Rom J Morphol Embryol 2026, 67(1):*in press* ISSN (print) 1220–0522, ISSN (online) 2066–8279 doi: 10.47162/RJME.67.1.y

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

REVIEW

Cleft lip/palate. From embryology to developmental pathogenesis

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Abstract

Cleft of the lip associated with or unassociated with cleft of the palate and cleft palate (CLP) represent frequent birth defects, and the mechanisms by which they occur are not completely clear. On the other hand, knowledge of the normal mechanisms underlying lip development and palatogenesis is still incomplete, and therefore a better understanding and consolidation of our knowledge in this field could help to study these deformities. Upper lip and secondary palate formation are complex processes, involving the subtle integration of several biological events, the disruption of which causes the clefting phenotype. Indeed, these developmental events imply a series of morphogenetic changes involving concerted cell survival, migration, growth, pattern generation, modulation of adhesiveness, death and differentiation. In recent years, genetically engineered animal models and *in vitro* palate cultures have greatly advanced our knowledge of the cellular and molecular pathways underlying normal orofacial morphogenesis, and abnormally developed CLP as well. Indeed, most of the morphogenetic events of craniofacial development are highly conserved amongst vertebrates, therefore animal models have revealed major insights into the mechanisms that take place in human orofacial development. Given their complexity, it is easy to imagine that the failure of the highly coordinated processes that guide the union of the lip and palate causes various forms of clefts. This article provides an overview of the embryological development of the lip and secondary palate, as well as the mechanisms underlying deviant development resulting in CLP, concentrating on the cellular and molecular characteristics.

Keywords: morphogenesis of the lip, palatogenesis, orofacial clefts, pathogenesis, biological bases.

Introduction

Cleft lip/palate (CLP) is a common congenital anomaly and the commonest head malformation, with a frequency of approximately one in 700 new births [1–5]. Children with CLP will require dedicated care into adulthood, involving multiple medical disciplines, including surgery, dentistry and orthodontics, otorhinolaryngology, and they will often experience speech and hearing problems, and possibly psychological distress [5, 6]. The etiology of CLP is variegated and intricate, with both genetic and environmental contributors, including the interplay of these factors at the level of gene–gene and gene–environment interaction [1]. The incidence of CLP varies widely in relation to geography, ethnic affiliation and socio-economic well-being [7, 8]. Generally, Asians and Latin Americans have the highest incidence, Caucasians have middle incidence, and Africans have the lowest incidence, therefore suggesting that the effect of individual susceptibility genes can differ significantly among diverse populations [1, 2]. CLP can be classified into two major groups: (*i*) cleft of the lip associated with or unassociated with cleft of the palate; (*ii*) cleft of the palate only [9]. The distinction between these two groups is important, as they are two distinct birth defects, with different causes and incidences between the sexes. Indeed, cleft of the lip unassociated with cleft of the palate and cleft of the lip associated with cleft of the palate share the same developmental defect, motivating their inclusion into a common group, and have been considered variants of the same malformation that only differ in severity. Indeed, as the morphogenesis of the lip and primary palate occurs before the formation of the definitive palate, a disturbance in the correct formation of the former can affect the fusion of the definitive, secondary palate, thus resulting in palate clefting [10]. About 70% of cases of cleft of the lip unassociated with cleft of the palate and cleft of the lip associated with cleft of the palate, and 50% of cleft of the palate only happen as solitary defects, without any other abnormalities, and thereby are usually called non-syndromic clefts; the other cases are included in syndromes which comprise additional abnormalities [9, 10]. More than 500 clefting syndromes are known, resulting from single gene mutations or chromosomal defects, or from teratogenic agents [1, 11, 12]. Albeit cleft of the lip associated with or unassociated with cleft of the palate and cleft of the palate only have been regarded as distinct defects [2, 13], every now and then both cleft of the lip associated with or unassociated with cleft of the palate and cleft of the palate only happen within the same family, thereby indicating the existence of overlapping, in-between forms [14]. Overall, cleft of the lip associated with or unassociated with cleft of the palate occurs with a male-to-female ratio of 2:1, whereas cleft of the palate only happens with an approximately 1:2 male-to-female ratio [1, 2, 5]. Furthermore, CLP presents an asymmetry of side occurrence, the left side being affected more commonly than the right with a 2:1 ratio [1]. Clinically, clefting may be one-sided or two-sided, and may range in extension, also including subclinical/very mild forms, such as tiny defects of the upper lip and the submucosal type of cleft palate [5, 6].

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Face morphogenesis starts at the sixth and ends at the twelfth week of embryonic life, and therefore causative factors responsible for abnormal development leading to clefting exert their effects in this period [3, 15]. Murine models have been widely used in the study of orofacial clefts [1, 3, 9, 13] revealing that orofacial development is orchestrated by an intricate molecular system with extensive interaction among distinct signaling pathways [16–21]. Although considerable achievements were obtained in determining the genes causing the syndromes associated with clefting, etiology of non-syndromic clefts is still unclear [1]. Knowledge of the origin of orofacial clefts imposes a complete awareness of the cell biology bases underpinning face morphogenesis and the manner in which they can be disrupted. CLP are frequent congenital defects and, in fact, because of the elevated intricacy of orofacial morphogenesis, developmental failure is concretely possible, thereby the quite high incidence of these defects would not be entirely unexpected. Nevertheless, an integrated cellular and molecular description of the entire process of lip and palate development is still incomplete. Therefore, with the aim of contributing to a better knowledge of the pathogenetic mechanisms of CLP, this paper offers a brief overview on the formation of the lip and primary and definitive palate in the embryo and focuses on the possible causes underlying the development of clefts, with special focus on cell biology features.

