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Review Articles

Palatogenesis and Palatoschisis. A Review on the Cellular and Molecular Mechanisms of Development

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The formation of the palate is a highly complex developmental process beginning with the growth of facial primordia and the subsequent fusion of the nasal and maxillary prominences, thus giving rise to the primary palate and upper lip. Development of the secondary palate occurs through the union of bilateral palatal shelves arising from the maxillary prominences that fuse with each other on the midline, thus leading to a continuous palate that definitively separates the oral and nasal cavities. If the intricate process underlying craniofacial morphogenesis is disrupted, an orofacial cleft may occur. The cellular and molecular bases of palate shelf fusion have not yet been clearly elucidated. Therefore, this review focuses mainly on the recent advances in knowledge about secondary palatogenesis, with special attention to the cellular and molecular mechanisms involved in the fusion of the apposing shelves as well as their failure to properly take place, thus generating clefting.

Key words: palatogenesis, cleft lip and cleft palate, medial edge epithelia (MEE), medial epithelial seam (MES), epithelial-mesenchymal transition, apoptosis, cell migration

Introduction

Cleft palate is a congenital deformity characterised by a defect in the roof of the oral cavity, resulting from abnormal embryonal development. Cleft lip and cleft palate are among the most common birth defects, affecting approximately 1 in 700 live-born babies [13, 16, 38, 48, 56]. The palate acts as a mechanical barrier separating the mouth from the nasal cavities, allowing breathing and simultaneous food intake. Structurally, it consists of a bony hard part anteriorly and a muscular soft part posteriorly, the latter functioning as a valve that closes the nasal airway, thus permitting swallowing and directing airflow during speaking [44]. Patients with cleft lip or cleft palate require significant care from birth to adulthood engaging many medical disciplines, including nursing, maxillofacial and plastic surgery, otolaryngology, orthodontics, speech therapy,

audiology, psychological and genetic counselling, and therefore they impose a substantial economic burden [56, 73, 78]. There is a wide variability in incidence of cleft lip or cleft palate in relation to geographic origin, ethnicity and socioeconomic status [15, 49, 71, 74]. In general, Asian or Latin American populations have the highest frequencies, Caucasian populations intermediate, and African populations the lowest, thus suggesting that the contribution of individual susceptibility genes can vary noticeably across different populations [13, 16]. However, the aetiology is heterogeneous, complex and multifactorial, involving both genetic and environmental factors, as well the interactions between them. Indeed, a further degree of complexity is provided by the interplay of both gene-gene and gene-environment interactions, contributing differently to the two main types of cleft in the clinical setting, non-syndromic and syndromic orofacial clefts [13, 70, 72, 76]. Clefts are usually classified as either cleft lip with or without palate involvement, or cleft that involves the palate alone [51]. Approximately 70% of cases of cleft lip with or without cleft palate, and 50% of cleft palate alone occur as isolated entities, with no other apparent associated abnormalities, and are therefore commonly termed isolated, non-syndromic clefts; the remaining cases are part of complex syndromes that, in addition to clefting, also include further anomalies [47, 51]. There is a 2:1 male to female ratio for cleft lip with or without cleft palate, and approximately a 1:2 male to female ratio for cleft palate alone [13, 16, 56]. Furthermore, syndromic clefting can be as a feature of chromosomal syndromes, inherited disorders affecting a single gene, or syndromes induced by teratogens, such as alcohol, tobacco smoke and drugs [11]. Approximately 500 syndromes associated with cleft lip or palate caused by a genetic defect have been identified [41]; the most common are Pierre Robin sequence, Van der Waude syndrome, DiGeorge/Velocardial syndrome, Stickler syndrome, Loeys-Dietz syndrome, Apert syndrome, Crouzon syndrome, and Treacher Collins syndrome [11, 77].

Cleft lip with or without cleft palate and cleft palate alone have historically been considered separate entities owing to their different developmental origins, and different epidemiology, genetics and family patterns [16, 47]. However, there is definitely a certain degree of overlap between all of these deformities. Since the formation of the upper lip/primary palate precedes the fusion of the secondary palate, disruption in the development of the upper lip/primary palate may compromise the correct contact between the secondary palatal structures and, therefore, cleft lip and cleft palate have a high co-morbidity [16, 30]. An integration of epidemiological, candidate gene and genome-wide essays as well as studies on animal models, has recently greatly deepened our understanding of the causes of both syndromic and non-syndromic clefts. However, due to the broad genetic heterogeneity, departure from the Mendelian inheritance models and the need for very large data sets, there has been less progress in our knowledge on the genetic contribution to the aetiology of non-syndromic clefts [13]. Mouse models have been commonly used in research on orofacial clefts due to the possibility of multiple genetic modifications and the high similarity between mouse and human facial morphogenesis [26]. Genetic manipulations in mice coupled with detailed morphological and molecular analyses of mutant mouse models has revealed that facial development is regulated by an extensive network of signalling molecules and transcription factors with abundant crosstalk between the distinct pathways, some of which are under post-transcriptional control [5, 21, 42, 43, 44, 64, 69, 75].

The following discussion will cover the cellular and molecular mechanisms of palatogenesis and the developmental alterations underlying cleft lip and cleft palate. Although cleft lip with or without cleft palate is the most common type of orofacial cleft, the cellular and molecular bases of upper lip/primary palate development have not been studied as thoroughly as those of secondary palate, possibly due to more limited usable models to study upper lip development [30]. Accordingly, this review covers in more detail secondary palatogenesis and clefting, with less discussion on the mechanisms underpinning lip/primary palate morphogenesis and clefting.

Facial Development

Face morphogenesis requires coordination of a series of complex events, including cell proliferation, migration, differentiation, and death [2, 16, 41, 64]. In humans, palate development starts around the 4th week of gestation with the growth, fusion and rearrangement of five primordia consisting of a core of mesenchymal cells originating mainly from the proliferation and migration of cranial neural crest cells, covered by an epithelium of ectodermal origin. At the rostral boundary of the primitive mouth (stomodeum) there is an unpaired median frontonasal prominence, while two paired maxillary prominences flank the primitive mouth and two paired mandibular prominences lie below (**Fig. 1**). As facial primordia grow by proliferation of their mesenchyme, the surface ectoderm bi-stratifies and undergo a critical differentiation to form a thin outer layer of flattened and tightly connected periderm cells characterised by a non-adhesive apical surface, thus allowing only highly controlled adhesion [2, 41]. Indeed, formation of the upper lip and secondary palate requires spatiotemporally regulated inter-epithelial adhesions and subsequent dissolution of the newly-formed intervening epithelial seams between the maxillary and medial/lateral nasal processes, and between the palatal shelves, respectively [41]. Initially, the facial prominences of the right and left sides are widely separated from each other, but they progressively move towards the midline [16]. Thickening around the nasal placodes divides the frontonasal prominence into paired medial and lateral nasal processes at the 5th week. Then, at the 6th week the continuous growth of the maxillary prominences medially pushes the medial nasal processes towards the midline that eventually coalescence around the 7th week and, meanwhile, the freely projecting bases of the medial nasal prominences fuse with the adjacent maxillary and lateral nasal prominences. The fused medial nasal processes form the inter-maxillary segment from which the philtrum of the upper lip, the primary palate, the upper central region of the jaw and the middle part of the nose are formed. Formation of the upper lip takes place through a fusion process that includes epithelial adherence between nasal and maxillary prominences, formation of a seam, and then its dissolution by apoptosis and/or epithelial-mesenchymal transition (EMT) [2, 16, 32]. A Pbx-dependent regulatory network seems to control this fusion mediated by the Pbx/Wnt/p63/Irf6 pathway promoting apoptosis [17], cross-talking with the Pbx/Snail/Smad/E-cadherin pathway leading to EMT [46]. The dual effect of the transcription factor Pbx is not surprising, as many lines of evidence indicate that apoptosis and EMT can be alternative, complementary or sequential processes that secure normal tissue morphogenesis [46] (also *vide infra,* in the discussion on the secondary palatogenesis). Failure in the fusion between the maxillary and medial/lateral nasal processes results in

cleft lip that can extend into the nostril and/or primary palate [2, 16, 26, 32, 67]. Extension to the nostril indicates that the defective fusion also involved the lateral nasal process [32]. The primary palate includes a small triangular area of hard palate anterior to the incisive foramen and the central maxillary alveolar arch with the four incisor teeth, and its development is completed together with lip closure by the end of the 6th week [41]. The lateral nasal processes form the nasal alae, while the maxillary prominences contribute to the lateral parts of the upper lip and, by merging with the mandibular prominences below, contribute to form the cheeks. The mandibular prominences also fuse at the midline to form the lower lip and jaw [16].

