The cleft palate and lip: embryology, genetics, environmental influences, and approaches to surgical repair

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Table of Contents

ABSTRACT

The cleft palate and lip is one of the most common birth defects that may or may not be syndromic. Clefting may manifest unilaterally or bilaterally with varying degrees of severity. In embryo, the upper and lower jaws were formed from the first brachial arches that descend from both sides and fuse. Many genetic loci and cell-signaling pathways have been identified with the fusion event, in which polar neural crest cells undergo the epithelial-to-mesenchymal transition. Genetic mutations, environmental teratogens, and nutrition have been associated with the cleft palate and lip. The extracellular matrix has been extensively studied to understand cell-cell communication and is crucial in tissue engineering. The gold standard today for palatal reconstruction remains to be an autogenous graft from the anterior iliac crest. Autogenous bone grafts have many disadvantages such as donor site morbidity. New approaches in tissue engineering involving stems cells, growth factors, and biomaterial scaffolding have been identified to avoid autogenous bone grafts. Mesenchymal cells may be harvested from dental tissue and adipocytes. Three-dimensional printing and computer-aided design are becoming widely used in oral surgery. More research are underway to overcome the challenges in soft tissue reconstruction of the soft palate.

Introduction

Dentists are medical practitioners who specialize in the oral cavity, including teeth, gum, and in some cases, the tongue, the mucosa lining in the oral cavity, and the maxilla and mandible bones of the jaw. Aside from general dentistry, the American Dental Association recognizes nine specialties in dentistry, including dental anesthesiology, dental public health, endodontists, oral and maxillofacial pathology, oral and maxillofacial radiology, oral and maxillofacial surgery, orthodontics and dentofacial orthopedics, pediatric dentistry, periodontics, and prosthodontics (National Commission and Recognition of Dental Specialties and Certifying Boards). In many cases, multiple specialists are required to work alongside the general dentist to treat one patient. In the case of orofacial clefting, a multidisciplinary team of surgeons, anesthesiologists, dentists, and orthodontists are usually required for a better outcome and quality of life (Paiva et al., 2019). Orofacial clefting compromises the integrity of the craniofacial complex, which then affects fundamental functions such as speech, mastication, deglutition, and aesthetics (Zhang et al., 2018). Depending on the severity, clefting often results in gaps in the alveolar bone, traditionally treated using osteoplasty via an autogenic bone graft (Vuletić et al., 2014). If the patient presents missing teeth, implants and orthodontic treatment are widely utilized. Although an autologous bone graft is currently considered the gold standard in osteoplasty, it still presents disadvantages that may be overcome using growth factor-aided tissue engineering and other regenerative methods of treatment (Vuletić et al., 2014).

Orofacial clefting is one of the most common forms of birth defects. Three main categories emerge from all clefting cases: isolated cleft lip and/or alveolus; isolated cleft palate; and combined cleft lip, alveolus, and palate. Each category is subdivided based on the severity of

the cleft as complete or incomplete, and unilateral or bilateral based on the number of clefts (Meng et al., 2009). Cleft lip with or without cleft palate (CLP) (Figure 1 a through d) is more common than isolated cleft palate without cleft lip (CPO) (Figure 1 e). Limited research has been done to identify how CPO differs from CLP in terms of etiology, genetic associations, and risk factors, because CPO is often excluded from studies or combined with cases of CLP (Burg et al., 2016). For CLP, North American Indians and Asians have the highest prevalence rates of 1 in 500 live births; Caucasian populations are observed to have intermediate rates of 1 in 1000 live births; populations from the African descent have the lowest rates of CLP prevalence of approximately 1 in 2500 live births. Japanese populations are found to have the highest rate of CLP occurrence (1 in 500 live births) among all Asian populations (Murthy et al., 2009; Omiya et al., 2014). Biological sex contributes significantly to CLP frequency, as it exhibits a 2:1 male to female ratio. For unilateral clefts, the left side is more prevalent with a 2:1 left side to right side ratio (Murthy et al., 2009). Isolated cleft palate without cleft lip (CPO) is the rarest form of oral clefting and is more common in females than males (Burg et al., 2016). Approximately 30% of orofacial clefts are syndromic and occur with the presence of other developmental abnormalities. Over 300 syndromes have been identified to associate with different forms of CLP. The remaining 70% of CLP cases are considered isolated or non-syndromic (Meng et al., 2009). Conditions involving orofacial clefting are relatively common and can result in a drastic decreased quality of life if not treated with surgical intervention and orthodontics. Research has revealed many possible causes for the cleft palate, including genetic and environmental factors. Teratogens, genetic abnormalities, and alterations in the extracellular matrix have been shown to strongly associate with newborns with orofacial clefting (Meng et al., 2009).

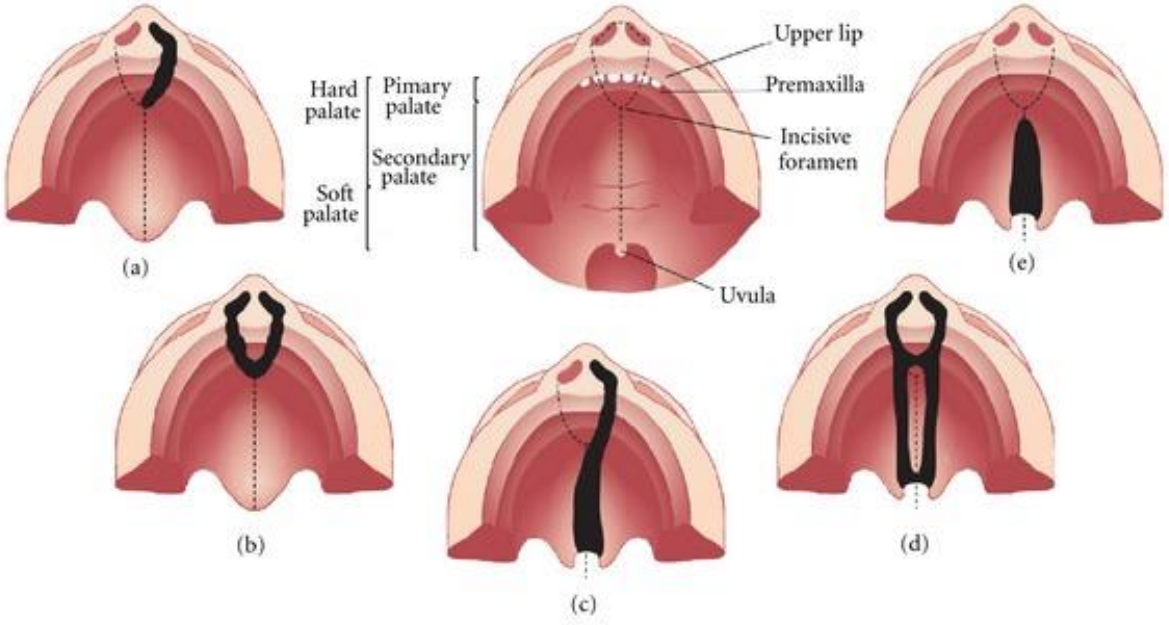
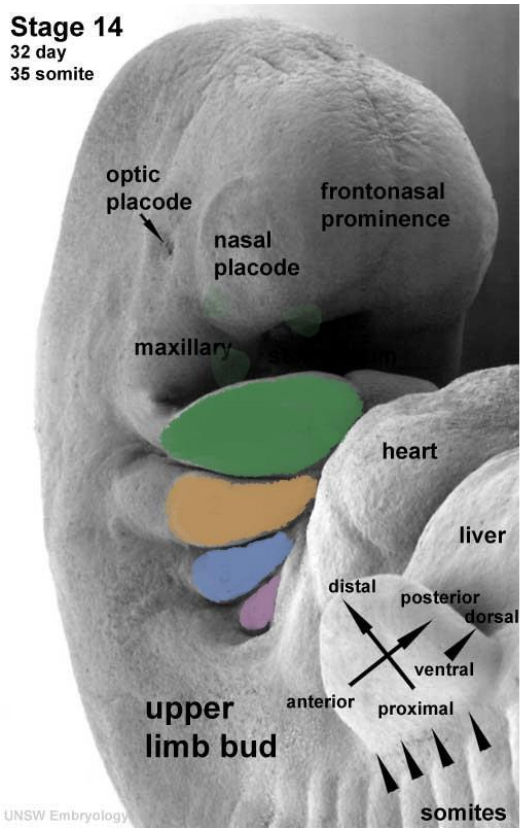