Upper lip formation and cleft lip

Face morphogenesis is a complex developmental event, engaging coordinated proliferation, migration, patterning, and differentiation of tissues of different embryological origins [22]. The interplay of key signaling pathways, involving mainly Wnt, fibroblast growth factor (FGF), sonic hedgehog (Shh) and bone morphogenetic protein (BMP) signaling is considered essential for driving facial primordia morphogenesis [23–25]. The development of the face begins with the arrival of cranial neural crest (CNC)-derived cells, that will constitute the majority of the mesenchyme of the primary facial processes, including an inner part made up of CNC-derived mesenchyme, covered by an epithelial lining originating from the ectoderm [15]. The unification of single processes to constitute the face may take place by two modalities. The first is called “fusion”, during which an epithelial seam is formed deriving from the union of apposing epithelial layers, thus forming a bi-layered structure. Once this intervening provisional structure is formed, the seam then degrades to create continuity between the fused prominences, with the mesenchyme forming a “mesenchymal bridge” [26]. The second mechanism, termed “merging”, utilizes cell proliferation and migration to unite separate structures through formation of an intervening groove that fills with mesenchymal cells, thus creating a smooth external surface [13, 26]. During the more elaborate process of “fusion”, occurring both in the morphogenesis of the lip and primary palate [27] as well as in that of the definitive, secondary palate [2, 28], epithelia become competent for adhesion unite in a strictly controlled manner. As facial prominences grow due to mesenchyme proliferation, the covering epithelium critically undergoes bi-stratification and differentiation, forming an exterior periderm layer characterized by a non-adhesive surface, important for a close control of the adhesiveness [15, 29]. The periderm is composed of flat, tightly packed and markedly polarized cells which cover the basal epithelium; due to the fencing function of their lateral cell–cell junctions, the apical surface of these cells is devoid of E-cadherin, therefore is unable to form adhesions with other cells [12, 18, 20]. In the fourth week, there are five different primordia that will form the face: the unpaired fronto-nasal process placed rostrally, and the double pair of maxillary and mandibular processes placed caudally [15]. Initially, the facial prominences are widely separated but gradually approach each other and move towards the midline [2]. In the fifth week, ectoderm of the fronto-nasal process bilaterally thickens to form the nasal placodes and, at the same time, mesenchymal cells proliferate around them, thus dividing the fronto-nasal process into two nasal processes, medial and lateral [15]. In the sixth week, the enlargement of the maxillary processes ends up pushing medially the medial nasal processes, thereby resulting in their fusion in the following week. At the same time, the rear extremities of the medial and lateral nasal processes merge with the maxillary processes at a tripartite zone, the so-named lambdoid junctional area [13, 15, 26, 27]. The fused medial nasal processes give rise to the philtrum, primary palate, median part of the maxilla and central nasal structures. The nasal wings arise from the lateral nasal processes, whereas the lateral parts of the upper lip arise from the maxillary processes [15]. Previously, the mandibular processes fused together medially to form the mandible and lower lip [2]. Failure of the primitive facial structures to approximate each other can result in wide separation of the prominences and thereby in malformations, such as hypertelorism and other facial dysmorphisms, including clefts, as observed in various craniofacial syndromes [2]. The intra-oral growth of the caudal ends of the medial nasal processes gives rise to the primary palate [2] comprising the most anterior area of the hard palate with the four upper incisors [29]. Morphogenesis of the upper lip and primary palate implicates the creation of an epithelial seam at the lambdoid junctional area, and its subsequent disintegration [2, 15, 27]. Defective fusion of the lambdoid junctional area leads to the formation of cleft lip which can extend to the palate, both primary and secondary [2, 15, 27, 30]. Several, potentially interacting occurrences may result in the development of cleft lip, as listed below [2]:

(*1*) Reduced growth of facial prominences, thereby hindering their meeting. This may be due to mesenchymal deficiency as a result of impaired migration or disproportionate growth and death of migrating CNC cells.

(*2*) Altered facial proportions. Subtle alterations in the shape or variations in the orientation of the facial primordia may lead to failure of contact between their surfaces and, indeed, differences in facial shape may represent a threshold factor for the development of cleft lip accounting for the varying frequency in different human populations [31].

(*3*) Defective adhesion of the lining epithelia. Adhesiveness of orofacial epithelia is strictly controlled, therefore changes in the apposed epithelia, mainly concerning their peridermal component, are necessary for them to fuse [32]. Indeed, failure of these changes, including periderm function, can result in clefting.

(*4*) Undue epithelial seam apoptosis, impaired strengthening of the fused parts and breakage following fusion. If excessive cell death occurs during dissolution of the epithelial seams, or there is inadequate mesenchymal migration and consolidation of the fused lip, a cleft may result due to post-fusion rupture [33]. The tractional forces of growth may pull apart the weak fusion site, thus causing its reopening which, if incomplete, gives rise to the so-called Simonart's bands [2, 33]. Furthermore, by preventing the migration of mesenchymal cells across the two sides and, therefore, consolidation of the fusion site, also failure of disappearance of the epithelial seam may result in breakage following fusion and cleft lip [34].