Secondary palatogenesis occurs from the 6th to the 12th week of development [2, 16, 26, 41]. It begins with the outgrowth of bilateral intra-oral projections known

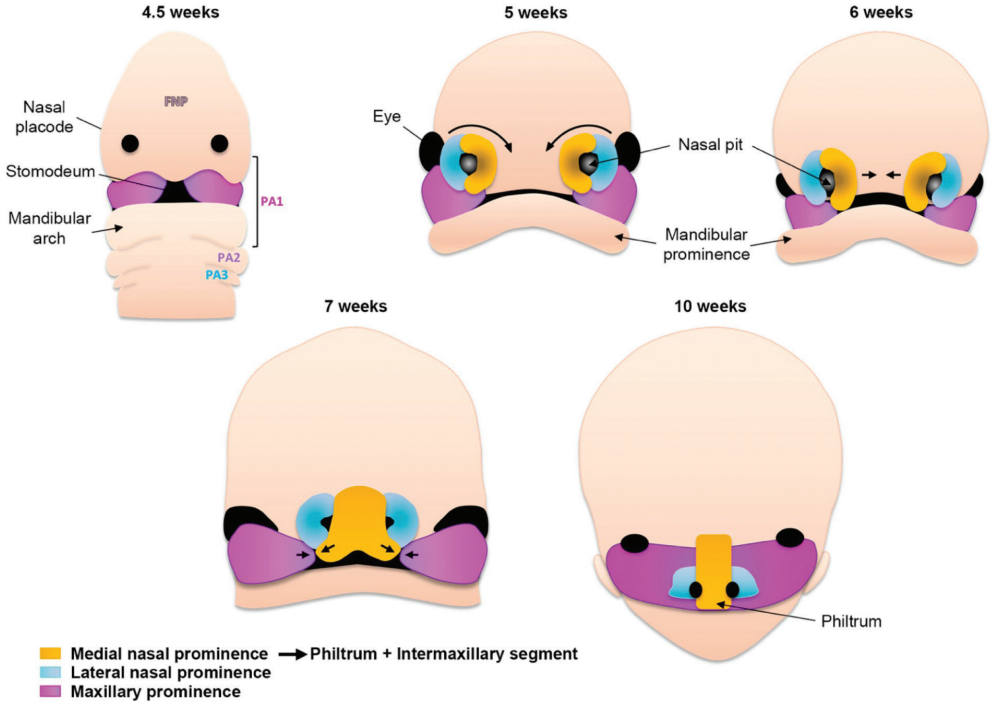

Fig. 1. Schematic drawing depicting human facial morphogenesis. At 4.5 weeks the facial primordia include the frontonasal prominence (FNP, *beige*) with nasal placodes placed rostrally to the stomodeum, the paired maxillary prominences (*dark pink*) flanking the stomodeum, and the mandibular prominences (*beige*) placed caudally; derivatives of the first pharyngeal arch (PA1) are indicated. At 5 weeks thickening of the frontonasal prominence around the nasal placodes results in medial (*yellow*) and lateral (*blue*) nasal prominences. Medial nasal prominences converge (6 weeks) and coalesce on the midline (7 weeks), thus giving rise to the intermaxillary segment and, therefore, to the middle of the nose and the philtrum of the lip (10 weeks), while the remaining upper lip arises from the maxillary prominences. The lateral nasal prominences form the nasal alae. From Antiguas, Paul and Dunnwald (ref. 2), reprinted by kind courtesy.

as palatal shelves arising from the oral side of the maxillary prominences (**Fig. 2A**). At first, they grow vertically downward along the sides of the tongue (**Fig. 2B**) but, at around the 7th week, as the mandible grows and lengthens and the tongue descends into the oral cavity creating space above it, they elevate above the dorsum of the tongue (**Fig. 2C**). Mouth opening, tongue protrusion and hiccup movements, and their associated pressure changes, probably facilitate palate shelf elevation [16]. In general, female human embryos elevate their palate shelves about 1 week later than males; this might possibly explain the higher female incidence of cleft palate alone, due to prolonged risk of developmental errors, or to greater exposure to teratogenic agents [16]. After elevation, the palatal shelves further grow horizontally towards the midline, and fi-

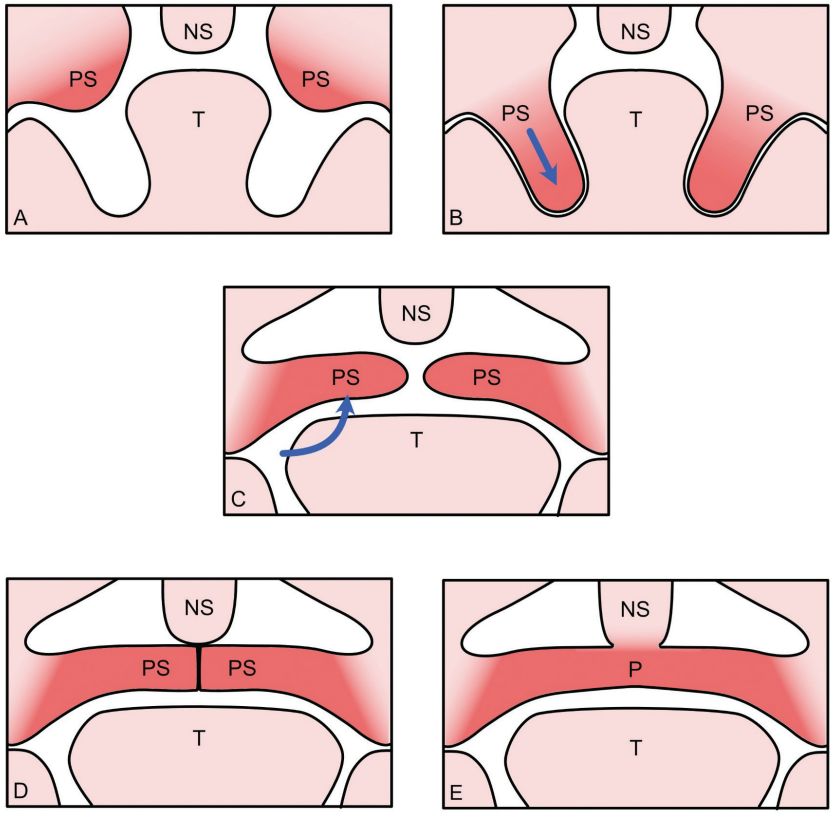

Fig. 2. Highly schematic drawing depicting the stages of secondary palate development. (A) At 6 weeks, palatal buds appear on the intra-oral sides of the maxillary processes, and initiate their growth downwards. (B) The palatal shelves grow down along the sides of the tongue that in this stage is placed high in the oral cavity, filling it almost entirely. (C) At 7 weeks, the tongue drops and flattens, thus allowing the palate shelves to elevate above the dorsum of the tongue until they reach a horizontal position, and grow further towards each other. (D) At 8 weeks, the shelves adhere to each other through their medial edge epithelia (MEE) at their tips, which then merge to form the medial epithelial seam (MES). (E) At 12 weeks, dissolution of the MES leads to mesenchymal confluence and complete fusion of the shelves to form a continuous palate, which also fuses with the overlying nasal septum. PS, palate shelves; T, tongue; NS, nasal septum; P, palate.

nally contact each other at the 8th week. The epithelia at the tips of the palatal shelves, called medial edge epithelia (MEE), adhere each other and merge to form a midline epithelial seam (MES) (**Fig. 2D**). Initial adhesion between the opposing shelves occurs in the middle of the palate shelves and proceeds anteriorly and posteriorly, similar to a "zipper" closing in both directions, ending at the incisive foramen anteriorly and the uvula posteriorly [2]. Between the 9th and the 12th weeks of gestation the epithelium of the MES disintegrates, thus leading to confluence of the mesenchymal stroma and palate continuity [2, 15, 16, 50] (**Fig. 2E**). Moreover, the united palate fuses anteriorly with nasal septum and the primary palate. The completion of the fusion process therefore leads to the definitive division of the oronasal space into separate oral and nasal cavities. Clefts of the palate can arise due to failure at any of the steps of palatogenesis, including palatal shelf growth, elevation, or fusion [2, 11, 16, 21, 26, 44, 64]. The primitively bi-layered epithelium covering the palatal shelves has different fates. Indeed, the mucosal lining of the oral side of the palate will differentiate into stratified squamous epithelium, while the epithelium of the nasal side will differentiate into respiratory-type pseudo-stratified, ciliated epithelium. The palatal mesenchyme will differentiate anteriorly into bone to form the hard palate, and posteriorly into muscle - thanks to myogenic cells derived from the mesoderm - to form the soft palate.

Molecular Regulation of Palatal Shelf Growth and Patterning

Growth of the palate shelves requires epithelial-mesenchymal interactions regulated by an intricate network of signalling pathways and transcription factors with extensive crosstalk between them, the disruption of which can be instrumental in the developmental pathogenesis of clefting. A wealth of genes have been implicated in the palatal growth of both animal models and humans, and it has been found that also a single gene mutation can lead to cleft. Although there are a plenty of molecules with a known role in palate development, the underlying principles of molecular signalling in palate morphogenesis can basically be attributable to the Shh, FGF, BMP, Wnt and - as we will see specifically later - TGFβ signalling pathways [19, 21, 42, 43, 44, 57, 64].