Figure 1. Types of orofacial clefting involving the palate. (a) Unilateral cleft lip with alveolar involvement; (b) bilateral cleft lip with alveolar involvement; (c) unilateral cleft lip associated with cleft palate; (d) bilateral cleft lip and palate; (e) cleft palate only; (a) through (d) represent different types of cleft lip with or without cleft palate (CLP); (e) is seen in isolated cleft palate without cleft lip (Brito et al., 2012).

Normal development and genetic involvement

The human palate is divided into a bony hard palate and a fibromuscular soft palate. The hard palate lies anterior to the soft plate. The incisive foramen is the anatomical marker that divides the hard palate into the primary and secondary palate. The primary palate is anterior to the incisive foramen and contains the maxillary incisors. The secondary palate is posterior to the incisive foramen and separates the nasal passage from the pharynx (Burg et al., 2016; Jankowski et al., 2016). The palate develops between the 4th and the 12th to 13th weeks after conception in the human embryo (Warren et al., 2012). This process begins with five pairs of bilaterally

symmetric protrusions, called branchial or pharyngeal arches that approach the midline on the ventral side of the embryo. The frontonasal prominences descend to form the external nose and the intermaxillary segment that contributes to the primary palate between the 5th to the 7th weeks of gestation. The prominences are derived from two ectoderm nasal or olfactory placodes as they enlarge and separates into the nasomedial and nasolateral processes. The nasomedial process descends and merges with the intermaxillary process (Graham, 2003; Jankowski et al., 2016). The first pair of branchial arches develops into the maxillary and mandibular processes, precursors of the upper and lower jaws, respectively. The maxillary processes fuse with the frontonasal prominences after the formation of the nose and the intermaxillary segment. In early facial development, cells involved may trace their lineage back to mesenchymal cells that are derived from the mesoderm encased in epithelial cells that either derived externally from the ectoderm or internally from the endoderm, depending on their physical location. However, neural crest cells are considered one of the largest contributors to facial development.



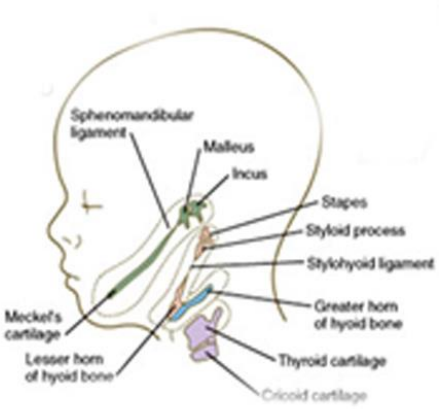


Figure 2. Scanning electron micrograph of Carnegie stage 14. Four pharyngeal arches are shown in green, tan, blue, and purple. One pharyngeal arch is not externally visible. The first pharyngeal arch develops into the maxillary and mandibular processes. Each arch consists of an internal endodermal pouch, a mesenchymal core (formed from the mesoderm and the neural crest cells), a membrane (from endoderm and ectoderm) and an external cleft (from ectoderm) (Hill, 2020). In humans, the first pharyngeal arch (green) differentiates into structures along the side of the face and the lower jaw, such as the Meckel's cartilage, the sphenomandibular ligament, and the malleus and the incus in the middle ear; the second pharyngeal arch (tan) differentiates into inferior structures such as the stapes in the middle ear, the styloid process, the stylohyoid ligament, and the lesser horn of hyoid bone; the third arch (blue) contributes to the greater horn of hyoid bone; the fourth arch (purple) gives rise to the thyroid and cricoid cartilage (Carlson, 2008).

Neural crest cells are highly proliferating and migratory in nature (Graham, 2003). Concurrent with the closure of the neural tube, the crest of the neural folds gives rise to the neural crest cells. In the beginning, neural crest cells appear as classic, tightly-bound epithelial cells with distinct apical-basal polarity. As shown in animal studies, the neural tube cells begin their metamorphic journey upon or before the closure of the neural tube in chick and mice embryos, respectively. The tight and adherens junctions and desmosomes start to disintegrate, and changes are observed in the cytoskeleton (Savagner, 2001). This marks the transition the neural crest cells undergo to adopt more mesenchymal properties, a process called an embryological epithelial-to-mesenchymal transition (eEMT). The mesenchyme is a special type of embryonic connective tissue with various destinations after differentiation. With more mesenchymal properties, the mesenchymal cells become known as ectomesenchyme. The eEMT transition allows the neural crest cells to better migrate laterally to the ventral side of the embryo. The ectomesenchyme will ultimately differentiate into the connective tissue skeletal structures of the face and determine facial appearance (Schneider et al., 2003). The outgrowth process is defined by the proliferation and differentiation of the neural crest cells. Many factors have been shown to affect the growth patterns of cranial neural crest cells, but they all contain a set of intrinsic and unchanging set of pattern of outgrowth that may not be overridden (Cox, 2004). These neural crest cells receive signals from multiple epithelial tissues, as demonstrated in microsurgical transplantation experiments with the pharyngeal endoderm (Couly et al., 2002) and frontonasal ectoderm (Hu et al., 2003). Secreted signals from the fibroblast growth factor (FGF), bone morphogenetic protein (BMP) families, sonic hedgehog (SHH), and components of the endothelial signaling pathway have been shown to influence the outgrowth of the frontonasal and maxillary processes (Clouthier et al., 2000; Richman et al., 2003).