Creation and disintegration of the epithelial seam during fusion of the upper lip

In the lambdoid junctional area, peridermal cells possibly start the contact between the apposing surfaces of the three prominences. Indeed, they undergo morphological and functional changes, becoming bulging and rounded, devoid of junctional polarization, and thereby they have become permissive to cause fusion to occur. However, they subsequently disappear during the establishment of the more developed seam consisting only of basal epithelial cells. The latter in turn is however destined to subside to create tissue continuity between the merging parts [35, 36]. As we will see, very similar events in the peridermal and basal layers of the inter-shelf seam also occur during secondary palatal shelf fusion [37–40]. Concurrent to the morphological changes occurring in the periderm, the signaling molecular network of basal cells is remodulated. Jagged 2 (Jag2)/Notch signaling is potentially responsible for the junctional polarization of peridermal cells and therefore for their non-adhesive function and thereby must be down-modulated to allow fusion to occur, while the wingless-type mouse mammary tumor virus (MMTV) integration site (Wnt) signaling is upstream of the p63/interferon regulatory factor 6 (Irf6) pathway, which also impact on periderm function and therefore must be regulated [35]. Wnt signaling remodulation is fundamental, because altered genes implicated in Wnt signaling, such as low-density lipoprotein receptor-related protein 6 (*Lrp6*), Wnt family member 9B (*Wnt9B*), and pre-B-cell leukemia transcription factor 1 (*Pbx*) transcription factors, can cause clefting [23, 41, 42]. However, once established, the definitively developed epithelial seam disappears *via* epithelial-to-mesenchymal transition (EMT), seam cell migration, convergence/extrusion or programmed cell death [2, 23, 24, 27, 35, 36]. Activation of the Pbx/Wnt/p63/Irf6 pathway favors cell death of the epithelial seam and its disappearance [23], and this pathway interacts with the Pbx/Snail/Smad/E-cadherin signaling which, instead, promotes EMT [24]. Shh is a growth-promoting signaling implicated in the formation of the lip, which interacts with BMP, FGF and Wnt signaling in reciprocal and complex circuits [43]. Apoptosis of cells in the epithelial seam, leading to its disintegration, may be caused by p63/Irf6 signaling, and it is conceivable that inadequacy of p63/Irf6-induced apoptosis and, consequently, retention of the seam cells can underlie the development of lip clefting [43]. Indeed, increased Shh activity, due to disruption of its receptor patched homolog 1 (Ptch1), has been found to reduce Wnt/p63/Irf6 pathway at the lambdoid junctional area, thus leading to decreased cell death, retention of the epithelial seam and lip clefting [43].

Secondary palate development and clefting

Morphogenesis of the secondary palate takes place through the growth and fusion of the two palatal shelves, thus generating a united palate that divides the oral and nasal cavities [2, 15, 29, 30]. Classically, some steps in secondary palatogenesis are identified: (*i*) vertical palate shelf growth; (*ii*) palate shelf elevation above the tongue; (*iii*) horizontal palate shelf growth; (*iv*) contact/adhesion and fusion at the midline (Figure 1, A–F).

The two palatal shelves form as intra-oral outgrowth of the maxillary prominences developing from their medial sides, and initially they extend vertically downward, on the sides of the tongue (Figure 1, A and B), which, in this early period of development, is located in the upper part of the oral cavity. The tongue subsequently repositions downward because of the growth and lengthening of the mandible, thereby creating space above it. Indeed, the palatal shelves abruptly reorient and become placed horizontally above the surface of the tongue in the seventh week (Figure 1C). Then, in the eighth week the palatal shelves grow further horizontally, moving closer together and finally coming into contact at the midline. The tips of the shelves are lined by a specialized epithelium, known as medial edge epithelium (MEE) (Figure 1C); the approaching MEEs eventually fuse together to form the midline epithelial seam (MES) (Figure 1D). Initially the palatal shelves adhere to each other at the central part of the shelves, then adhesion moves in the anterior and posterior direction until they reach the incisive foramen forward and the uvula rearward [15]. From the eighth week until the twelfth week, the MES epithelium undergoes disruption (Figure 1E), showing fragmentation with formation of epithelial "pearls" and basement membrane (BM) degradation [44]. Eventually, every remnant of the MES will disappear entirely, thereby leading to stromal confluence and continuity between the former shelves [2, 7, 15, 28] (Figure 1F). Furthermore, the united palate merges with the primary palate and the median nasal wall, thus participating in the separation of the nasal cavities. Clefting of the secondary palate can develop as a consequence of failure occurring in all phases of palatogenesis: vertical growth, horizontal orientation and growth, and shelf adhesion and merging [2, 11, 15, 17, 20, 21, 30].

The epithelia lining the palatal structures have diverse differentiation fates: the mucosal lining the oral side will become a stratified, keratinizing squamous epithelium, whereas the nasal mucosa will be characterized by a respiratory-type, columnar epithelium. The mesenchyme of the palate will transform into the osseous hard palate anteriorly; it will also contribute to the formation of the muscular soft palate posteriorly, due to the arrival of myogenic cells derived from the mesoderm.

Below, we will examine the individual steps of secondary palatogenesis, as well as the alterations that may occur resulting in clefting.