The central player in the organisation and regulation of palatal shelf growth is definitely Sonic hedgehog homolog (Shh) signalling that cross-talks with Fibroblast growth factor (FGF) and Bone morphogenetic proteins (BMPs) (**Fig. 3**). Shh is already expressed in the early oral epithelium prior to palate shelf outgrowth [42, 59]. Abrogation of Smoothened (Smo), i.e. the transducer of Shh signalling, results in defective shelf growth and cleft palate, thus highlighting a critical role of Shh signalling for palate development. The mesenchymal expression of the transcription factors Foxf1/2 and Osr2 is activated by Shh, which also regulates the expression of the cell cycle activators Cyclin D1/2 in the palatal mesenchyme, thus sustaining its proliferation and growth [40]. The epithelial expression of Shh is largely dependent on the mesenchymally expressed FGFs, with FGF10 inducing, whereas FGF7 repressing Shh expression in the palate epithelium. Shh positively regulates FGF10 expression through the Osr2 transcription factor in the mesenchyme and, therefore, FGF10 and Shh act in a positive feedback loop to maintain each other's expression [42]. Moreover, a further Shh/Foxf/FGF18/Shh feedback loop has been identified in which the transcription factors Foxf1/2 downstream of Shh control Shh expression

in the epithelium [65]. In addition, maintenance of Shh expression also requires the mesenchymal transcription factors Msx1 in the anterior palate, and Pax9 in the posterior palate. Indeed, Msx1 is restricted to the anterior part of the developing palate and regulates anterior palate mesenchyme proliferation through activation of BMP4 which, in turn, signals to the epithelium to maintain Shh expression. While BMP4 expression in the anterior palate is dependent on Msx1, and each stimulates the expression of the other, BMP4 expression in the posterior palate is dependent on Pax9. BMP4 is fundamental to maintain Shh expression which, in turn, also induces BMP2 expression in the mesenchyme that promotes palatal growth. Pax9 is also upstream of Osr2 and FGF10. Therefore, Pax9 controls the Osr2/FGF10/FGFr2b/Shh, Msx1/BMP4/Shh and the BMP4/Shh pathways, with both Pax9 and Shh signalling converging on Osr2 transcription factor (**Fig. 3**). In summary, Pax9 regulates two major

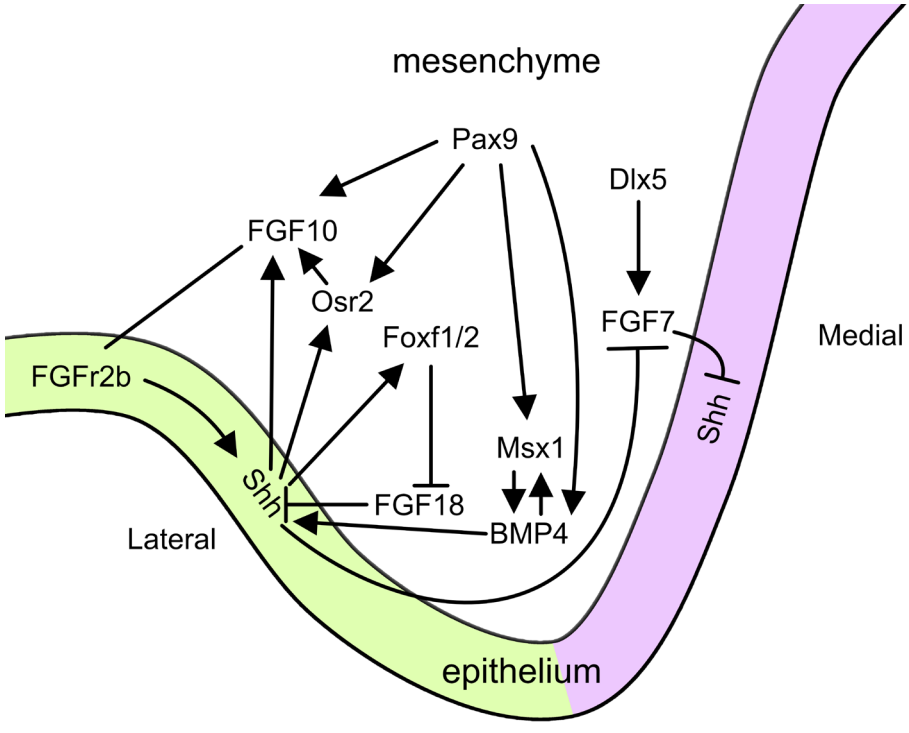

Fig. 3. Molecular control of palatal shelf development. Highly simplified diagram of signalling interactions in a developing palate shelf with the medial side on the right. Shh is expressed in the epithelium and acts in a feedback loop with the FGF10 expressed in the mesenchyme to self-maintain their expression, thus ensuring palatal growth. Another feedback loop between Msx1 and BMP4 also helps to maintain Shh expression in the anterior palate. Pax9 regulates these feedback loops and, furthermore, acts upstream of BMP4 to sustain Shh expression in the epithelium of the posterior palate. Another feedback loop involving the mesenchymal transcription factors Foxf1/2 also regulates Shh signalling. Expression of FGF7 in the mesenchyme is maintained by the transcription factor Dlx5, and both are restricted to the medial side of the palate where FGF7 and Shh repress the expression of each other. Arrows represent induction, blunt arrows indicate inhibition.

feedback loops that control growth and patterning in the developing palate, one involving FGF, the other involving BMP4, both aimed at maintaining Shh expression in the epithelium and, therefore, the consequent effect of stimulating palate shelf growth [43]. In addition to Msx1 and Pax9, other transcription factors including Shox2, Barx1, Mn1 and Tbx22 are differentially expressed along the anteroposterior axis, with Shox2 being restricted to the anterior palate, and Barx1, Mn1 and Tbx22 expressed in the posterior palate mesenchyme, where Mn1 acts upstream of Tbx22 to regulate posterior palatal growth [42]. Wnt5a is another signal affecting palate shelf growth with higher levels in the anterior region and a graded anteroposterior expression. Wnt5a appears to be a requirement for palatal mesenchymal cell proliferation and migration, mediated through non-canonical Wnt pathway and, indeed, its deficiency in mice causes cleft palate resulting from impaired palate shelf growth [27].

Lip and Palate Development and Clefting

Cleft lip with or without cleft palate and cleft palate alone (**Fig. 4**) result from defective embryonic morphogenesis between the 6th and 12th week of human gestation [38]. Cleft lip may arise from disturbances in cell proliferation, survival and migration of the neural crest-derived cells to facial primordial buds resulting in impaired growth, or in defective fusion between the nasal and maxillary processes [7, 42]. Cleft palate

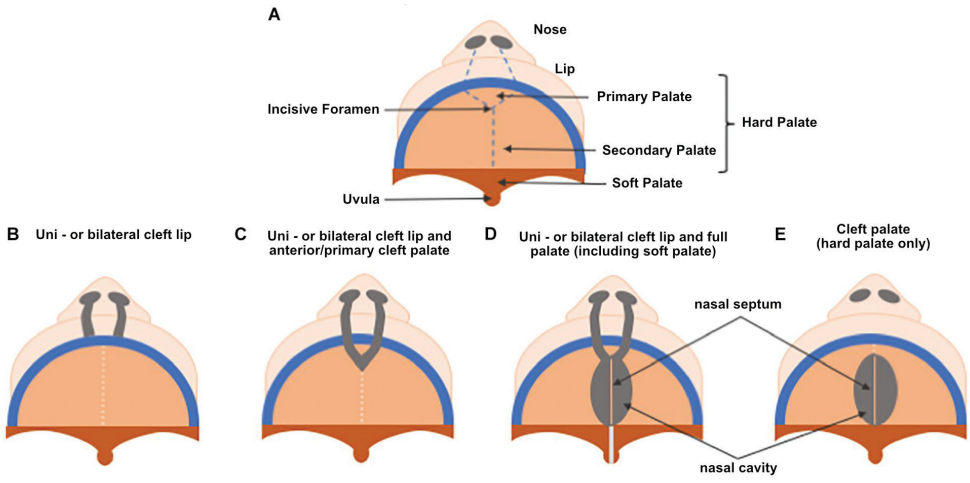

Fig. 4. Schematic representation of some clinical forms of cleft lip and/or palate in humans. There are various classification systems for cleft lip and cleft palate. For example, cleft lip can be classified as unilateral or bilateral; incomplete or complete; in the latter, the cleft involves the entire thickness of the upper lip and, in addition, the alveolar ridge and primary palate are often involved. Cleft palate can be unilateral or bilateral, and incomplete or complete (refs. 11, 64). In the diagram shown here, the defects are depicted as bilateral, with different degrees of involvement. (A) Normal anatomy of lip and palate. (B,C) Bilateral cleft lip, without (B), and with involvement of the primary palate (C). (D) Bilateral cleft lip with cleft of both the primary and secondary palate, comprising the soft palate. (E) Cleft palate involving only the secondary hard palate. Kindly reprinted from Paiva, Maas, dos Santos, Granjeiro, Letra (ref. 56).

may result from disorders at any stage of secondary palatogenesis, i.e. from impaired palatal shelf growth; delayed or failed shelf elevation; failure of shelf fusion or lack of degeneration of the MES; failure of mesenchymal consolidation and/or differentiation; or post-fusion rupture [16, 42, 50].