Because the cranial facial tissue is derived from multiple cell lines with different growth rates, the interaction between the epithelial and mesenchymal cells is deemed to be crucial in development. The differences in growth rate manifests as the maxillary and mandibular prominences rapidly proliferate and consolidate while the frontonasal prominence divides in a comparatively consistent rate. Different types of facial dysmorphology arise depending on the severity, timing, and the type of cells affected by genetic and/or environmental influences. Perturbations may act directly on neural crest cells or act upon the signaling pathway between the neural crest cells and their neighboring ectodermal, endodermal, mesodermal epithelium, and mesenchyme (Cox, 2004). For example, *Tbx1* knockout mice exhibit disrupted signaling from the pharyngeal arch endoderm and mesoderm and the differentiation processes of neural crest cells in the arches. Facial and cardiovascular abnormalities were found in *Tbx1* haploinsufficent mice, consistent with clinical observations with patients with DiGeorge syndrome, a relatively common form of birth defect affecting craniofacial development (Baldini, 2002). DiGeorge syndrome displays in a board spectrum of clinical manifestations, with approximately 70% of cases presenting one form of abnormality of the palate. In one study, submucous cleft palate was found to be the most prevalent in Chilean patients, constituting approximately 20% of all cases of newborns with DiGeorge syndrome (Rozas et al., 2019).

Before the medial nasal and the maxillary processes fuse, scattered apoptosis must occur in order to allow the fusion of the primary and the secondary palates (Sun et al., 2000; Holtgrave et al., 2002). This apoptosis process serves several functions. Take the fusion of the primary palate as an example, dying cells make room in the pre-contact area for the eventual merger of the medial nasal and the maxillary processes. These dying cells protrude and weaken the cell-to-cell contact in the epithelia and allow the region to bulge out. The initial contact, recognition,

and consolidation is facilitated by filopodia induced on the epithelial surface before the eEMT process begins within the epithelial cells at the fusion site (Figure 3) (Cox, 2004).

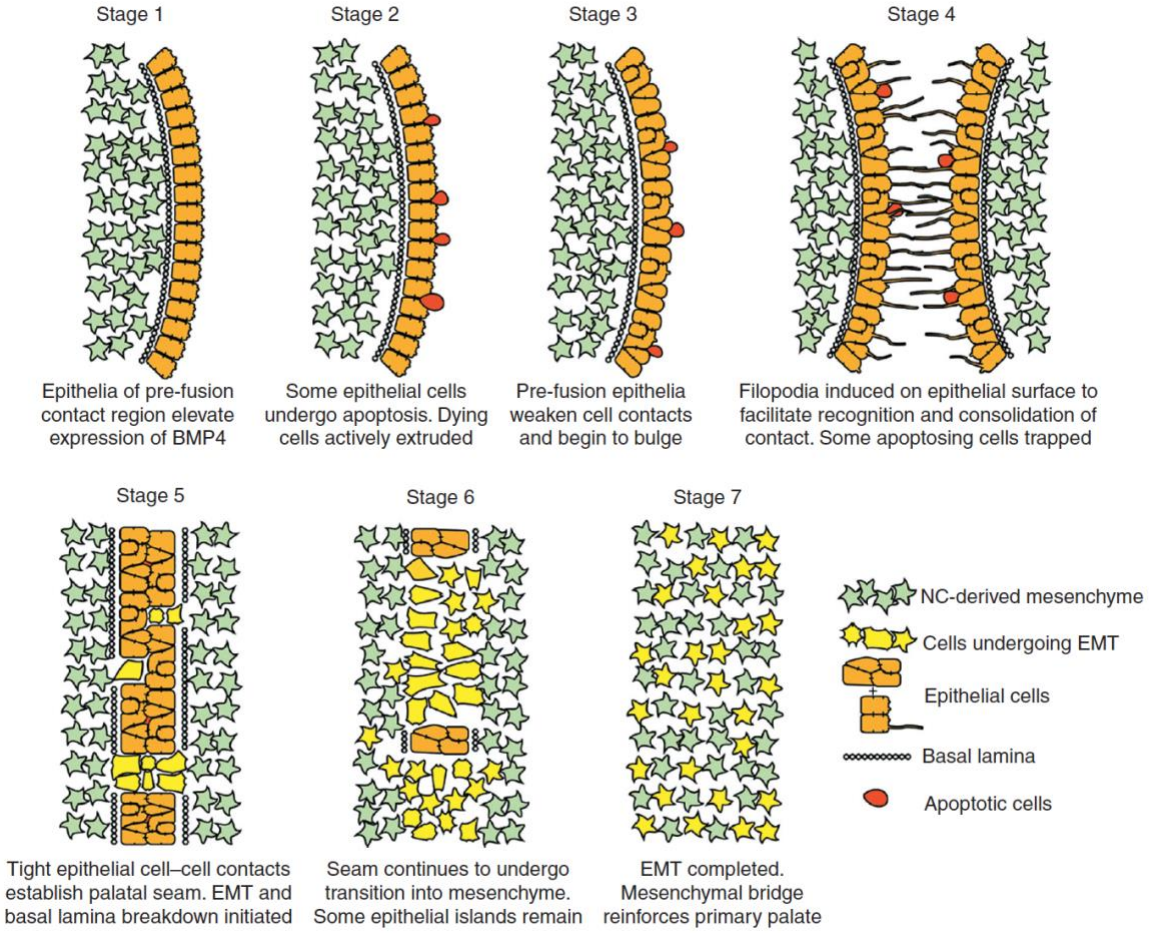


Figure 3. The seven stages of primary palate fusion. The increased expression of BMP4 during stage 1 allows the epithelial cells at the pre-fusion contact zone to undergo a cascade of cellular changes including apoptosis (stage 2), apical surface bulging (stage 3), and filopodia formation (stage 4). Adherent junctions, right junctions, and desmosomes (stage 5) are formed at contact site, followed by the epithelial-to-mesenchymal transition (EMT) and the breakdown of basal lamina. When EMT is complete, the primary palate should consist of confluent mesenchymal cells (Cox, 2004).