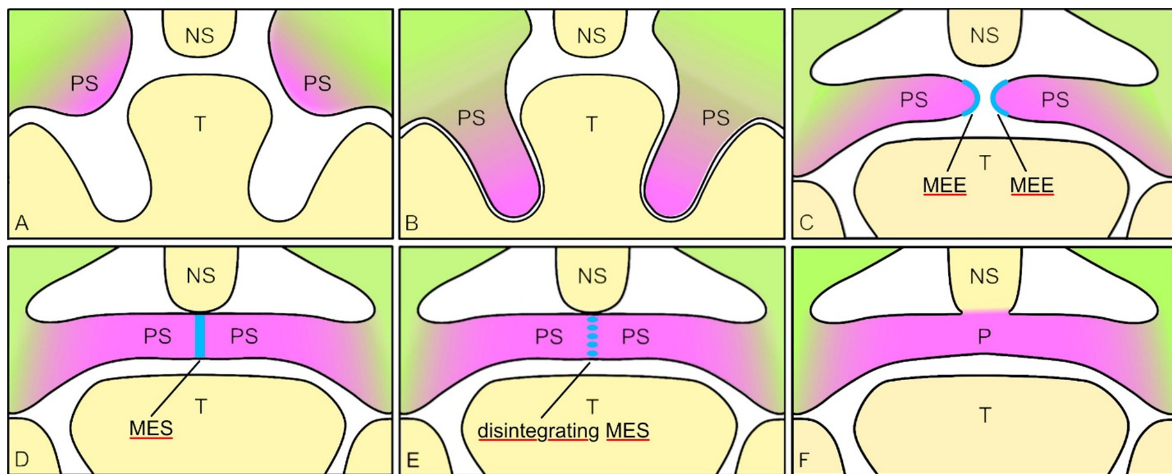

Figure 1 – ***Scheme illustrating the steps of development of the definitive, secondary palate. (A) In the sixth week, the palatal primordia emerge intraorally from the maxillary prominences and begin to grow vertically. (B) The palate shelves extend downward, while the tongue is situated centrally, elevated in the oral cavity at this time, occupying it almost completely. (C) As soon as the tongue lowers – as a result of the lengthening of the mandible – a space is created above it. This enables the palate shelves to elevate above the tongue, attaining a horizontal alignment by the seventh week, while continuing to progress toward one another. At this point, MEE (in blue) is the term used to indicate the tip epithelium of the shelves, a specialized portion of the epithelial lining that becomes competent for adhesion with the opposing MEE to generate a two-epithelial seam. (D) MES (in blue) is, therefore, a two-layered epithelium formed when the two shelves adhere to each other through their opposite MEEs in the eighth week. (E) From the ninth to the twelfth week, MES epithelium undergoes disruption, showing progressive fragmentation. (F) The united palate that connects above with the NS is formed by complete disappearance of the MES and formation of a "mesenchymal bridge" leading to stromal confluence between the shelves in the twelfth week. Cell migration, EMT, apoptosis, convergence/extrusion, or a combination thereof can all contribute to disruption of the MES and its removal (see the main text). EMT: Epithelial–mesenchymal transition; MEE: Medial edge epithelium; MES: Midline epithelial seam; NS: Nasal septum; P: Palate; PS: Palate shelves; T: Tongue.***

Palatal shelf growth

The palatal shelves consist of a mesenchymal central part comprised of CNC-derived cells, covered by an epithelial lining made up of basal epithelial cell layer and a superficial peridermal cell layer. Their development begins as intra-oral outgrowths arising from the ventral surfaces of the maxillary prominences (Figure 1A). In animal models, failure to form the palatal shelves is a rare, serious defect resulting from disruption of the signal transduction pathways underlying the crosstalk between the presumptive palatal bud epithelium and the underlying mesenchyme, an epithelial–mesenchymal interaction indispensable for the formation and growth of the shelves. Much more frequent in animal models is insufficient growth of the palatal shelves, due to impaired cell proliferation or excessive death.

Molecular mechanisms in palate shelf growth

Growth of the palate shelves is controlled by an intricate molecular signaling system extensively interconnected, involving mainly Shh, FGF, BMP and Wnt signaling [17–21, 45]. Shh is the most crucial participant and is expressed in the bud epithelium where it interacts with FGF and BMP signaling. Indeed, epithelial Shh expression is mainly controlled by FGF produced in the mesenchyme, whereby FGF10 increases Shh expression, while FGF7 suppresses it. Furthermore, participating within a positive feedback circuit, Shh enhances mesenchymal expression of FGF10, thereby maintaining reciprocal expression [18]. Shh also activates the mesenchymal expression of the transcription factors forkhead box protein F1/2 (Foxf1/2) and odd-skipped related transcription factor 2 (Osr2) that also participate in the control of Shh expression and, in addition, also regulate the mesenchymal expression of cyclins D1 and D2, thereby promoting the growth of the mesenchyme [45–47]. Furthermore, Shh expression also depends on homeobox protein MSX1 (Msx1)/BMP4 pathway anteriorly, while it depends on paired box 9 (Pax9)/BMP4 pathway posteriorly in the palate [48]. On the other hand, Shh also induces the mesenchymal expression of BMP2, thereby favoring growth of the mesenchymal cells [48]. Wnt5A also seems to be important for palate shelf growth, since its insufficiency in mice causes reduced palatal shelf growth and leads to cleft palate [49].