As we have seen, the upper lip arises from morphogenesis of the frontonasal prominence and fusion with the maxillary processes, whereas at a different time the secondary palatal shelves grow inside the primitive mouth from the maxillary processes. In some cases cleft palate may result entirely by a localised failure in the palate developmental program, but this does not happen in other cases. Fusion between the secondary palatal shelves occurs much later in embryogenesis than upper lip closure, and it is known that failure of lip formation can secondarily affect palatal shelf contact and cause cleft palate [32]. According to Ferguson, cleft secondary palate as a consequence of the cleft lip would occur in most cases because the tongue tip would become trapped above the cleft pre-maxilla, thus maintaining its high early position in the oral cavity, thus hindering palate shelf elevation and resulting in cleft secondary palate [16]. Furthermore, since palate development occurs concurrently with growth and expansion of the whole craniofacial complex, abnormalities of structures more or less in the vicinity of the palatal shelves can hinder the process that leads to contact between the opposing shelves, thus resulting in secondary cleft palate [5, 7, 42]. Therefore, in some cases of cleft palate the defect is not intrinsic to the palatal shelves, but results from different and unrelated morphological anomalies in surrounding or remote anatomical structures [14, 16], usually as a part of a syndrome comprising other malformations. It is known that initially the two palatal shelves grow downward, lateral to the tongue; at this point, the tongue is narrow and tall, almost completely filling the oral cavity; only during the 7th week the two palatal shelves dramatically change their positions and elevate to a horizontal position above the dorsum of the tongue [45] (**Fig. 2B, C**). Abnormal persistence of the upwardly displaced tongue will therefore result in a physical obstruction to palatal shelf elevation and, therefore, to cleft palate [5]. One of the better known examples of cleft palate as a secondary consequence of other craniofacial malformations is the Pierre Robin sequence, in which the lower jaw is either small (micrognathia) or set back from the upper jaw (retrognathia), resulting in failure of tongue descent, and thus causing a physical obstacle to palatal shelf elevation by the displaced tongue [5].

Below, we discuss the single phases of normal palatogenesis in relation with examples of cleft palate, mostly deriving from animal model studies.

Palatal shelf formation

Failure of palatal shelf formation is a rare, severe defect resulting from abnormal molecular networks operating between the palatal bud epithelium and mesenchyme, and involving Shh, BMP and FGF, occurring during early steps of palatogenesis. Shh is a key early signal that drives palatal shelf outgrowth through signalling from the epithelium to the underlying mesenchyme to promote palatal growth. As we have seen Shh, FGF and BMP functions in feedback loops that promote cell proliferation and, therefore, growth of the palatal shelves [5, 58, 59]. As one might expect, FGFr2b mutation affects the initial development of the palatal shelves and results in complete cleft palate [58]. On the other hand, Shh signalling is required for the activation of several important transcription factors in the mesenchyme, including Msx1, Foxf1/2 and Osr2

[65]. In animal studies, targeted mutation of Msx1, Osr2 or Shox2 generates cleft palate caused by altered mesenchymal proliferation [21]. Both Osr2-/- and Pax9-/- mouse embryos exhibit cleft palate and significant reduction in FGF10 expression in the developing palatal mesenchyme [68].

Palatal shelf elevation

Around the 7th week of gestation the palatal shelves rapidly elevate into a horizontal position above the tongue, thus suggesting that they have an intrinsic capability to elevate. There remains controversy concerning the mechanisms responsible for palatal shelf elevation. It has been proposed that the intrinsic shelf elevation force might be produced either by the generation of turgor pressure following hydration of the extracellular matrix or, alternatively, by proliferation, migration or contraction of the palatal shelf mesenchymal cells [50]. Many evidences indicate that the shelf elevation force is related to the presence of hyaluronan in the mesenchymal extracellular matrix. Hyaluronan is a glycosaminoglycan capable of binding a large amount of water, and therefore it could generate osmotic pressure [50]. The role of mesenchymal cell proliferation/migration in the palatal shelf elevation is more controversial, particularly considering the rapidity with which shelf elevation occurs. However, the production of an elevating force could be related to changes in cytoplasmic microfilament apparatus of the mesenchymal cells. Indeed, palatal shelf mesenchymal cells before elevation appear elongated and polarised, the cells nearest the basement membrane being perpendicularly aligned to the it. After shelf elevation, these cells became more rounded, possibly indicative of cell contraction, and this could be the means of generating the shelf elevation force, also indicating that actin-based contractility could be involved [50]. Since the elevation of the palate shelves is a rapid event, compared to alternative processes such as differential cell growth/migration, the actin-driven contraction model fits better than others. Osr2 regulates palatal mesenchymal cell proliferation and palatal shelf elevation [68], and it has been reported the occurrence of delayed palate shelf elevation in Osr2-/- (38), as well as in PDGFc-/- (12) mutant mice.

Failure of elevation due to abnormal adhesion and the role of periderm. A specific cause of failure of palate shelf elevation is the adhesion/fusion of the growing palatal shelves with the tongue or mandible. Under normal conditions, palatal shelves do not fuse with other oral structures, and this function is ensured by the presence of the non-sticky periderm layer [2]. Any factor that interferes with the differentiation and maintenance of the periderm can cause premature, abnormal adhesions. Peridermal cells differentiate from the basal layer through finely tuned molecular signalling. Basal cells express the transcription factor p63 that maintains the proliferative potential of the basal layer [60], but also activates Jag2/Notch signalling through FGFr2b, and induces Irf6 expression [42]. In supra-basal cells p63 becomes down-regulated, as Notch signalling represses p63 and, on the other hand, Irf6 promotes proteasome-mediated p63 protein degradation, thus determining the periderm specification. Therefore, in these cells Irf6 becomes strongly expressed, whereas p63 is down-regulated, as Irf6 converges with Jag2/Notch signalling to drive periderm differentiation through feedback down-regulation of p63. In this way, periderm differentiation of supra-basal cells is induced and maintained by Irf6 and Jag2/Notch signalling acting synergistically [41]. It follows that a malfunction of these pathways no longer ensures protection from unwanted adhesions, and therefore inappropriate fusion of other structures with the

palate shelves can prevent their elevation and cause cleft palate [7]. For example, in mice that do not express FGF10 - which is upstream of FGFr2b - the palatal shelf epithelium fuses with the tongue and the epithelium covering the mandible, thus preventing palatal shelf elevation. In these mice there is a severe reduction in the expression of Jag2 that disrupts the Jag2/Notch signalling and, therefore, periderm differentiation [10]. In humans, a gene that has been associated with inappropriate adhesions is Tbx22 and, indeed, mutations of this gene have been reported in families with X-linked cleft palate and ankyloglossia [10].

We will return to the question of the periderm when we discuss the formation and breakdown of the MES.

Contact and adhesion between the palatal shelves

Once elevated into a horizontal position, the palate shelves further grow until they contact each other at the midline (**Fig. 2C, D**). Failure of palatal shelves to meet after elevation is the most common type of cleft palate defect documented in animal studies [7]. In addition to its function in regulating the fate of the MEE/MES (*vide infra*), TGFβ3 is also critical for proper proliferation of the cranial neural crest-derived palatal mesenchyme in the palatal buds [7], thus underscoring the crucial role of TGFβ3 signalling in controlling the entire process of palatogenesis [21]. Mice lacking TGFβr*2* in the palate shelf mesenchyme develop a cleft palate due to reduced horizontal extension of the shelves [28]. Similarly, embryos lacking PDGFc activity show delayed elevation and hypoplastic palatal shelves that are unable to meet [12]. After their elevation and further growth, the palatal shelves must quickly acquire the competence to adhere and fuse. These are crucial steps taking place through a sequence of events, including contact, adhesion and merging of the two opposing MEEs, thus creating the single MES [21] (**Fig. 2D**). Competence for palatal shelf adhesion is precisely regulated. As we saw, before contact the MEE epithelium of the palatal shelves is composed of two layers: (a) the inner, basal layer of cuboidal cells sitting on a basement membrane; and (b) the outer layer of flattened peridermal cells. These two epithelial layers have different fates during palatal fusion. The basal epithelial layers of each MEE are destined to adhere to each other and to fuse, thus forming the MES which becomes stabilised by cell-cell junction systems formed between the adhered cells [14, 26]. While there is a general agreement that the cells of the basal layer will form the MES, the role of peridermal cells is not so clear. The presence of a continuous periderm layer on the MEE acts as a non-sticking barrier and, therefore, removal of the periderm appears to be a prerequisite for the merging of the two MEEs and palatal fusion [2]. It was initially thought that the periderm detached from the tips of the palatal shelves before they came into contact, so that initial contact between the shelves would be achieved *via* exposed MEE basal cells [18, 20]. Indeed, classical morphological studies seemed to indicate that, just prior to adhesion between the apposing shelves, the nuclei of the periderm cells became pyknotic, the cells detached and died by apoptosis [18,20]. However, other investigators have demonstrated that the initial contact between palatal shelves and some degree of weak adhesion instead takes place between the periderm cells of the apposing shelves, mediated *via* chondroitin sulphate proteoglycan expressed on the filopodia produced by the periderm to increase the surface area available for interconnection [26]. Therefore, whereas some peridermal cells may indeed shed away and die by apoptosis prior to contact between palatal shelves [14, 54], most of them persist in

