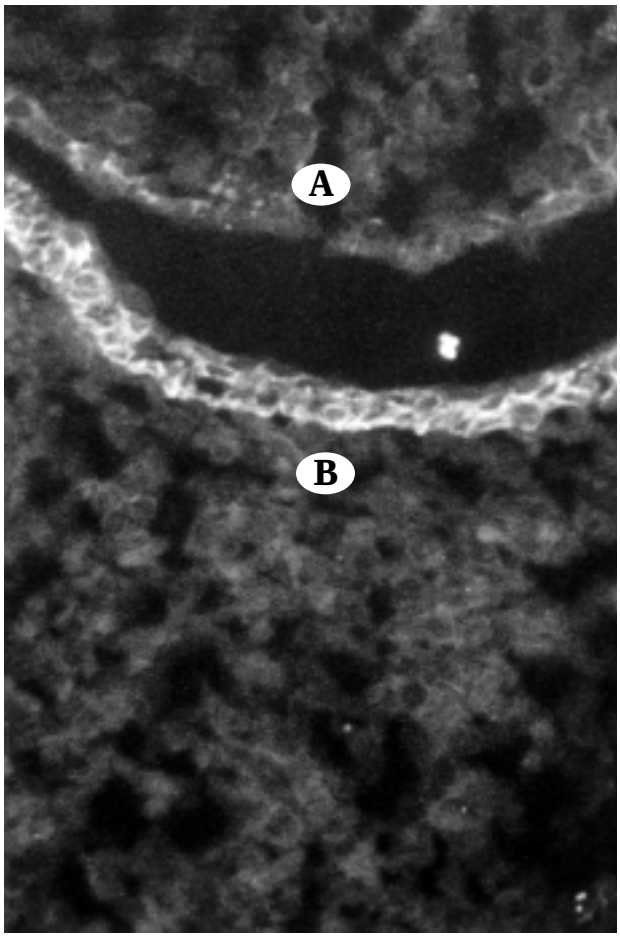


Figure 18. The medial edge epithelia of palatal shelves failing to label immunocytochemically for type IX collagen before shelf elevation. A = palatal shelves; B = epithelium covering floor of the mouth. x 560. Courtesy of Professor M.W.J. Ferguson.

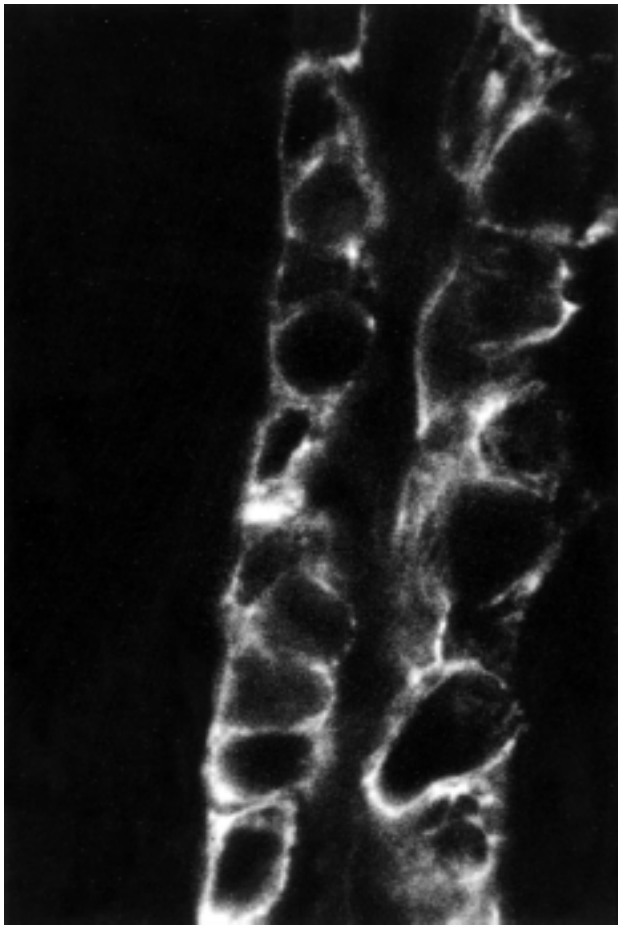


Figure 19. The medial edge epithelia of palatal shelves labelled immunocytochemically for type IX collagen at a time when medial edge epithelial differentiation occurs as determined by recombination experiments. x 500. Courtesy of Professor M.W.J. Ferguson.