Palatal shelf elevation

Of all the stages of the palatogenesis process, palate shelf elevation is the least known and the most enigmatic. As we have seen, at first, palatal shelves elongate vertically (Figure 1B); around the seventh week, when the lower jaw increases in height and the tongue moves downwards, they rapidly rise horizontally above the surface of the tongue (Figure 1C). The rise of the shelves is delayed in females compared to males, and this could account for the higher frequency of cleft palates in females. Elevation of the palatal shelves implicates the coordinated actions of fetal movements, including tongue movements and head extension, expansion of the cranial base and broadening of the lower maxilla, as well as intrinsic shelf capability to elevate [33]. Morphological studies in animals found that the mechanisms by which the anterior and posterior portions of the palatal shelves rise horizontally are different, whereby the anterior region can merely flip up, while central and rear parts would be remodeled through horizontal protrusion of the medial wall, and synchronous recession of the underneath portion [20]. Moreover, the whole lingual part of the shelves growing

vertically would move close to the medial line, while the terminal ends of the shelves would end up underneath and sideways [20]. The forces responsible for shelf rise may originate from either the turgidity and hydrostatic pressure resulting from extracellular matrix (ECM) hydration or, otherwise, from the proliferative, migratory, or contractile activities of mesenchymal stromal cells [28]. Several findings suggest the importance of the accumulation of hyaluronan in the interstitial matrix of the stroma, with subsequent binding of extensive amount of water, thus generating hydrostatic pressure [28]. The relevance of growth and/or movement of the mesenchymal cells in determining horizontal displacement of the palatal shelves is less clear, particularly considering the quickness with which shelf elevation takes place. Research regarding the arrangement of cytoplasmic actin fibers, the ECM, and the elongation of cells indicates that tensile force builds up within the palatal mesenchymal cells. Furthermore, it has been observed that actin fibers align towards the upper medial wall in the intermediate and rear segments of the palatal shelves before they elevate, thereby providing a possible elevating force [20]. The generation of such strength might be associated with shifts in the arrangement of cytoskeletal microfilaments within mesenchymal cells. In fact, before elevation, the mesenchymal cells within the palatal shelves are characterized by a polarized and elongated shape, particularly those closest to the BM, which are oriented perpendicularly to it. Following the elevation of the shelves, the cells tend to exhibit a rounder shape, likely indicating that they have contracted. This observation may point to the mechanism behind the generation of the lifting force, implying that actin-driven contractility could play a role in this process [28]. A recent study conducted on murine strains shows that palate shelf elevation begins with the formation of posterior bilateral bulges that progress anteriorly and medially over the tongue until elevation is reached and, moreover, confirms that the two shelves can elevate asynchronously [50]. These dynamics coupled with mesenchymal cell orientation and increased phospho-myosin and actin, therefore consistent with actomyosin contraction, are possibly responsible for the rapid reorientation of the shelves above the surface of the tongue [50]. Indeed, due to the rapid horizontal movement of the palatal shelves, this aligns more closely with the contraction-driven model than with alternative explanations like the mesenchymal cell's gradual differential growth or migration as potential sources of elevating force. Nonetheless, it is feasible that the elevation of the palatal shelf in the middle and posterior areas may result from cellular contraction, while variations in the structure and composition of the ECM within the mesenchyme play a role in the remodeling of shelf tissues [20].

Obviously, the timing of palate shelf elevation is of paramount importance, as a delayed elevation of the shelves may represent a susceptible context in which additional negative genetic/environmental factors could place the individual above the threshold for developing clefting [50]. Moreover, one particular reason for the unsuccessful elevation of the palatal shelves is the adherence or fusion of these developing structures to other intraoral elements. Typically, palatal shelves remain free from fusion with surrounding oral components, a process facilitated by the existence of the non-adhesive periderm layer [15]. Factors interfering with periderm differentiation from the primitive basal layer and its subsequent maintenance can cause premature, abnormal adhesions of the shelves, thus preventing their elevation. Peridermal cells arise from the basal layer *via* a precisely regulated process of molecular signaling. The basal layer cells produce the transcription factor p63, which is crucial for preserving the proliferative capacity of the basal layer [38]. Additionally, they stimulate Jag2/Notch signaling *via* fibroblast growth factor receptor 2b (FGFr2b) and promote the expression of Irf6 [18]. In supra-basal cells, the expression of p63 decreases due to the suppression caused by Jag/Notch signaling. Concurrently, Irf6 facilitates the proteasome-mediated degradation of p63 protein, contributing to p63 suppression and, therefore, to the specification of the periderm. Indeed, in these cells, the expression of Irf6 is significantly increased, while p63 is reduced. This occurs as Irf6 interacts with the Jag2/Notch signaling pathway to promote the differentiation of the periderm, whereby both Irf6 and Jag2/Notch are involved with a feedback mechanism that down-regulates p63. In summary, the differentiation of supra-basal cells into periderm is triggered and sustained by the combined action of Irf6 and the Jag2/Notch signaling pathways [29]. Therefore, a malfunction in these signaling pathways fails to provide protection against undesirable adhesions, which can lead to the improper fusion of the palatal shelves to other intra-oral structures. This fusion can impede their horizontal reorientation, ultimately causing palate clefting.