the MEEs and migrate out downward and upward along the oro-nasal axis, where they will participate in the formation of the epithelial triangles observed in the MES [14, 9, 26, 60]. Many studies have revealed an essential involvement of TGFβ3 signalling in this process as well as in all subsequent phases of fusion [5, 42]. TGFβ3 expression is specifically expressed in MEE cells, including the periderm layer, already prior to palatal shelf adhesion [10, 43]. Notably, in TGFβ3-/- mice filopodia are absent, chondroitin sulphate proteoglycan is not expressed, the periderm cells fail to migrate away, and a cleft palate occurs [26, 62]. Indeed, according Taya, O'Kane and Ferguson [62], the production of TGFβ3-induced filopodia might be considered as diagnostic of increased cell motogenic/migratory activity of the MEE. Such increased activity would also be important for the rapid interdigitation of opposing palatal MEE cells following contact, to secure a firm fusion [62]. On the other hand, enhanced motogenic and migratory activity would also have a role in the subsequent disruption of the MES, through MEE cell migration towards the oral and nasal surfaces. Thus, according these studies, TGFβ3 could intervene in all phases of palate fusion by stimulating inter-palatal MEE cell adhesion and by enhancing cell migration both during MES formation and during its disruption [62]. Therefore, it should be highlighted that the periderm probably has an instrumental role in MES formation in response to TGFβ3, as in absence of TGFβ3 peridermal cells fail to migrate out of the MES [41, 62]. Thus, current data does not support the hypothesis that periderm undergoes complete sloughing/apoptosis prior to palate shelf adhesion [18,20], but rather are consistent with the periderm playing an active role in maintaining epithelial integrity and stability through palate fusion [41].

As we have seen, proper periderm differentiation and maintenance are key in palatogenesis by preventing abnormal adhesion of palatal shelves to other oral structure. Mice lacking Jag2, FGF10, Irf6, or Grhl3 gene function exhibit aberrant adhesion or fusion of palatal shelves to mandible and/or tongue and, therefore, develop a cleft palate [44]. Indeed, molecular networks including FGF10/FGFr2b/Jag2/Notch and p63/Irf6 signalling, as well as Grhl3 transcription factor, are essential in driving differentiation of the periderm [5, 43, 44] (**Fig. 5A**). Irf6 is a direct target of p63, and p63 has been shown to positively regulate FGFr2b and Jag2 expression [42]. Animals with malfunctioning Jag2, Fgf10, Irf6, and Grhl3 genes have abnormal intra-oral adhesions and a cleft palate phenotype [5]. The central player in this molecular network is Irf6, whose loss of function in humans has been associated with clefting in Van der Woude syndrome, as well as in cases of non-syndromic clefting [38]. P63 is expressed in basal cells of the MEEs and becomes down-regulated in supra-basal cells when periderm differentiation is achieved [42]. As we have already mentioned, during ongoing epithelial stratification, the transcription factor p63 activates FGFr2b/Jag2/Notch signalling and the expression of Irf6, which in turn down-regulate p63 in the supra-basal cell layer, thus inducing p21-mediated cell cycle exit and allowing periderm differentiation [42, 44]. Therefore, both Jag2/Notch ad Irf6 signalling seem to be activated by and negatively feedback to p63 to direct periderm differentiation/maintenance [42]. On the other hand, it has been seen that the expression of Irf6 is also crucial for MES dissolution. Indeed, once formed, the MES must be eliminated to obtain mesenchymal confluence and palate continuity. Biological systems often use similar mechanisms to achieve different outcomes and, indeed, as we shall see in the following paragraph, the same Irf6/p63/p21 pathway used for periderm differentiation is reutilised to facilitate MES dissolution [41, 42, 44].

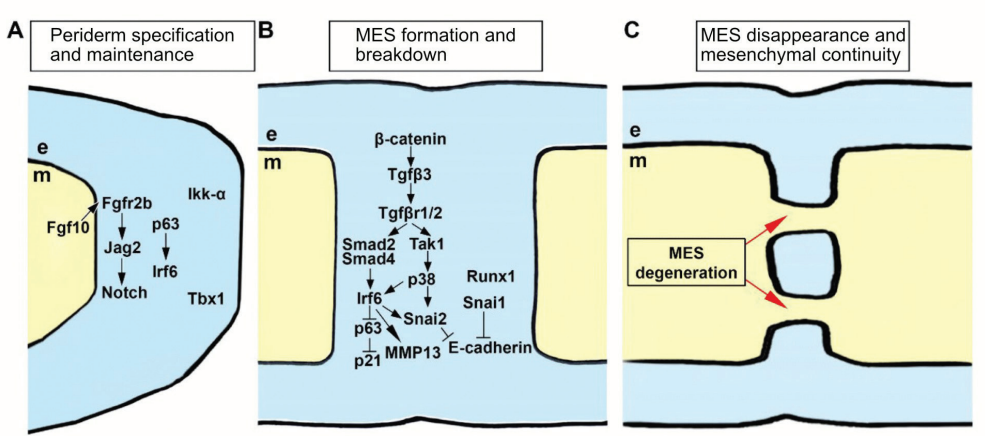

Fig. 5. Highly schematic representation of the molecular and morphological stages of palatal shelf fusion: from periderm differentiation and maintenance in the MEE (A), through the union of separate MEEs into the single MES and its initial breakdown (B), to MES disappearance and generation of a continuous palate (C). (A) Molecular control of epithelial/periderm differentiation in the MEE cells prior to palatal shelf contact. The MEE is formed by basal cells and a thin layer of periderm resulting from stratification of basal cells. Active FGF10/FGFr2b/Jag2/Notch and p63/Irf6 signalling together to Ikka and Tbx1 promote the differentiation and maintenance of the periderm, thus ensuring that inappropriate adhesions of the MEE do not occur. (B) The apposing palatal shelves have come into contact and, in order for them to adhere to each other and form the MES the periderm must migrate away. However, once formed also the MES must be eliminated. TGFβ3 signalling - probably under the control of Wnt/β-catenin - plays a fundamental role in these processes through both Smad-dependent and -independent pathways that activate Irf6/p63/p21 signalling, thus leading to cell-cycle arrest that favours apoptosis as well as MMP activation that mediates basement membrane disruption and extracellular matrix remodelling. TGFβ3 and Irf6 also activate Snail to loosen E-cadherin-based cell-cell adhesions, thus favouring motility/EMT of MEE cells. Runx1 is expressed in the MEE and is required for anterior palate fusion [5]. (C) The MES breaks up into epithelial islands, and becomes substituted by infilling mesenchymal tissue, until its complete disappearance, thus ensuring palate continuity. See the text for further explanation. Kindly reprinted from Won, Kim, Won, Shin (modified, from ref. 64).

Formation and breakdown of the MES

Fusion of the secondary palate requires juxtaposition of the MEE cells that cover the tips of the palatal shelves, and thereby the transformation of the separate MEEs into a single MES (**Fig. 6**). While adhesion between the opposing MEEs is taking place, some peridermal cells may become trapped between the adhered basal cells, where they may continue to migrate towards the oral and nasal epithelia or die by apoptosis [14]. However, once formed, the MES appears to be composed primarily of juxtaposed basal MEE cells. Morphologically, it consists of a seam comprised of two or three layers of epithelial cells, surrounded on both sides by an intact basement membrane. The constituent cells appear to interdigitate and form new cell-cell adhesion systems that stabilise the forming seam [15]. Nevertheless, the newly formed MES must be removed to allow mesenchymal continuity throughout the fused palate. The breakdown