epithelia of palatal shelves labelled immunocytochemically for type IX collagen before shelf elevation (Fyfe and Ferguson, 1988). Although it was once proposed that the palatal mesenchyme could signal epithelial differentiation directly by cell-to-cell contact, mesenchymal-epithelial cell contacts are very rare during palatogenesis (Ferguson, 1988). It seems that ECM molecules may provide the signal and work has been undertaken to assess the role of type IX collagen (Ferguson, 1988). Figure 18 shows that, at the earliest stages before shelf elevation, the medial edges of the palatal shelves label poorly for type IX collagen compared with floor of the mouth epithelia. Figure 19 shows the medial edge epithelia of palatal shelves labelled immunocytochemically for type IX collagen at a time when medial edge epithelial differentiation occurs as determined by recombination experiments. At this stage, type IX collagen appears around the surfaces of the medial edge epithelial cells. It is believed that the control of the synthesis of type IX collagen is influenced by growth factors (Ferguson, 1988).

Ferguson (1988), using immunocytochemical labelling with antibodies against epidermal growth factor receptors, has demonstrated the presence of such receptors on the mesenchymal cells adjacent to the MES of fusing palatal shelves. Epidermal growth factor (EGF), or its embryonic homologue known as transforming growth factor α (TGF- α), is known to inhibit palatal medial edge epithelial cell death in the presence of mesenchyme (Tyler and Pratt, 1980). Furthermore, it has been shown that the synthesis of ECM molecules (including type IX collagen) is stimulated by factors such as TGF–α and TGF–β and is inhibited by fibroblast growth factors (FGF) (Sharpe and Ferguson, 1988; Ferguson, 1988; Sharpe et al., 1993). When palatal shelves are organ-cultured with EGF, the medial edge of the palatal shelf shows a nipple-like bulge, medial edge epithelial cell death is absent, and the mesenchyme possesses increased quantities of ECM molecules (Jelnick and Dostal, 1974; Nanda and Romeo, 1975; Cleaton-Jones, 1976a; Ferguson, 1988). It has been proposed, therefore, that the palatal shelf mesenchyme produces growth factors that either directly signal epithelial differentiation or, by stimulating ECM production, indirectly influence differentiation through this matrix. Ferguson (1988) has suggested that EGF receptors show regional heterogeneity and that the receptors only appear beneath the medial edge of the shelves when the epithelial seam is degenerating.

Hyaluronan is also critical during breakdown of the MES, providing a suitable matrix for some of these cells to undergo epithelial-mesenchymal transformation in order to subsequently migrate to the oral and nasal aspects of the palate. Hyaluronan produced by *Has* 2 is vital for epithelial-mesenchymal transformation during heart morphogenesis (Camenisch et al., 2000) and is proposed to function in a similar manner in the developing palate.

Recent studies have highlighted the importance of TGF–β during fusion of the palatal shelves. TGF-$\beta_{1, 2}$ and $_3$ expression during mouse palatogenesis has been extensively studied (both temporally and spatially) and results suggest that TGF-βs act as regulators at palatal shelf fusion. Immediately before palatal fusion, TGF-β_3 expression is localised in the medial edge epithelium (Pelton et al., 1990: Fitzpatrick et al., 1990). Shortly afterwards, TGF-β_1 expression is also detected in the medial edge epithelium but TGF-β_2 expression can only be seen in the mesenchymal cells. Furthermore, for TGF-β_3 knockout mice, the TGF-β_3-null mutant fetuses develop cleft palate so that all TGF-β_3-null pups die shortly after birth (Proetzel et al., 1995). The TGF-β_3 knockout mouse is characterised by appearing to have no other morphological anomalies (excepting the lung). TGF-β_1 knockout mice, however, do not develop cleft palate (Shull et al., 1992; Kulkarni et al., 1993) and