Apoptosis is crucial for all of the subsequent steps in the epithelial seam formation, which is why the mice model deficient in *Apaf1*, a gene coding for an apoptotic factor, reveal phenotypes including midline facial cleft and cleft palate (Cecconi et al., 1998). Following induced apoptosis, the remaining epithelial cells adopt cell boundaries that are less defined at the medial edge epithelia (MEE) (Souchon, 1975; Martínez-Alvarez et al., 2000). As primary palate develops in mice, the epithelial cells in the maxillary, medial nasal and lateral nasal processes express *BMP4*, a member of the transforming growth factor-β (TGF-β) superfamily. The expression of this gene becomes restricted to the region of pre-fusion contact region and persists as the epithelia adheres to each other and form the epithelial seam (Ashique et al., 2002; Gong et al., 2003). Members of the TGF-β superfamily, including BMP4 and TGF-β3 induce apoptosis in embryonic tissue and are considered to play a crucial role in development in general. BMP signaling activates downstream loci such as the homeobox transcription factors, MSX1 and MSX2. Deficiency of *MSX 1* in human is found to lead to a syndromic form of CLP while common polymorphisms within the *MSX1* locus may cause non-syndromic forms of CLP (Lidral et al., 1998; Ashique et al., 2002). It is suggested that the epithelial cells respond to the genetic signals randomly, allowing an adequate number of cells to die to make room for the merging processes. At the same time, enough cells must survive to make the epithelial-mesenchymal transition (Cox, 2004). An antagonist to TGF-β3, called *NOGGIN* (*NOG*) is rapidly downregulated in the epithelial cells of the medial nasal where fusion contact occurs (Sela-Donenfeld et al., 1999). In the maxillary pre-fusion contact sites, *SHH* is found to be downregulated. SHH has been demonstrated to serve different functions in the cell cycle across different contexts. In the context of palatogenesis, it is known as an antagonist to BMPs, thus it must decrease in order for the epithelial cells to become preceptive to cell death signals from

BMPs. Its role to control the adhesive properties of the cell membrane is also proposed (Ashique et al., 2002; Cox, 2004).

The epithelial-mesenchymal transition is crucial for normal palatogenesis because the epithelial seam initially formed during the fusion of the processes (medial nasal, maxillary, and lateral nasal) from each side is not strong enough to hold the two sides together through the subsequent developmental events. The development of the face imposes enough torsional force on the seam to separate the facial prominences. In order to gain more tensile strength, the epithelial cells must differentiate into a confluent, thickened mesenchymal cell layer (Diewert et al., 1992). The merging process involves an initial contact of the opposing epithelial cells as well as the formation of a bilayer epithelial seam. Nectin1, a product of the *PVRL1* gene, produce Nectins, a type of immunoglobulin-type cell-cell adhesion molecule. Nectin1 is found to be upregulated in facial ectoderm, palatal epithelia, and neural tissue as it introduces adherent junctions in the contact site with the help from E-cadherins (Takai et al., 2003). The three protein isoforms encoded by *PVRL1* also help with forming cellular projections such as filopodia on the basal surface on the epithelial cells during the break down of the basal lamina during the EMT process. The Nectin1 ectodomains will eventually be cleaved by members of the ADAM (a distintegrin and metalloproteinase) family, an important player in the extracellular matrix transformation during palatogenesis (Kim et al., 2002; Tanaka et al., 2002; Cox, 2004).

The human palate is divided into the hard and the soft palate, with the hard palate further divided into the primary and secondary palate. The fusion of the palate occurs as the five pairs of pharyngeal arches approach the midline, recognize each other, and merge into one. Important cellular changes must occur to adapt to their new life as a consolidated unity. Appropriate apoptosis, changes in cell shape, development of filopodia, and the epithelial-to-mesenchymal

transition (EMT) are considered some of the most crucial transitions. In the end, the epithelial seam where the palate came together should consist of a thick layer of confluent mesenchymal cells. Many genes have been found to be involved in this process, such as the fibroblast growth factor (FGF), bone morphogenetic protein (BMP) families, sonic hedgehog (SHH), Tbx1 gene, homeobox transcription factors (MSX1, MSX2), NOGGIN (NOG), Nectin1 (PVRL1 gene). In addition, extracellular changes will also occur to accommodate at the contacting surface and will be covered in the subsequent sections. These changes are crucial for both normal embryonic development and research in bioengineering for novel, regenerative approaches to correct the cleft palate.

Environmental factors and cell stress

Cleft lip and palate may occur due to environmental factors such as suboptimal nutrition and exposure to teratogens. Many nutrient deficiencies and excesses have been found to be associated to CLP. Deficiencies in cholesterol, thiamin, riboflavin, niacin, pyridoxine, folate, cobalamin, ascorbic acid, zinc, magnesium, and myo-inositol are known to increase the risk of CLP. Vitamin A and iron are associated with the increased risk of CLP when either deficient or in excess. Excess glucose was associated with CLP instances (Krapels et al., 2006). Although the underlying mechanism of how these nutrients affect palatogenesis is largely unknown, many possible pathways have been suggested. The homocysteine pathway could be interrupted when involving riboflavin, folate, pyridoxine, cobalamin, and zinc as cofactors and/or substrate. The oxidative pathway has been shown to affect palatogenesis (Krapels et al., 2006). Oxidation states of enzymes, substrates, and cofactors are crucial to cell signaling, function, and gene expression. Glucose and homocysteine are oxidants, and ascorbic acid and glutathione are antioxidants, all of

which would interfere with the oxidative pathway. Iron, cobalamin, and folate are involved in the hematopoiesis pathway, another possible candidate for causing defects during palatogenesis. Gene expression may be altered during the developmental process through epigenetic events associated with niacin and folate and/or through changing the genomic stability when involving magnesium, folate, and zinc. These genetic changes affect transcription and translation, thereby altering biochemical pathways and hormone production (Krapels et al., 2006).

Among all nutrients associated to orofacial clefting, vitamin A is one of the most studied compounds in palatogenesis. Retinoic acid (RA) is a vitamin A derivative and has many functions in gene regulation. Retinoic acid receptors (RAR) and retinoic X receptor (RXR) are nuclear receptors that are known to form dimers with each other as well as other nuclear receptors to regulate cell proliferation, differentiation and apoptosis (Zhang et al., 1992; Forman et al., 1995). High doses of RA inhibits the expression of *sonic hedgehog* (*Shh)* by eliminating polarizability and growth of the frontonasal and maxillary processes (Helms et al., 1997). In normal development, medial edge epithelial (MEE) cells do not undergo apoptosis until the palatal shelfs are in the horizontal position. However, when an exogenous level of RA was introduced in embryonic mice, the MEE cells were observed to slough off from the periderm, preventing further differentiation and closure of the palatal shelves. High levels of RA also induced apoptosis in the tongue, thus preventing it from playing its normal role in elevating the palate horizontally through the movement of the hyoglossus muscle (Tsunekawa et al., 2005; Okano et al., 2007).

Environmental toxins, such as 2,3,7,8-tetrachlorodibenzo-p-doxin (TCDD), a by-product in paper manufacturing, metal smelting and waste incineration, was found to cause cleft palate in mice (Wang et al., 2019). TCDD is suggested to share a signaling pathway with all-trans-retinoic

acid (atRA) because TCDD fails to induce cleft palate when atRA signaling is impaired (Jacobs et al., 2011).