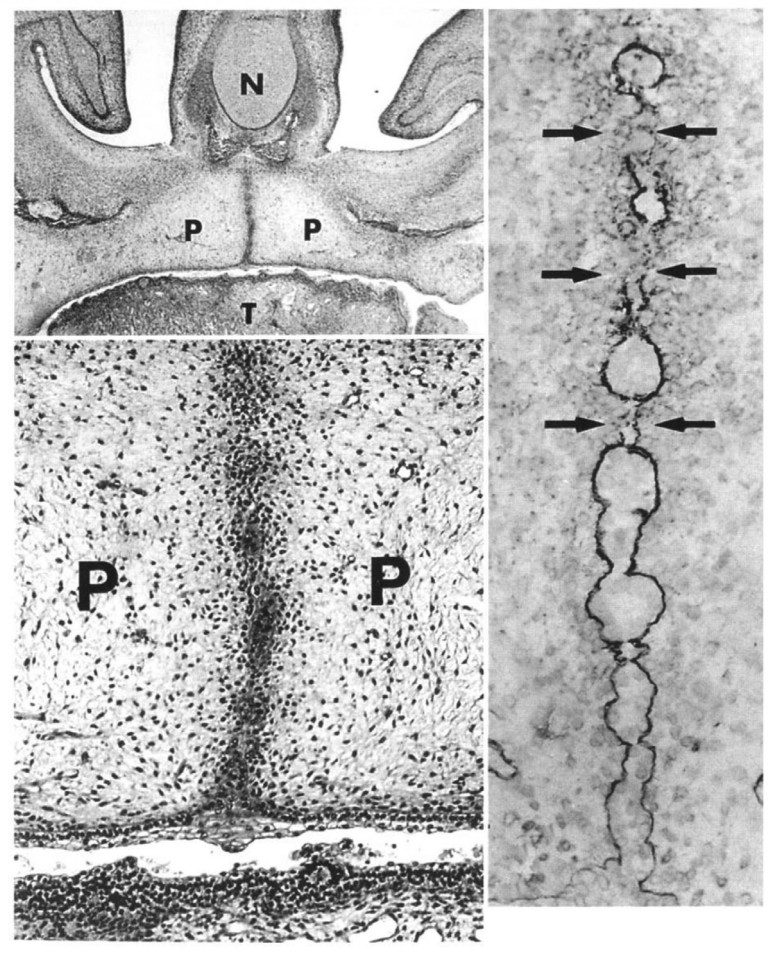

Fig. 6. Fusing palate of a 9-week-old human foetus. (A, upper left; B, below left) Haematoxylin and eosin-stained coronal section from the anterior palate region, showing well established fusion between the palate shelves with the intervening MES exhibiting noticeable breakdown (magnified in B). (C, right) An adjacent section from the same sample stained with antibodies to collagen type IV shows the MES surrounded by a basement membrane exhibiting evident areas of disruption (arrows). Correspondingly, the MES shows fragmentation/disintegration and formation of epithelial islands. Basement membrane disruption, which is a consistent finding during MES breakdown, is indicative of tissue rearrangement, but does not specifically address any of the hypothesised mechanisms for MES dissolution. As a matter of fact, basement membrane degradation may be compatible with epithelial-mesenchymal transition (EMT), apoptosis, or even migration of MES cells. Indeed, while basement membrane degradation is necessary to allow the translocation of transitioning cells from the epithelial compartment to mesenchyme during EMT (refs. 18, 25), basement membrane degradation can also occur as a consequence of apoptosis, the so-called "cataptosis" (ref. 9). Finally, extracellular matrix-degrading metalloproteinases have been suggested to play a role in the initiation of MES breakage that occurs during collective epithelial migration, another proposed mechanism for palate fusion (ref. 62). P, palate shelves; N, nasal septum; T, tongue. From Guarino et al. (ref. 25), reprinted with permission of Elsevier.

of the MES is morphologically characterised by thinning of the epithelium thickness, basement membrane disruption, breaking up of the seam into epithelial islands, mesenchymal penetration between them and, eventually, the complete disappearance of every trace of the intervening epithelium [15, 18, 20, 34, 45] (**Fig. 5C, Fig. 6B, C**). Kim and coworkers [37] proposed an interesting model of MES formation/dissolution. They demonstrated the creation of a transient multilayered MES in which palatal fusion proceeds through concurrent convergence, cell intercalation and displacement, apoptosis and extrusion of the MES epithelium along the the oro-nasal axis and, ultimately, MES breakage into epithelial islands [37, 41] (**Fig. 7**).

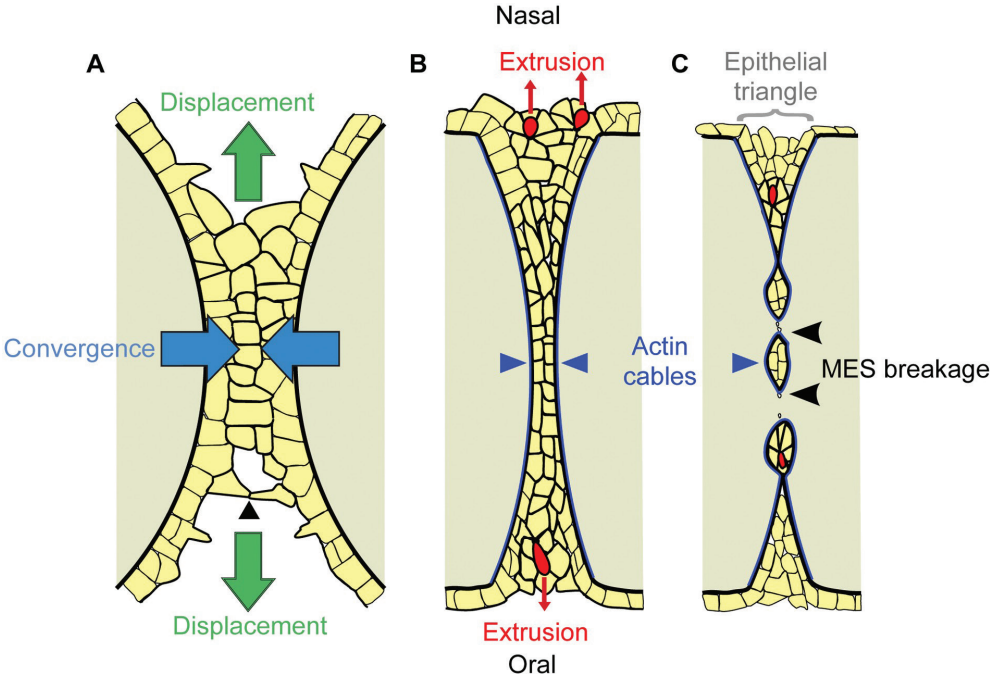

Fig. 7. Convergence and extrusion model by Kim and colleagues (ref. 37), proposed to explain the formation and breakdown of the (MES). (A) Formation of the MES is initiated by cellular protrusions which transiently create epithelial bridges (arrowhead), to establish contact between the shelves and give rise to a multilayered MES epithelium that then converges towards the midline, in conjunction with cell intercalation and oral and nasal cell displacement of MEE cells. (B) The formation of actin cables and multicellular rosettes is associated with extrusion of cells on the oral and nasal surfaces of the palate. (C) Contraction of the actin cables leads to breakage of the seam into islands, and further oro-nasal cell extrusion contributes to the formation of epithelial triangles. Kindly reprinted from Kim, Lewis, Singh, Ma, Adelstein, Bush (ref. 37).

The signals responsible for MES degradation are not yet fully understood, but there is evidence that TGFβ3 signalling still plays a prominent role. Indeed, TGFβ3 could promote MES degeneration by inducing cell cycle arrest and apoptosis, reducing epithelial cell adhesion, and favouring cell migration and extracellular matrix remodelling [26]. After binding to its membrane receptors, TGFβ3 activates Smad2/

Smad4 and the p38 MAPK pathways which, through the Irf6/p63 signalling, regulate p21 expression in the MES cells [5] (**Fig. 5B**). Indeed, quite similar to its function in periderm differentiation, Irf6 expression would cause down-regulation of p63 and an increase in p21 expression in the MES cells, which contributes to their cell cycle exit, thus favouring apoptosis and, therefore, degeneration of the midline seam [42]. In addition, TGFβ3 signalling also favours Snail-mediated disruption of epithelial adhesion and MMP-dependent breakdown of extracellular matrix. Actually, some of the events downstream of TGFβ3 are crucial to both the formation and dissolution of the MES (**Fig. 7**). Parallel activation of Snail family transcription factors downstream of TGFβ3 could, on one hand facilitate periderm sloughing and migration by loosening MEE basal cell/periderm cell adhesion through down-regulation of E-cadherin [44], and on the other hand it could contribute to promoting MEE cell apoptosis [5, 52]. Furthermore, Snail-driven E-cadherin-dependent loosening of cell-cell adhesion could be functional to the facilitation of cell intercalation, displacement or migration occurring either during the formation or the breakdown of the MES. Therefore, it is possible that Snail-mediated down-regulation of E-cadherin could, on the one hand favour the migration and apoptosis of the periderm in the approaching MEEs as well as basal cell displacement/intercalation to form the MES and, on the other hand, it could promote the disintegration of the MES itself after its formation by facilitating MES basal cell migration [44, 52]. Moreover, TGFβ3-dependent E-cadherin down-regulation could also underlie MES breakdown into small epithelial islands, a consistent step in MES dissolution [55] (**Fig. 6B, C, Fig. 7C**). E-cadherin is required for palate shelf fusion, but it is down-regulated by TGFβ3, thus indicating a complex role of this adhesion molecule in the fusion process. Mutations of CDH1/E-cadherin, which deletes the extracellular cadherin repeat domains needed for cell-cell adhesion, have been associated with cleft lip/palate in families with hereditary diffuse gastric cancer [67]. Furthermore, TGFβ3 is implicated in the remodelling of the extracellular matrix through the regulation of matrix metalloproteinase (MMP) function, including MMP13 [67]. Indeed, TGFβ3-induced MMP activation is responsible for the basement membrane disruption observed during dissolution of the MES, as well as for the reorganisation of the interstitial matrix necessary to achieve confluence of the palatal stroma after the disappearance of the MES, thus forming a united secondary palate [14].