Palatal shelf contact and adhesion

The palatal shelves are lined by a covering epithelium which generally does not allow contact and fusion with adjacent structures, due to the presence of the non-adhesive periderm cell surface. After their elevation, palatal shelves further grow horizontally and, at the same time, the lining epithelium at their tips is specified to become competent to establish interconnection between the two shelves. This area of epithelium is of crucial importance is the MEE (Figure 1D) [17]. The MEE consists of a basal epithelial layer and an outer layer of periderm, and each of these two epithelial layers will undergo distinct outcomes during the fusion of the palate. It is certain that the basal layers of the MEEs are intended to stick to one another and consolidate to create a unified MES, which becomes stabilized by new cell–cell junctions which form between the two opposite basal layers of the MEEs [30]. While there is overall agreement that MEE basal cells generate the MES, the outcome of peridermal cells has been relatively unclear. It was traditionally thought that the periderm would die and shed before the two shelves came together [51]; however, newer research indicates that the first interaction between the two apposing MEEs takes place specifically through vital periderm cells [38–40, 44]. Indeed, it has been found that peridermal cells bulge, emanate filopodia and establish a weak adhesion with the periderm of the opposite shelf, involving chondroitin sulfate proteoglycan present on the filopodial extensions, which are formed to broaden the surface area usable for connections [30, 39, 40]. Moreover, some authors [44] have shown that periderm cells subsequently migrated to the oral and nasal regions in the merging MEE/MES, playing a role in the development of the epithelial triangles of the fully established MES.

Therefore, peridermal cells initiate the contact between the MEEs, but subsequently they must migrate out of the MEEs [30, 38, 44] to permit the merging of just the basal cells into the MES.

The molecular basis of palatal shelf fusion is centered on the regulation of FGF/Jag2/Notch, p63/Irf6 and transforming growth factor β3 (TGFβ3)/Irf6/p63/p21 molecular pathways [19, 21], with Irf6 and Jag2 signaling being the main players in regulating periderm differentiation, maintenance and fate. As we have seen, p63, Irf6, and Jag2/Notch function together in a controlling system characterized by a negatively acting feedback loop [12, 13, 18, 20, 29]. Initially, p63 promotes the formation of periderm by stimulating the expression of Irf6. The heightened activity of Irf6 is thought to deactivate the initiating signal from p63 by triggering its degradation. This process ultimately restricts cell proliferation and facilitates the differentiation of the periderm. Subsequently, p63 signaling plays a role in preserving the integrity of the periderm by activating the Jag2/Notch pathway [12], continuing this process until the palatal shelves develop and contact one another. In other words, an increase of both Irf6 and Jag2/Notch pathway appears to be essential for the development and preservation of the periderm, and this takes place as a result of their interaction with p63 function. However, once contact and initial adhesion have taken place, peridermal layer needs to be eliminated from the apposed MEEs to facilitate proper adhesion/fusion, a process that is regulated by TGFβ3-mediated down-regulation of p63 and therefore of Jag2/Notch which leads to controlled migration of peridermal cells from the MEE/MES, facilitating direct contact between the basal cells alone [21, 44]. An essential role in all phases of palatogenesis is therefore effected by TGFβ3 signaling [16, 18]. This growth factor causes a decrease in p63 expression, which subsequently regulates Jag2 and influences its impact on the periderm. This process ultimately results in the periderm's removal from the MEE/MES [38]. Jag2, originally present in the palatal epithelium, becomes reduced in the MEEs just before they fuse into the MES. This reduction occurs simultaneously with the onset of periderm bulging and migration [38]. TGFβ3 knockout mice exhibit a reduction in bulging of the periderm [39, 40], the levels of p63 and Jag2 are maintained [38], filopodia and chondroitin sulfate proteoglycan are lacking and removal of peridermal cells does not occur, thus resulting in the development of palate clefting [30, 52]. Conversely, the enhanced migrating capacity facilitated by TGFβ3 may contribute to the subsequent breakdown of the MES whereby TGFβ3-driven migration is probably an important mechanism of MES disappearance through the promotion of the movement of MES basal cells into the oral/nasal mucosae [53]. Therefore, TGFβ3 plays multiple roles at different stages of palate merging, enhancing the adhesion between the apposing MEE cells, facilitating the migration of the periderm during the formation of the MES and, as we shall see, determining its subsequent breakdown [21, 52].

MES creation and disintegration

The union of the palatal shelves to form the definitive palate needs the crucial rearrangement of the two distinct MEEs into a single unified MES (Figure 1, C and D), formed by the fusion of basal MEE cells that interdigitate and tightly connect each other. Nonetheless, after its formation, the MES need to be eliminated to ensure the continuity of the stroma across the fused shelves by establishing a "mesenchymal bridge" between the two previously separate shelves. The disruption of the MES is characterized by BM disintegration, progressive dissolution and fragmentation of the MES into epithelial "pearls", the encroachment of mesenchymal tissue around these structures, and, eventually, the total eradication of any epithelial remnants of the previous seam (Figure 1, E and F) [7, 21, 51, 54, 55].

TGFβ3 has a crucial role in MES disruption, as the TGFβ3/Irf6/p63/p21 signaling could potentially diminish the adhesion of epithelial cells thereby promoting cell migration, favor the restructuring of the ECM, the arrest of the cell cycle and apoptosis, and EMT [21, 30, 54, 56–58]. Indeed, the elevation of p21 expression would aid in the cessation of the cell cycle and enhance apoptosis in MES cells, a crucial element in the degeneration of the MES [16, 18]. Moreover, TGFβ3 signaling also prompts the remodeling of the ECM, including BM dissolution, and Snail-directed E-cadherin-dependent dismantling of cell–cell adhesion, with TGFβ3 and Snail signaling promoting either apoptosis [16, 21] or EMT [56]. E-cadherin plays a crucial role in the fusion of palate shelves; however, its expression is reduced by TGFβ3 and, additionally, mutations in E-cadherin have been linked to cleft lip and palate in families affected by hereditary diffuse gastric cancer [59]. TGFβ3 may be partially regulated by Wnt/β-catenin, as malfunction of Wnt/β-catenin pathway leads to reduced expression of TGFβ3 and cleft palate [60].