The Extracellular matrix in palatogenesis

The extracellular matrix (ECM) plays an important role in embryonic development, homeostasis, and tissue repair. The increasing knowledge about the ECM is beginning to bridge the gap between the traditional surgical methods and the tissue regeneration approach for repairing the cleft palate. In the ECM, there are structural molecules attached to the cell membrane and soluble factors (Paiva et al., 2019). One broad category of membrane protein, called the secretome, is in charge of interacting with the ECM and secreting molecules to the ECM. These molecules could either be in soluble forms or secreted into vesicles called extracellular vesicles (EVs). Matrisomes describe a board category of proteins found inside the secretome, which consist of ECM-proteins, also known as the core matrisomes and ECM-associated proteins (matrisomes-associated). The core matrisomes contain fibrous proteins and proteoglycans, while the matrisomes-associated proteins include ECM-related proteins, soluble factors, and ECM regulators (enzymes). Fibrous proteins, such as collagen and elastin provide the matrisomes with structural support, and the fibronectin, laminin, nidogen, and vitronectin carry out adhesive properties. These macromolecules have been found to communicate with each other and bind to growth factors (Raghunathan et al., 2019). The matrisomes-associated proteins function to modulate the ECM and are encoded by approximately 700 genes, making up 4% of the human genome (Hynes et al., 2012).

The other component of the core matrisomes, proteoglycans, are proteins conjugated to glycosaminoglycans (GAGs). GAGs are highly negatively-charged molecules that attract

positively-charged sodium ions, and subsequently, water, which maintains viscosity and preventing desiccation. It was found that the ECM also contains a high level of hyaluronic acid or hyaluronan (HA), a GAG without sulfate (Garantziotis et al., 2019).

During early stages of development, tissue repair, and disease, the ECM components transition from their initial state to a tissue-specific makeup. This transitory state, called the provisional matrix, is formed by fibrin, fibrinogen, fibronectin, HA, and versican, a large chondroitin sulfate proteoglycan. HA interacts with CD44, a membrane receptor, to provide structure, or "glue" that brings together all other components in the pericellular space. The provisional matrix is considered viscoelastic, a property that allows the ECM to create space for cell migration. This is why the migration route of the neural crest cells express high levels of versican (Barker et al., 2017; Chester et al., 2017). Tenasin, another type of ECM protein, is expressed in embyroic cells involved in the neural crest cell migration pathways in mammalian. Tenasin is found to be upregulated in response to epithelio-mesenchymal interactions and is highly restricted during vertebrate development (Riou et al., 1992; Barker et al., 2017; Chester et al., 2017). During development, the palate elevates due to its intrinsic "internal shelf force," at which time, HA is found to be the most abundant GAG in palatal ECM. Specific enzymes on the cell surface are found to produce HA. It is worth noting that these enzymes are unique to the tissue of the embryonic palatal mesenchyme (derived from neural crest cells) and epithelium and exhibit a differentiated expression of the involving genes (Galloway et al., 2013; Paiva et al., 2019). Fibronectin, another component of the provisional matrix, is observed to be elevated in areas of cell migration during palatogenesis and also responsible for palatal shelf elevation (Schwarzbauer et al., 2011; Tang et al., 2015).

Soluble factors are well-known as a form of cellular communication. Cell surface proteins and receptors receive signal from soluble factors from the ECM and help achieve cell-cell interactions including juxtracrine, autocrine, paracrine, and endocrine signaling (Ansorge et al., 2018). Many different forms of cell-cell interactions occur during development. Local mediators, such as peptides and growth factors are common in controlling cellular activities. Morphogens, a type of mediators, are known to induce specific cell differentiation in a specific spatial pattern using its varying concentration gradient (Inomata, 2017). Recently, microRNAs (miRNAs), a class of small regulatory non-coding RNA molecules, have been identified as key regulators in palatogenesis. MiRNAs have shown to play a role in both normal development as well as cleft palate formation. MiRNAs may act as post-transcriptional repressors, or the "fine-tune" mechanism, of certain gene expression involved in the palatogenesis pathway (Schoen et al., 2017).

Mechanisms of ECM remodeling

The ECM environment is dynamic, both during development and later in life. Post-transcriptional modifications, including collagen-collagen, collagen-ECM, and ECM-ECM, are often referred to as ECM cross-links. ECM cross-links are important interactions for structural support in the microenvironment. When first formed, cross-links are immature and prone to proteolytic degradation; cross-links will improve stability once they generate insoluble proteins polymers and establish a stronger collagen network with better biomechanical properties. The modeling and remodeling of the ECM is determined by the soluble or EV-associated proteases secreted into the ECM or membrane-bound proteases, considered a class of cross-linkers (Paiva et al., 2019; Sanderson et al., 2019).

During palatogenesis, the development of the facial primordia is achieved by the remodeling of the ECM. Many genes and enzymes have been shown to participate in this process, most of which belong in the metzincin family of metalloproteinases (Stöcker et al., 1995). One member of the family, the vertebrate matrixins (MMPs) are most studied for their ability to degrade all ECM components (Bond, 2019). During development, MMPs participate in the process of morphogenesis through their ability to modify the components in the existing ECM, allowing cell migration and differentiation, tissue resorption, and cell-cell interactions. The ECM remodeling process is crucial for palatal shelf orientation and the epithelial-to-mesenchymal transition (EMT) during palatal fusion (Brown et al., 2002). The tissue inhibitors of metalloproteinases (TIMPs) are another class of enzymes that are upregulated and distributed in a similar spatial pattern to the MMPs in cells of the epithelial basement membrane (Morris-Wiman et al., 2000). TIMPs are shown to be associated with ECM structural integrity and rigidity by inhibiting the MMPs and similar enzymes such as the ADAM (A Disintegrin And Metalloproteinase) and ADAMTS (A Disintegrin-like And Metalloproteinase with ThromboSpondin motifs) (Sahebjam et al., 2007; Paiva et al., 2019). Palatal fusion involves the disintegration of the basement membrane, the EMT process, and the migration and adhesion of the differentiated mesenchymal cells to the adjacent side, all of which involve matrix metalloproteinases. The involved epithelial cells are shown to also express genes encoding matrix metalloproteinases (Horejs, 2016). It is worth noting that research has shown possible compensatory mechanisms for these crucial genes in palatogenesis, because the single knockouts for many genes in the TIMPs and MMPs family do not lead to development of cleft palate (Paiva et al., 2014). While individual, loss-of-function MMPs may be compensated for, the loss of multiple specific MMP genes in combination may interfere with normal palatogenesis. It is also

suspected that MMP genes may play a role in interacting with and modifying other genes involved in the palatogenesis pathways (Paiva et al., 2019).

Proteomics has been the most popular strategy in characterizing ECM components in both normal development and pathological conditions. One of the biggest challenges to proteomics in the past has been the solubilization and protein recovery, until an optimized protocol was developed (Paiva et al., 2019). The proteins in the ECM matrix can be now digested into peptides before analyzed using mass spectroscopy, and web tools are used to annotate and quantify ECM proteins relative to each other. This new development contributed to faster results and analyzing the changing expression of ECM proteins during development and remodeling (Naba et al., 2017).