The mechanism of palatal closure should ensure the strength of palatal fusion, such as the resistance to the muscular forces of the tongue and cheeks [6]. This would initially be provided by the establishment of firm epithelial cell-cell adhesions and desmosomes during the formation of the MES [20], and later strengthened by progressive mesenchymal infilling during MES breakdown, thus permitting merging and continuity of the core stromal component of the palatal shelves and consolidation of the fusion [6].

The mechanism behind the disappearance of the MES

The cellular mechanisms and dynamics that lead to the disappearance of the MES have been the subject of discussion and debated among three main, non-exclusive hypotheses [2, 5, 14, 21]: (a) epithelial-mesenchymal transition (EMT) of the MES that could allow the intervening seam epithelium to migrate and become incorporated into the

mesenchyme of the palatal stroma; (b) death of the MES cells by apoptosis that would lead to MES disintegration; (c) migration of MES cells in the oro-nasal directions which would allow their incorporation into the epithelial lining of the oral and nasal cavities.

Below we will analyse these hypotheses individually, and also mention models of MES removal that combine two or more cellular mechanisms.

Epithelial-mesenchymal transition (EMT)

In his comprehensive paper published in 1988, Ferguson [15] carefully described the fusion of the secondary palate shelves and the morphological features of MES dissolution. He also noted that some MES cells migrated into the palatal stroma where they became indistinguishable from other mesenchymal cells, and interpreted these cells as a specific subpopulation of "basal stem cells able to migrate" [15]. In their study published the following year, Fitchett and Hay [18] reexamined the mechanism of MES dissolution, and demonstrated that MES cells elongated into the adjacent mesenchyme through basement membrane discontinuities, lost epithelial characteristics, and acquired characteristics quite similar to migrating fibroblasts. The authors definitively interpreted the finding as an example of epithelial-mesenchymal transition (EMT). The possibility of a role of TGFβ3 in EMT during palatal fusion has been extensively investigated [10, 29, 34]. TGFβ3 could be the main EMT inducer by signalling *via* both Smad-dependent and PI3K or p38 MAPK pathways, thus leading to activation of the key Snail family transcription factors necessary for down-regulating E-cadherin, and thereby EMT induction [29, 67]. On the other hand, MMP13 expression is strongly induced by TGFβ3, and it is possible that this metalloproteinase specifically expressed in the MEE/MES and in adjacent mesenchyme during palatal fusion, could initiate EMT by promoting basement membrane degradation, thus influencing cell-cell and/or cell-matrix interactions [4]. According to this model, the disruption of the basement membrane is a significant and early event, because it could be instructive to initiate EMT and, on the other hand, it would allow the passage of transitioning cells into the mesenchymal compartment. However, regardless of its significance, basement membrane degradation is indeed consistently observed during MES degradation (**Fig. 6C**). In brief, according to the EMT hypothesis the MES would undergo progressive disintegration through disruption of the basement membrane, loss of cell-cell junctions and the epithelium-specific molecules E-cadherin and cytokeratin, while gaining vimentin intermediate filaments. Then, transitional cells would migrate into the stroma and become indistinguishable from the mesenchymal cells [18, 25]. EMT is known to occur in embryogenesis [35], play a role in pathological conditions including fibrosis [34], cancer histogenesis and progression [24, 25], and can be easily reproduced under experimental conditions [23]. The EMT-based model became very popular, and the establishment of the concept of EMT as the prevailing mechanism of palate fusion led to a wealth of studies attributing roles to different molecules, including TGFβ3, Lef1, Smads, Rho, PI3K, MMPs, Twist and Snail as possible mediators of palatal EMT [29, 55, 67]. However, subsequent studies have produced inconclusive results regarding a significant role of EMT in MES dissolution, and there is the possibility that, if the above-mentioned molecules may indeed play a role in palatal fusion, it could not necessarily be related to EMT [21].

Apoptosis

The longest-standing model to explain the disappearance of the MES has been programmed apoptotic cell death and, even today, many evidences support a major role for apoptosis in MES removal [5, 8, 9]. Overall, many findings fit well with the apoptotic death model [64]. For example, cell proliferation is rarely observed in the MES, and many MES cells are TUNEL- and active caspase 3-positive during palatal fusion, indicative of ongoing apoptosis [14, 64]. Moreover, TGFβ3 signalling seems to play an important role in favouring MES apoptosis, and it is known that lack of TGFβ3 in mice embryos allows palatal shelves to adhere at the midline, but not to fuse due to the persistence of the MES [34]. Periderm migration out the MEE/MES is known to be important to trigger basal MEE cell death [9], and TGFβ3 signalling is known to play a significant role in both periderm migration and cell cycle arrest of the MEE cells [64]. Irf6 is a fundamental factor responsible for induction and maintenance of periderm differentiation in the MEE, and it is a direct target of p63 [22] and, in fact, p63 and Irf6 function in a regulatory feedback loop to control both epithelial proliferation and periderm differentiation [22]. In the MES, Irf6 is up-regulated through both TGFβ3/Smads and TGFβ3/p38 MAPK pathways, and leads to down-regulation of p63 and, therefore, increased p21 expression [64]. This mechanism is believed to favour cell cycle exit and, together with activation of Snail, to promote apoptosis and subsequent degeneration of the MES [5, 64]. Maintenance of oral periderm integrity also depends on Jag2-Notch signalling, and TGFβ3 is crucial for the down-regulation of Jag2, likely a another key mechanism by which TGFβ3 disrupts periderm function, thus facilitating MEE basal cell adhesion and fusion [64]. In conclusion, TGFβ3 signalling would allow periderm migration out of the MES, thus causing on one hand complete palatal fusion, on the other hand it would reduce the proliferative potential of MES basal cells, thereby favouring MES cell loss through apoptosis (**Fig. 5**). According to Cuervo and Covarrubias, the MES essentially degenerates by programmed cell death triggered by the adhesion between the apposing shelf epithelia [9]. Prior to the fusion, periderm cells cover the MEEs and act as a barrier for direct contact between the opposing MEE basal cells, which actually is a requirement for MEE cell death activation. Thus, periderm should shed away or migrate out of the MEE/MES to allow contact between the apposing basal cells, and thereby activate their apoptosis. These authors also found that activation of cell death promoted the degradation of the basement membrane underlying the dying MES cells, a process that they call “cataptosis”, and suggested that dying cells would directly activate MMPs, including MMP13, eventually responsible for the basement membrane degradation [9].

Cell migration

Migration of cells of the MES along the midline towards the oral or nasal surface epithelia is another mechanism that has been proposed to explain the disappearance of the MES [6]. According Carette and Ferguson [6], MES cells would migrate nasally and orally out of the seam where they are recruited into, and constitute the epithelial triangles on both the oral and nasal aspects of the fusing palate. Subsequently, these transitory migrating cells would become incorporated into the oral and nasal epithelia on the surfaces of the palate [6]. However, other studies seem to indicate that the majority of cells that migrate along the midline towards the oral and nasal surfaces are peridermal cells, rather than basal MEE cells. Indeed, Cuervo and Covarrubias

[9] demonstrated that epithelial triangles at oral and nasal ends of the MES do not appear to result from basal MEE cell migration, but rather from periderm cell migration, and that the migration of periderm cells out from the MEE is necessary to initiate and complete normal shelf fusion [9]. According to the authors, TGFβ3 could play a role in promoting the migration of peridermal cells along the oro-nasal axis which then accumulate at the bottom and top of the MES to form the epithelial triangles, possibly important for sealing the ends of the MES [9]. A recent paper demonstrated the importance of actomyosin dynamics in palate fusion whereby the MES is removed through actomyosin-dependent collective cell migration of epithelial trails and islands of basal cells in order to allow mesenchymal confluence, independently of the occurrence of programmed cell death, since blocking apoptosis did not prevent MES removal [63]. Indeed, by novel static- and live-imaging, these authors detected a unique form of collective epithelial migration, whereby the MES would be removed through streaming migration of collections of epithelial cells to reach the oral and nasal epithelial surfaces. This mechanism of MES elimination depended exclusively on the contractility of actomyosin filament system generating a peristaltic-type propulsive force, and not by apoptosis, as genetic suppression of the intrinsic apoptotic regulators BAX and BAK did not prevent successful MES disappearance [63].

Combined model hypotheses

For three decades there has been a heated debate as to whether MES cells became mesenchymal through EMT, died by apoptosis, or migrated into the oral or nasal surface epithelia. However, it is also possible that more than one of these mechanisms, or even all of them, are physiologically used to remove the MES.

Using an organ culture system, Jin and Ding [31] observed the migration of MES epithelial cells towards the nasal side – but not towards the oral side – of the fusing shelves, and simultaneously demonstrated the presence at some distance from the midline of β-galactosidase-labelled cells in the mesenchymal stroma of the palate shelves, indicative of their epithelial origin and, therefore, of the occurrence of a mechanism of palate fusion based on both migration and EMT.