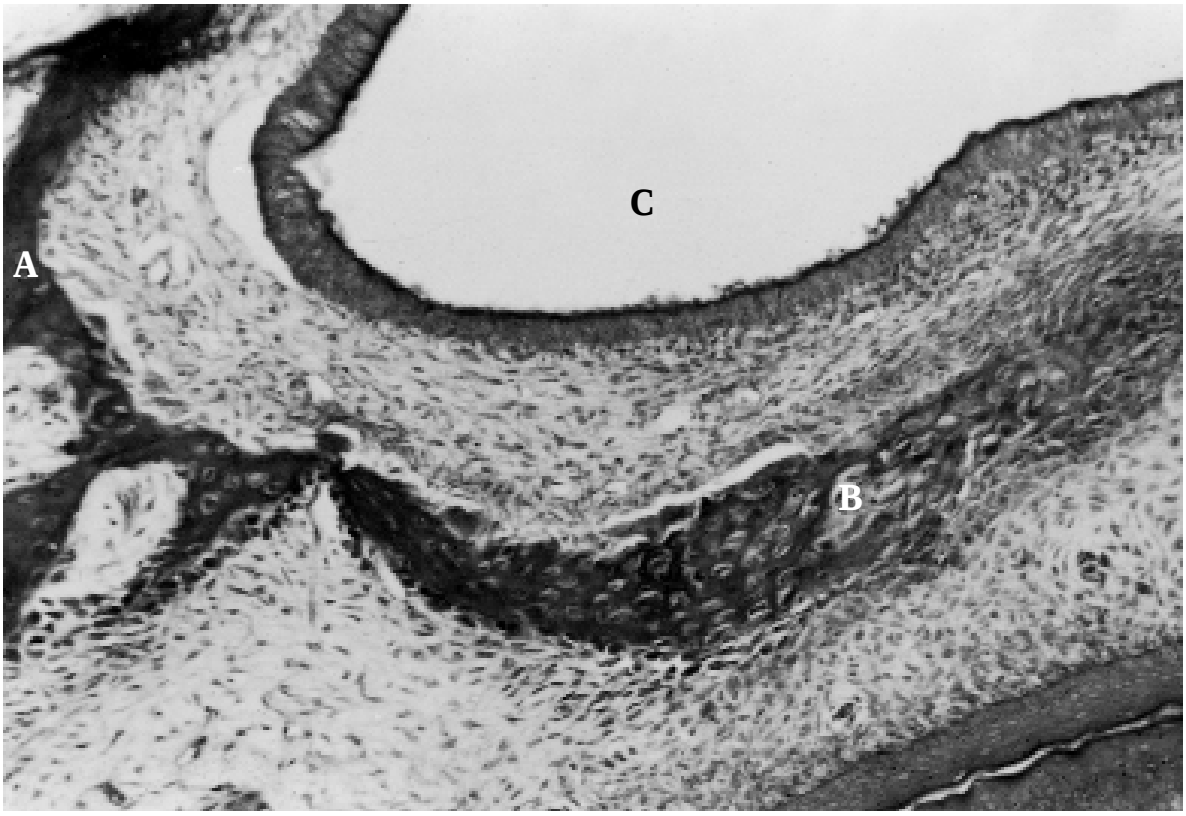


Figure 20. Coronal section through the developing hard palate showing early ossification. A = developing body of maxilla; B = bone extending from body of maxilla into palate; C = nasal cavity. (Masson's trichrome). x 160.

cleft palate, along with many other types of abnormalities, is observed (but at lower incidence rates) in TGF-β_2 knockout mice (Sanford et al., 1997). That TGF-β_3 plays an important role in palatal shelf fusion is also shown by the fact that palate fusion fails to occur *in vitro* when the activity of TGF-β_3 is inhibited by antisense oligonucleotide or by neutralising antibody (Brunet et al., 1995). More recently, Taya et al. (1999) reported that mutation of the TGF-β_3 gene results in cleft palate formation and that, when palates from transgenic mice with TGF-β_3 deletions are grown in organ culture such that shelves were placed in homologous (+/+ vs +/+, -/- vs -/-, +/- vs +/-) or heterologous (+/+ vs -/-, +/- vs -/-, +/+ vs +/-) paired combinations, pairs of -/- and -/- shelves failed to fuse while pairs of +/+ and =/+ shelves showed complete disappearance of the MES whereas -/- and +/+ shelves retained some remnants of the MES. They also studied the ability of TGF-β_3 family members to rescue the fusion between -/- and -/- palatal shelves *in vitro* by adding to the culture medium recombinant human TGF-β_1, porcine TGF-β_2, recombinant human TGF-β_3, recombinant human activin, or porcine inhibin. It was reported that, for untreated organ culture -/- palate pairs that would be expected to show complete failure to fuse, TGF-β_3 treatment induced complete palatal fusion whereas TGF-β_1 or TGF-β_2 produced near normal fusion and activin and inhibin had no effect. The mechanism whereby TGF-β_3 rescued the fusion was claimed to be related to the appearance of filopodia-like process on the surface of the MES cells that are coated with material resembling proteoglycan.