Below we will briefly discuss the cellular events thought to underlie MES dissolution. It should be kept in mind that these mechanisms are not mutually exclusive, and may act in concert, or one may follow the other.

Cell migration

In 1992, for the first time, Carette and Ferguson [53] proposed a cell migration-based model for MES degeneration, thereby single MES cells would migrate towards the oral/nasal sides of the merging palate shelves to become incorporated into the lining mucosal epithelia. However, a later study actually reported the downward and upward migration of single MEE cells on both the oral and nasal sides, but the migrating cells would be exclusively periderm cells, not basal cells. Moreover, the authors of this essay illustrated that the epithelial triangles found at the oral/nasal edges of the established MES were formed exclusively by periderm cells [44]. A model has also been suggested where collective cell migration occurs when certain MES cells undergo partial EMT changes, enabling them to function as motile leader cells which pull groups of adherent cells [55]. Moreover, it has been recently demonstrated the importance of actomyosin-generated forces during this type of migration involving movement of collective cell groups [61].

EMT

EMT has emerged as one of the most widely recognized and extensively researched mechanisms believed to underlie MES disappearance [62]. An ultrastructural survey by Fitchett and Hay [51] depicted cells of the MES apparently undergoing EMT emanating pseudopodia protrusions, typical

attributes of motile mesenchymal cells, and according to the model proposed by the authors, these cells were in the process of moving into the stroma and becoming entirely mesenchymal. The BM appeared to be disintegrated at the sites of presumptive EMT, and it was hypothesized that the absence of intervening BM and thus contact of MES cells with a new set of matrix components, might favor EMT [51]. Therefore, according to this model, BM disruption would be an early and instructive event, as it would initiate the mesenchymal phenotype transition. In fact, BM breakdown is frequently noted during the degradation of the MES, regardless of its actual meaning [51, 62]. EMT is a process of pivotal importance in embryogenesis [51], in pathological conditions including fibrosis and cancer [62, 63], and can be readily replicated and studied in controlled experimental settings [64]. TGFβ3 might have a role in EMT possibly associated with MES disappearance [54] and, furthermore, TGFβ signaling has the potential to trigger both EMT and apoptosis within the same type of cell [56, 57].

Programmed cell death

Numerous studies have consistently backed the idea that programmed cell death (apoptosis) serves as a mechanism for the disappearance of MES [17, 44]. A recent study demonstrated that suppression of molecular apoptotic mediators led to complete cleft palate in up to 45% of the triple knockout mice [65], thus definitely attesting the real importance of apoptosis in palatal fusion. Some authors [44] have suggested that the tight adhesion observed between the basal cells of the opposing MEE during shelf fusion may initiate their programmed cell death, which concurrently leads to BM breakdown, termed. These authors propose the term 'cataptosis' to indicate this phenomenon, and suggested that, as a consequence of apoptosis, the matrix metalloproteinases (MMPs) responsible for BM degradation were locally activated. Thus, according to these authors, in contrast to the periderm cells that move away from the seam into the epithelial triangles where they ultimately undergo apoptosis, the basal cells of the MEE seem to die in situ within the MES. In summary, according to this view, palate shelf fusion could happen like this: palate shelves approach each other until a first contact is established, and this would induce periderm cell migration out of the MEE; progressive inter-shelf adhesion appears therefore to be controlled by periderm cell migration out of the MEE/MES; increasing adhesion between the shelves possibly originates from MEE basal cell intercalation, and it is conceivable that cell death is activated precisely at this moment, when a closer contact between epithelial basal cells is established and, in turn, this would cause BM degradation [44].

Convergence/extrusion

During tissue fusion, it has been demonstrated that apoptosis can result in forced exit of dying cells from tissues. When a cell within epithelial tissue experiences apoptosis, it can be eliminated from the epithelium by neighboring non-apoptotic cells through an actomyosin-generated force which would result in the extrusion of the apoptotic cell [66]. Kim *et al.* [58] introduced a novel model for the formation and dissolution of the MES that incorporates this occurrence. This model is based on the convergence of the MEE palatal shelf epithelia coupled with multilayering, intercalation, displacement, apoptosis, migration, and elimination of the MEE cells and, ultimately, MES fragmentation and formation of "pearls" [29, 58]. In this model, actomyosin contractility plays an essential role in driving these cell dynamics, mediated by Rho kinase and myosin light chain kinase that would phosphorylate and activate non-muscle myosin. Thus, according to this model, through this series of events where apoptosis is combined with cell migration and cell extrusion, the MES would become discontinuous, until its ultimate disappearance [57].

Conclusions

Normal morphogenesis of the orofacial structures involves complex tissue interactions in which initially separate tissue masses eventually fuse together. The developing facial primordia are composed of CNC-derived cells which rely on their microenvironment for appropriate migration, proliferation, patterning and differentiation, and of an epithelial lining of ectodermal origin which undergoes a crucial differentiation event, the formation of the peridermal layer. In addition to pinpointing a wealth of genes essential for orofacial development, numerous studies have started to reveal the intricate network of interacting molecular signaling pathways that play a role in the formation of the upper lip and the development of the palate. Face morphogenesis is fueled by the regulated activity of a diverse array of growth factors, transcription factors, and signaling molecules that have been demonstrated to impact the behavior of cells and tissues, including some crucial fusions of tissue parts. The occurrence of CLP can be caused by any of these developmental processes being disrupted.