Ahmed and colleagues [1] proposed a mechanism of MES disintegration whereby MES cells would sequentially undergo cell cycle arrest, EMT-mediated cell migration and apoptosis in response to TGFβ3. These data suggest that TGFβ3 induced different phenotypic changes at different times functional to palatal MES dissolution: cell cycle arrest, repression of E-cadherin-based cell–cell adhesion and migration and, finally, apoptosis of post-EMT migrated cells. Indeed, before undergoing apoptosis, MES cells showed gradual phenotypical alteration, changing from cohesive epithelial to fully migratory fibroblastoid, indicative of EMT.

Studies by Benson et al. [3] are consistent with a model of MES degradation where seam epithelial cells would undergo EMT and/or death by apoptosis, thus leading to confluence of the palatal stroma. In addition to TGFβ3, a further signal for initiating palatal fusion and EMT could be provided by members of the Ephrin family [3]. Indeed, TGβ3 and Ephrin signalling could cooperate in seam dissolution: both would induce a migratory phenotype in cultured MEE cells [3] and, in addition, TGFβ3 could also promote apoptosis [1, 54].

Ke and colleagues [36] investigated the role of Irf6 in the molecular mechanisms underlying palate fusion using palatal shelf organ culture. The authors found that

TGFβ3 up-regulated Irf6 which, in turn, increased Snail, thus promoting EMT. On the other hand, Irf6 could also lead to apoptosis *via* the p63/p21 signalling cascade. These results indicate the TGFβ3/Irf6 pathway can lead to different results, EMT or apoptosis, and that both mechanisms could contribute to MES dissolution during normal palatal fusion.

According to Nakajima and colleagues [53], multiple mechanisms would contribute to removing the MES to form a single, continuous palate. Coincident with the strong expression of TGFβ3 in the MES, both EMT and apoptotic changes could be observed among the cells at the midline, and, in addition, cells of the MES could also migrate collectively as clustered aggregates into the oral and nasal epithelial layers. Basically, the MES would undergo collective cell migration, EMT, and eventually apoptosis, which might be a form of post-EMT apoptosis ("lethal" EMT). Therefore, some of the MEE-derived mesenchymal cells would be lost by apoptosis, but the remaining ones, characterised by sustained high expression of TGFβ3 and TGFβ receptors, would be adopted into the palatal stroma. Meanwhile, crowding force due to epithelial migration would cause cell extrusion at the epithelial triangles, releasing MEE cells to the oral and nasal surfaces of the palate. Due to these multiple biological events, the number of cells of the MES is decreased, thus causing discontinuities in the seam and formation of epithelial islands, until complete disappearance [53].

Logan and coworkers [45] proposed a new model that incorporates features of partial EMT along with collective cell migration. In such a model, TGFß and Ephrin signalling would induce a partial EMT in a subpopulation of MES cells, but nevertheless the cells would appear to move together as a cohesive sheet. Indeed, these EMT-transformed cells would serve as leader cells of a movable cell collection. This scenario would also fit with the breaking of the epithelial seam into islands before its complete dissolution. Each island could be a population of relatively epithelial-like cells attached to more fibroblast-like leader cells. The resulting motile collective units would migrate following leader cells that provide the moving force.

Recently, hypotheses have been proposed based on the formation and dissolution of the MES by cell convergence and extrusion. These studies reveal an essential role for actomyosin contractility-driven convergence and cell intercalation in the formation of the MES, and subsequent cell displacement and extrusion during MES breakdown. These models mechanistically connect most of the cellular behaviours previously observed palatal fusion, including early extension of filopodial protrusions by periderm cells, cell shape changes, cell displacement and cell migration in the oro-nasal directions during MES formation and breakdown, apoptotic cell death and actomyosin-driven MES convergence [42] (**Fig. 7**). Indeed, Kim and coworkers [37] examined cell behaviour during palatal fusion using a combination of genetic lineage labelling, tissue-specific gene inactivation, and live imaging. The authors reveal an essential role for actomyosin contractility, cell intercalation and displacement, apoptosis and MES cell extrusion at the oro-nasal surfaces. Whereas an argument against apoptosis as the major mechanism for MES dissolution argued that massive cell death of the MES cells would weaken the fusion site and potentially lead to separation of the palatal shelves [61], by using live imaging Kim et al. [37] demonstrated a process of MES cell extrusion, during which converging MES cells form rosettes, and the cells in the centre of these rosettes are squeezed out by multicellular actin cables. Therefore, apoptosis in the MES does not involve the simultaneous death of all cells that could weaken the

fusion site, but rather it would occur through the extrusion of the apoptotic cell by its neighbouring cells that remain viable. The forces for the convergence and extrusion events would be provided by actomyosin contractility requiring Rho kinase- and myosin light chain kinase-mediated activation of non-muscle myosin [37, 42](**Fig. 7**).

In conclusion, according to the most recent views, MES disappearance could require actomyosin contractility as well as by TGFβ3/Irf6-regulated cell cycle arrest/apoptosis and MMP13-mediated extracellular matrix breakdown [42]. On the other hand, some other molecules, in particular the transcription factors Runx1 [53] and Snail [52], could be required for MES formation/breakdown during palatal fusion. Snail family transcription factors have been implicated in EMT by directly repressing cell-cell adhesion components, therefore it is possible that Snail, downstream or in cooperation with TGFβ3/Irf6 signalling, works by loosening adhesion between either periderm and MEE basal cells to form the MES, or between basal MEE cells in the formed MES, thus contributing to its ultimate dissolution [42, 64](**Fig. 5B**), perhaps even through the induction of actual EMT [35] (**Fig. 8**).

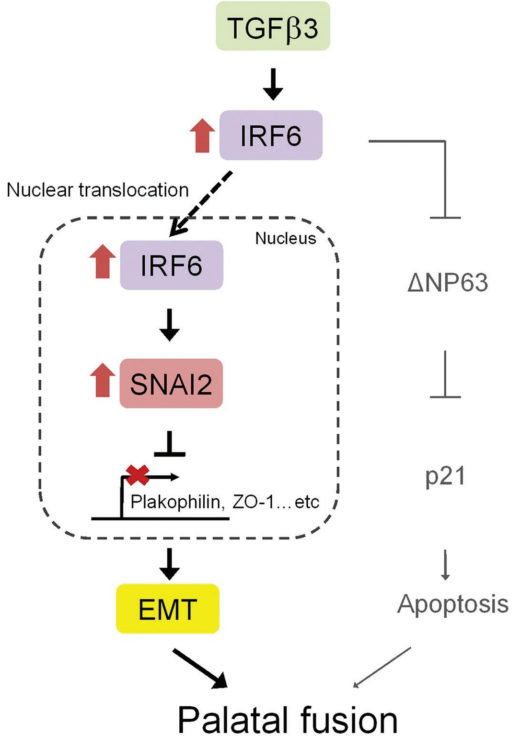

Fig. 8. Diagram depicting TGFβ3/Irf6 signalling pathway leading to EMT or, alternatively, apoptosis during palatal fusion. TGFβ3 up-regulates the expression of Irf6 and enhances its nuclear translocation, which then would increase the expression of Snail transcription factors twhich, in turn, could induce EMT of the MES cells. In addition, Irf6 down-regulates p63, which will result in induction of p21 expression, thus favouring MES cell apoptosis. Both of these events could occur in the process of palatal fusion, and contribute to MES disintegration. Kindly reprinted from Ke, Xiao, Chen, Lo, Wong (ref. 36).

Conclusions

Orofacial defects including cleft lip and cleft palate are among the most common congenital birth anomalies, and are caused by failure of the facial/palatal processes to grow or fuse properly during the first trimester of gestation. The cellular and molecu-

lar mechanisms governing normal palatogenesis and their failure in orofacial clefting have not definitively been elucidated. On the other hand, the care of patients with cleft lip and cleft palate continues to be a cause for concern, and therefore, prevention of these deformities remains the ultimate objective of research. Thus, advances on the mechanisms underlying orofacial clefting as well as their relationships with genetic and environmental factors are the key to preventing these disfiguring birth defects. Moreover, further study of the events involved in palatogenesis could not only improve our understanding of the developmental pathogenesis of these deformities, but could also provide clinical and prognostic information. For example, knowing the molecular defect underlying the cleft in a given patient can provide information in the healing of the palate after surgical repair and therefore the risks for post-operative complications, as it is known that approximately 10% of patients develop wound complications following surgical repair of cleft palate. The observation that patients with Van der Woude syndrome, which in most cases is caused by Irf6 mutation, had worse surgical outcome based on surgical wound healing complications, compared with patients with non-syndromic cleft [33], suggests the importance of a detailed knowledge of the etiological mechanism underlying orofacial clefting. Currently, numerous genes and molecular pathways involved in normal palatogenesis and pathological clefting have been identified. As an increasingly number of molecular studies are rapidly improving our knowledge on the signalling networks underlying palate development, their integration with notions on genetic-environmental factors and tissue morphogenetic events leading to clefting could translate into the creation of new strategies for the prevention, treatment and prognosis of these defects.

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