ECM remodeling plays an important role in embryonic palatogenesis. Growth factors and other molecules involved in the ECM remodeling progress suggest promising future directions for novel ways of palatal reconstruction and regeneration. Two of the most important families of genes involved in ECM remodeling are the MMPs and TIMPs, both enzymes of the metalloproteinase family. The MMPs are shown to be involved in ECM degradation, making room for cell migration and differentiation during palatal fusion. The TIMPs are shown to inhibit the activity from MMPs as well as other similar enzymes in the ECM. Research failed to show cleft palate development due to MMP single knockouts, suggesting a compensatory mechanism *in vivo*. However, the loss of function of a combination of specific MMPs are likely to cause impaired palatal development in embryo.

Methods of palatal reconstruction

Palatal reconstruction is the term used to define “any intervention able to restore the barrier between the oral and nasal cavities, and physiological functions” (Paiva et al., 2019). The most traditional method of repair for the cleft palate and lip involves plastic surgery for the lip and a bone graft for the palate. Orthodontist treatment is often needed and delivered in multiple stages throughout development. Depending on each unique patient’s case, a multidisciplinary team of surgeons, dentists, speech pathologists, geneticists, and nutrition experts may be involved in delivering care. Without adequate care, the cleft palate with or without cleft lip may result in difficulty in deglutition, breathing, speech, and hearing. Surgical repair of the cleft lip, cheiloplasty, usually occurs 3 months after birth, followed by palatoplasty, surgical repair of the cleft palate, in the first 6-12 months of life. Bone grafts, when needed, are usually delivered between 8-11 years of life. Orthodontist treatment may be necessary anytime between the second year of life and early adulthood. The variability in the type and the timing of the treatment is dependent on the severity of the cleft and the extension of the tissue loss (Paiva et al., 2019).

Among all different types of bone grafts, the autogenous bone grafts are considered the gold standard for repairing the alveolar bone and reconstructing the palatal structure. In some cases, multiple grafts, divided into treatment stages, may be necessary. Craniofacial bone and noncraniofacial bone present different embryonic origins. As illustrated above, craniofacial bones and cartilages were formed from the mesenchymal cells that derived from the neural crest cells, while the bones in the axial skeleton came from the somites and the lateral plate mesoderm. Most of the bones of the skull are flat bones and are known to undergo intramembranous ossification, a process in which mesenchymal cells are directly converted to bone. In contrast to intramembranous ossification, endochondral ossification involves an extra step as mesenchymal

cells becomes cartilage first before converted into bone. Many parts of the appendicular skeleton in the body, such as the femur, are categorized as long bones and would undergo endochondral ossification in embryo (Zhang et al., 2018).

Craniofacial and noncraniofacial bones show different homeostatic mechanisms, and membranous bone grafts were found to retain volume better than endochondral bone grafts (Zins et al., 1984). That's why the most commonly used donor sites include the anterior iliac crest, proximal tibia, mandibular symphysis, calvaria, and ribs, some examples of bones derived from the mesoderm. One of the biggest disadvantages of autogenous bone grafts stems from the limited bone volume available to harvest as well as potential morbidity at the donor site. Post-surgical complications may involve symptoms such as chronic pain, paresthesia of the thigh, and hypertrophic scarring. Unsuccessful repairs are often associated with the loss of the graft due to inflammation, bone resorption, and the development of oronasal fistulas (Borba et al., 2014).

Over the years, studies have investigated many alternatives to repair the cleft palate. Following the first generation of palatal reconstruction using autogenic bone grafts, the second generation of palatal reconstruction utilizes biomaterials and growth factors. Osteoconductive biomaterials including hydroxyapatite and tricalcium phosphate were introduced as an alternative to allogeneic, xenogeneic, and alloplastic grafts. Growth factors such as the BMPs, "natural adjuvant" platelet concentrates, denominated platelet-rich plasma (PRP), and platelet-rich fibrin (PRF) were used in combination with various biomaterials (Paiva et al., 2019). Specifically, BMP-2 has demonstrated an increase in the production of mature bone both *in vitro* and *in vivo* (Shimakura et al., 2003). Clinical studies demonstrated that BMPs are as efficient as autologous bone graft for the repair of the cleft palate and alveolar bone (Hammoudeh et al., 2017).

Cell-based therapies have been investigated as another alternative to repair the cleft palate and alveolar bone. These methods involve stem cells that are able to differentiate into active osteoblasts to promote bone growth and regeneration (Fallucco et al., 2009; Paiva et al., 2019). Two studies done in 2018 and 2019 found no statistical difference between the role of BMP2 and the tissue-engineered bone replacement materials in repairing the palate and alveolar clefts (Kamal et al., 2018; Paiva et al., 2019; Scalzone et al., 2019). One meta-analysis conducted in 2018 compared iliac crest bone grafts (ICBG) with BMP-2, acellular dermis matrix membrane, cranium, and rib grafts; when BMP-2 was bound to absorbable collagen sponge, it showed a similar cleft repair efficacy to ICBG; covering ICBG with acellular dermis matrix was shown to increase bone retention for unilateral cleft patients; mixing ICBG with plasma may increase bone retention for skeletally mature patients but not for younger patients; and that the mandible graft is more effective than cranium and rib grafts for alveolar cleft reconstruction; ICBG is still shown to be one of the best courses of treatment based on patient outcomes (Wu et al.).

The third generation of palatal reconstruction was made possible due to the recent advancements in three-dimensional (3D) cell culture techniques. Tissue and ECM remodeling and palatal fusion occur in a three-dimensional environment, so 3D cell cultures more closely mimic realistic cell morphology, physiology, and pathology. This technology not only offers novel ways to study and observe embryonic development, but also another alternative to palatal reconstruction without the need of scaffolding. Three-dimensional cell cultures are considered 4D when time is taken into consideration (Paiva et al., 2019). To create a 3D cell culture, one may utilize cell aggregates, spheroid, or organoids (Alhaque et al., 2018).