In some cleft syndromes, the developmental abnormality may arise due to defective generation, migration or proliferation of mesenchymal CNC cells that form the facial primordia, or in the subsequent tissue patterning and modeling to generate the specific shape of the primitive prominence. The fusion of the lip and palate can occur abnormally and cause cleft syndromes if the genes implicated are mutated, and these mutated genes may also participate in clefting unassociated with syndromes [67] (Table 1). Finally, the definitive fusion of the facial structure also requires morphogenetic movements involving the epithelial lining of the fusing tissue masses, and these changes in the epithelia are also governed by genes, such as *IRF6* and *TP63*, which, when altered, can cause clefting (Table 1). Epithelial cell dynamics, including migration, EMT, apoptosis, and convergence/extrusion are involved in developmental fusions, and it cannot be ruled out that more than one, or even all of these mechanisms are operative in the completion of seam disappearance and, therefore, definitive orofacial fusions. Obviously, defective gene-driven failure of these processes could similarly lead to a clefting phenotype.

Early postnatal surgery is the traditional approach to treating CLP, usually with the lips repaired already in the first few weeks and the palate before the first year of life [6]. However, interventions may be extensive and require being divided into different phases. Moreover, they may cause complications and sequelae needing lifelong multi-disciplinary care, thus highlighting the need for innovative

treatments. Therefore, it is highly desirable that new research approaches, especially in combined application, will improve our understanding of CLP, thus allowing for better clinical management and prevention.

Table 1 – *List of some clefting syndromes, their mutated gene and pathogenetic/clinical features*

Syndrome	Involved gene	Pathogenetic/clinical features
Van der Woude syndrome	*IRF6*	Disturbance of periderm formation and cell adhesion. Cleft lip or cleft palate, lower lip pits, hypodontia.
Popliteal pterygium syndrome		Overlapping to Van der Woude syndrome. In addition, popliteal pterygia, digital and genital anomalies.
DiGeorge/Velocardiofacial syndrome	*TBX1*	Defective neural crest/pharyngeal arch development. Cardiac and renal abnormalities, thymus and parathyroid gland hypoplasia, micrognatia, cleft palate, low-set ears.
Pierre Robin sequence	*SOX9*, *BMPR1B*	Defective mandibular growth resulting in failure of palate shelf elevation. Micrognatia, glossoptosis, cleft palate, airway obstruction.
Stickler syndrome	*COL2A1*, *COL11A1*, *COL11A2*	Connective tissue disorder involving mostly fibrillar collagens. Midfacial hypoplasia, cleft palate, frequent association with Pierre Robin sequence, ocular anomalies, hearing loss, arthritis.
Treacher Collins syndrome	*TCOF1*	Reduced generation of neural crest cells due to excessive apoptosis. Zygomatic bone hypoplasia, micrognatia, cleft palate, lower eyelid and external ear abnormalities.
Loeys–Dietz syndrome	*TGFβR1*, *TGFβR2*	Connective tissue disorder due to malfunctioning TGF*β* signaling. Hypertelorism, arterial aneurysms, cleft palate, scoliosis, craniosynostosis.
Apert syndrome	*FGFR2*	Altered osteogenic process resulting in craniostenosis. Midfacial hypoplasia, cleft palate, syndactyly, mental disability, fusion of the cervical vertebrae.
Crouzon syndrome		Altered osteogenic process resulting in craniostenosis. Midfacial hypoplasia, cleft palate, normal intelligence, proptosis.
Ectrodactyly-ectodermal dysplasia clefting syndrome	*TP63*	Altered proliferation–differentiation balance of the keratinocytes. Ectodermal dysplasia, limb malformations, cleft lip or cleft palate.

Mutations in some genes known to cause syndromes associated with cleft lip or cleft palate may also contribute to non-syndromic orofacial clefts (*e.g.*, mutations in *IRF6*, *FGFR2*, *TBX22*; among many others, genetic mutations commonly identified in non-syndromic clefts affect *BMP4*, *FGF8*, *FOXE1*, *MSX1*). For a more extensive list, information and related references, see Dixon *et al.* (2011) [1] and Reynolds *et al.* (2020) [67]. BMP4: Bone morphogenetic protein 4; BMPR1B: Bone morphogenetic protein receptor type 1B; COL2A1: Collagen type II alpha 1 chain; COL11A1: Collagen type XI alpha 1 chain; COL11A2: Collagen type XI alpha 2 chain; FGF8: Fibroblast growth factor 8; FGFR2: Fibroblast growth factor receptor 2; FOXE1: Forkhead box E1; IRF6: Interferon regulatory factor 6; MSX1: Msh homeobox 1; SOX9: Sex determining region Y (SRY)-box transcription factor 9; TBX1: T-box transcription factor 1; TBX22: T-box transcription factor 22; TCOF1: Treacle ribosome biogenesis factor 1; TGF*β*: Transforming growth factor beta; TGF*β*R1: Transforming growth factor receptor 1; TGF*β*R2: Transforming growth factor receptor 2; TP63: Tumor protein p63.

Conflict of interests

The author declares no conflict of interests.

Acknowledgments

I would like to thank Fabio Nangeroni for his generous assistance and useful suggestions.

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Received: June 29, 2025

Accepted: May 14, 2026