Once fusion is complete, the hard palate ossifies intramembranously from four centres of ossification, one in each developing maxilla and one in each developing palatine bone (Sperber, 2001; Berkovitz et al., 2002; Meikle, 2002). The maxillary ossification centre lies above the developing deciduous canine tooth germ and appears in the eighth week of development. The palatine centres of ossification are situated in the region forming the future perpendicular plate and appear in the eighth week of development. Incomplete ossification of the palate from these centres defines the median and transverse palatine sutures. There does not appear to be a separate centre of ossification for the primary palate in Man (in other species there being a separate "premaxilla"). Figure 20 provides a coronal section through the developing hard palate to show early ossification.

Clinical considerations

Malformations of palatogenesis may result in the appearance of clefts (Sperber, 2001; Berkovitz et

al., 2002; Meikle, 2002). Clefts of the palate, like those of the lip, are multifactorial malformations, involving both genetic (polygenic) and environmental factors. Clefts may result from disturbances of any of the processes involved during palatogenesis, i.e. from defective palatal shelf growth (e.g. Abbott et al., 1990); delayed shelf elevation or failure of elevation (e.g. Ferguson, 1981a); defective shelf fusion or lack of degeneration of the MES; or failure of mesenchymal consolidation and/or differentiation (e.g. Abbott and Birnbaum, 1989).

The mildest form of cleft is that affecting the uvula, such a disturbance occurring relatively late in the process of palatal malfusion. Disturbances occurring during the early phases of palatal fusion can result in a more extensive cleft involving most of the secondary palate. Should the cleft involve the primary palate, it may extend to the right and/or left of the incisive foramen to include the alveolus, passing between the lateral incisor and canine teeth. Cleft palate may be associated with cleft lip, though the two conditions are independently determined. Dental malformations are commonly associated with a cleft involving the alveolus. A submucous cleft describes a condition where the palatal mucosa is intact, but the bone/musculature of the palate is deficient beneath the mucosa. Less problematic than clefts (but more common) is the retention of epithelial remnants in the midline that eventually become cystic.

Hypotheses to explain the mechanisms responsible for cleft palate formation range from genetic predisposition (e.g. Bonner and Slavkin, 1975) to the administration of teratogens (e.g. Fraser and Fainstat, 1951). Ferguson (1981b) has also proposed that the expression of a cleft palate is a manifestation of phylogeny - birds develop a physiological cleft and the oral and nasal cavities are not separated (e.g. Shah and Crawford, 1980). Recent research indicates that retinoids in excess have a teratogenic effect, producing clefts of the palate and the abnormal appearance of "islands" of cartilage in the mesenchyme (Emmanouil-Nikoloussi et al., 1999; Emmanouil-Nikoloussi et al., 2000). More recently, work by Gunston, Moxham and Emmanouil-Nikoloussi (unpublished data) shows that all-trans retinoic acid (RA) is the most teratogenic isomer of RA in terms of rat palatal abnormalities, that the time of administration of RA is more critical than dose, but that immunohistochemical labelling for cartilage ECM molecules fails to detect ectopic cartilage within the palates. Ectopic localization of Sonic hedgehog protein (*Shh*) in the developing rostral neural tube is also associated with craniofacial defects (Nasrallah and Golden, 2001). This is thought to be due to disruption in normal genes expression patterns (e.g. *wnt-3a, wnt-4, Pax-6, HNF-3(and Ptc*).

Studies using transgenic mice suggest that many homeobox genes and transcription factors are involved in palatogenesis. For example, Satokata and Maas (1994) have highlighted the possible significance of *Msx1* and Winograd et al. (1997) of *Msx2*. Tissier-Seta et al. (1995) have suggested a role for *Barx 1*. Gendron-Maguire et al. (1993) and Rijli et al. (1993) suggest that Hoxa2 is important and Martin et al. (1995) have implicated *Mhox*. Peters et al. (1997) have determined a role for Pax9 and Mo et al. (1997) have reported on the significance of *Dli* and *Dli3*. Finally, Takihara et al. (1997) suggest that there is expression of *rae28* during palatogenesis and Takagi et al. (1998) *deltaEF1*. Additionally, many cytokines (and their receptors) are also involved in palate development. For example, TGF-alpha/EGF receptors, TGF-β_2, and TGF-β_3 have important functions (Miettinen et al., 1999; Sanford et al., 1997; Proetzel et al., 1995; Kaartinen et al., 1995; see also above). Furthermore, importance has also been claimed for activin-βA, activin-receptor type II and follistatin (Matzuk et al., 1995a, b, c). Lohnes et al. (1993, 1994) have shown a role for retinoic acid receptor gamma during palate development and Kurihara et al. (1994) have reported on endothelin. Orioli et al. (1996) have suggested an involvement of sek4/nuk1.

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