Palatal reconstruction using stem cells, biomaterials, scaffolds, and signaling molecules could be divided into two main approaches. The "top-down" approach takes place *in vitro* and involves producing functional tissue using proliferating cells within scaffolding biomaterials. The "bottom-up" approach takes place *in vivo* and produce modular tissue units (spheroids) from adult stem cells that are responsible for synthesizing their own ECM (Baptista et al., 2018). Research in cleft palate repair using bone bioengineering is still in its infancy stage, with very few studies using animal models. However, more studies have been conducted for repairing alveolar clefts or mid-palate cleft. Several studies have shown a successful mid-palate repair in animal models using human stem cells. For example, one study uses the rat model and demonstrated filling a palatal defect using an autogenous engineered graft. The authors used fat-derived stem cells that were differentiated into osteoblasts/osteocytes and seeded onto a poly-L-lactic acid absorbable scaffolds (Conejero et al., 2006). A more recent study demonstrated the use of a type of autogenous multilayered palate substitute with bone and oral mucosa tissue to repair palatal defects in the rabbit model. In this study, adipose tissue-derived mesenchymal stem cells (MSCs) were divided into individual cell layers and seeded onto fibrin-agarose hydrogels to induce differentiation into osteogenic cells. Fibroblasts and keratinocytes were also seeded onto the same gel. The oral mucosa layer was placed on top and compressed to fuse the mucosal stroma (fibroblasts) with the osteogenic layer. Partial bone differentiation was observed. The authors suggest the multilayered approach may result in an increase in maturation time when compared to monolayered approaches *in vivo* (Martín-Piedra et al., 2017).

Types of stem cells proposed for craniofacial reconstruction

Bone-marrow stem cells (BMSCs) are one of the most studied topics in regenerative medicine, and their cell properties are relatively well-known. However, it remains unknown in terms of the specifics of how to prepare BMSCs *ex vivo* before their differentiation process and clinical application (Shanbhag et al., 2019). Scaffold-free BMSCs are considered safe to use to repair alveolar clefts in CL/P patients, but not more extensive bone defects (Bajestan et al., 2017). However, the use of BMSCs still requires a donor site (primarily at the iliac crest) and do not overcome the challenge of donor site morbidity observed in regular autogenous bone grafts, even when minimally invasive techniques are used (Paiva et al., 2019).

Because the use of BMSCs can still lead to donor site morbidity, researchers started looking for alternative sources for mesenchymal stem cells (MSCs). The MSCs found in adult dental tissues display cranial neural crest cell (NCC) properties and are more closely related to cells involved in palatogenesis in the embryo than BMSCs from other areas in the body (Dixin et al., 2018). In the mouth, human MSCs could appear in tissues with both odontogenic and non-odontogenic origins, and may be harvested during a wide range of surgical procedures. Among cells from non-odontogenic origins, those that display MSC and osteogenic properties have been observed in gingival connective tissue, also known as gingival mesenchymal stem/progenitor cells (GMSCs) (Yang et al., 2013), oral periosteum of the palate, the lower and upper vestibule (Ceccarelli et al., 2016), palatal connective tissue (Pall et al., 2017), and adipose stem cells from buccal fat pad (Farre Guasch et al., 2010). A recent study in 2019 demonstrated the retention of stem cell properties of palatal periosteum-derived MSCs (Naung et al., 2019). In a registered clinical trial aimed to repair human alveolar clefts, the researchers found no statistical difference among three random groups: 1) using anterior iliac crest (AIC) bone and a collagen membrane;

2) lateral ramus cortical plate with buccal fat pad derived mesenchymal stem cells (BFSCs) mounted on a natural bovine bone mineral; and 3) both AIC and BFSCs cultured on natural bovine bone mineral with a collagen membrane (Khojasteh et al., 2017). However, this approach still does not resolve the issue of limited amount of tissue available as presented in other types of autogenous grafts.

Five different types of MSCs have been identified in various locations in dental tissues: dental follicle progenitor stem cells (DFPSCs), stem cells from apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs), dental pulp stem cells (DPSCs), and stem cells from exfoliated deciduous teeth (SHEDs) (Baniebrahimi et al., 2019). Because it is a natural part of development to exfoliate SHEDs, they are considered the most obtainable odontogenic tissue with little to no harm to the donor site. The pulp tissue may be harvest between 5 and 12 years of age, when a child's deciduous teeth are replaced by permeant ones, and this procedure is not considered to be associated with significant ethical implications (Taguchi et al., 2019). Furthermore, SHEDs are highly proliferative, display a capacity to differentiate across multiple linages, and are known to secret immunomodulatory molecules. Similar to SHEDs, DPSCs may be harvested during the extraction procedures of third molars, a procedure often done in young adults and adolescents, presenting another example of sources to obtain MSCs with little to no known ethical implications (Yamada et al., 2019). Both SHEDs and DPSCs allow cell sheets (Lee et al., 2019) and 3D spheroid cultures (Wang et al., 2010). Both types of stem cells display high regenerative potential because they express high levels of secretome as well as many types of paracrine soluble molecules and EVs. It is worth noting that they present potential in many different areas of regenerative medicine, including cells involved in the immune system, neurons, and vasculature (Kichenbrand, 2019; Paiva et al., 2019).

Compared to using MSCs and BMSCs, SHEDs were proposed to be better alternatives for craniofacial bone repair in one study. The SHEDs were primed using FGF-2 and/or hypoxia to improve angiogenesis (Novais et al., 2019). Systematic reviews evaluating methods using bioengineered PDSCs and SHEDs to repair bone defects in both humans and mice showed promising results (Leyendecker Junior et al., 2018). In European countries, biobanks were established to collect and store healthy exfoliated teeth as a lower-cost alternative to umbilical cord banks. However, stems cells obtained from PDSCs and SHEDs still require *ex vivo* manipulation, an unavoidable time-consuming and costly procedure as another challenge the scientific community is still yet to overcome (Paiva et al., 2019).

Another proposed source of MSCs comes from the *orbicularis oris* muscle incised during the cheiloplasty procedure of CL/P patients. The *orbicularis oris* muscle is a circular muscle that surrounds the lips and is responsible for lip movements. It is usually discarded after the initial surgery repair of the cleft lip. Research demonstrated that these muscle cells have the potential to express classical MSC cell surface proteins as well as differentiate into multiple different tissue lineages *in vitro*, including bone, fat, cartilage, and skeletal muscle (Paiva et al., 2019).

Adipose-derived mesenchymal cells (AMCs) are another potential candidate for bone regeneration. The biggest advantage of AMCs are their accessibility and availability in large amounts. AMCs were demonstrated to have similar growth kinetics and cell senescence as BMSCs isolated from the same donor (De Ugarte et al., 2003). Various types of scaffolding have been investigated to work with AMCs in regenerating craniofacial bone in animal models. For example, one of the more recent studies showed bone regeneration in a critical-sized calvarial defect in mice using silk scaffolding (Jin, 2014).

Technology in bioengineering and regenerative medicine

Computer-Aided Design (CAD) is widely used in many areas in dental medicine, such as the planning of implants, crowns, and orthodontist treatments, and facial reconstruction is no exception. Due to the complexity of the craniofacial structures, minor bone resorption could lead to less than desirable outcomes from reconstructive surgery. CAD provides more accurate strategies to craniofacial reconstruction (Zhang et al., 2018). Titanium scaffolds have been suggested for an alternative candidate in craniofacial reconstructive surgery due to their nonabsorbable properties (Terheyden et al., 2004). Titanium cages filled with granules, cancellous bone chips, or bone blocks in conjunction with adipose-derive stem cells (ASCs) have demonstrated success in repairing large craniofacial defects (Zhang et al., 2018).

Thanks to the evolving technology that gave rise to three-dimensional (3D) printing, 3D biomimetic scaffolds were made possible in regenerative medicine. Scaffolds serve as a method of delivery of progenitor cells and growth factors in surgical sites, mimicking the ECM composition of craniofacial bone (Zaky et al., 2014; Teven et al., 2015). A well-designed scaffold should be easy to implant *in vivo*, and support cellular adhesion and proliferation (Zaky et al., 2014). Many types of material have been proposed and investigated for possible clinical application, each representing unique strengths and drawbacks. Three broad categories arise from all scaffolds that have been investigated for craniofacial regeneration: polymer-based scaffolds, calcium phosphate-based scaffolds, and composite scaffolds. Within the polymer-based scaffolds, natural polymers such as chitosan and silk fibroin have been demonstrated to produce promising results; synthetic polymers such as poly(lactic acid) and poly(glycolic acid) and poly(e-caprolactone) have been synthesized to support osteoblastic functions. Calcium phosphate-based ceramic scaffolds includes hydroxyapatite and β-tricalcium phosphate materials

(Teven et al., 2015). Composite scaffolds usually comprise both polymer-based and calcium-phosphate-based materials to take advantage of the best in both worlds. The ceramic-based scaffolds are superior in their biocompatibility, osteoconduction, and mechanical strengths. Polymer-based scaffolds are slower in degradation rate and relatively easier for structural manipulation (Rezwan et al., 2006).

Currently, 3D printing techniques allow researchers to print cells directly, biomaterials with cells, or scaffold-free cell aggregates. For tissue regeneration, three types of 3D printers are commercially available: inkjet printers, laser-based printers, and microextrusion printers (Zhang et al., 2018). While laser-based printers and microextrusion printers have been used to fabricate tissues such as vascular trees and cellularized skin, inkjet printers are the only ones that have been shown to be able to successfully fabricate bone tissue. Inkjet bioprinters are relatively time-efficient, use a "drop-on-demand" process, and can be customized to the individual patient's needs each time (Azuma et al., 2014). For rapid functional recovery, it would be beneficial to regenerate hard and soft tissue simultaneously, an area of craniofacial regenerative medicine that is still under investigation. It has been suggested that dental tissue may be regenerated simultaneous with mandibular or maxillary bone in the future (Zhang et al., 2018).

One of the biggest challenges in 3D bioprinting is the limitation on the size of the structure. Hydrogel, the most popular injecting material, fails to provide stable structural support for bone and/or tissue structures that are of clinically relevant size (Chang et al., 2011). To overcome this challenge, a new tissue-organ printer (ITOP) has been developed to generate larger tissue structures suitable for regenerative medicine. The ITOP achieves mechanical stability by printing cell-laden hydrogel integrated with biodegradable polymers onto sacrificial hydrogels. CAD imaging data is used to establish the correct shape of the tissue. The anatomical

defect is scanned and entered into a computer program to ensure cells are dropped into correct locations. Microchannels are incorporated into the tissue to diffuse nutrients to printed cells to overcome the previous challenge of having a diffusion limit of 100-200 um for cell survival. The developers of ITOP was able to demonstrate fabricating mandible and calvarial bone, cartilage, and skeletal muscle (Kang et al., 2016).

Soft tissue regeneration in the palate

Not all CLP patients present with the cleft in the soft palate. Soft palate clefts are associated with difficulty in speech, swallowing, sucking, and the inability to separate the nasal cavity from the oral cavity (velopharyngeal dysfunction). Traditional surgical repair usually involves closing the cleft and reconstructing the muscle *levator veli palitini* (LVP), the major muscle of the soft palate (Boorman et al., 1985). However, approximately 10-30% of patients fail to achieve adequate velopharyngeal function post-surgery, mainly due to three main factors. The muscles in the soft palate presents intrinsically low regenerative capacity compared to skeletal muscles on the limb; the formation of the cleft in the soft palate in embryo usually leads to the dysfunctional organization of the muscles; and the development of fibrosis post-surgery (Carvajal Monroy et al., 2012).

In normal development, five pairs of muscles should arise to form the soft palate: the *tensor veli palatini* (TVP), the LVP, the *palatopharyngeus* (PP), the *palatoglossus* (PG), and the *uvulae* (U). All muscles, with the exception of U, which is an intrinsic muscle without bony attachment, extend from separate bony structures but share the same insertion at palatal aponeurosis (PA), near the center of the soft palate. When a cleft forms in the soft palate, PA is divided in half, each shifted to the lateral sides of the soft palate, giving rise to an abnormal

insertions for the four pairs of aforementioned soft palate muscles (Figure 5). Because the muscles now have two instead of one skeletal attachment, they present limited isometric contractions (Hubertus Koch et al., 1999). Without treatment, the cleft muscles could further widen the gap as they pull the two halves of the soft palate superiorly and laterally (Fara et al., 1970). The LVP may undergo atrophy due to the lack of stimulation (Cohen et al., 1994).

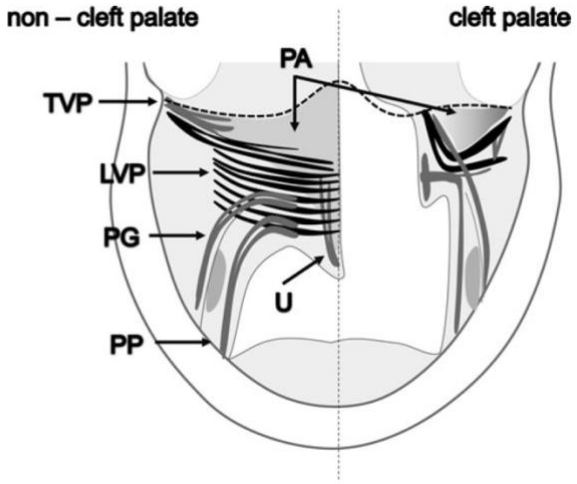


Figure 5. The comparison of the soft palate muscles in normal development vs. in a cleft palate patient. The soft palate consists of five pairs of muscles: the *tensor veli palatini* (TVP), the *levator veli palitini* (LVP), the *palatopharyngeus* (PP), the *palatoglossus* (PG), and the *uvulae* (U). Palatal aponeurosis (PA) is the normal insertion of four pairs of muscles with the exception of U. PA is split into two structures if cleft is present in the soft palate. Cleft palate muscles are often underdeveloped and show disorganized myofibers due to the abnormal insertion near the posterior border of the hard palate (Carvajal Monroy et al., 2012).

All skeletal muscles may be divided into categories of slow or fast twitch. Slow twitch muscle fibers have a low activation threshold and are resistant to fatigue while fast twitch muscle fibers have a higher activation threshold and are more fatigable. Slow twitch muscles are more likely to undergo a larger proportion of aerobic metabolism while fast twitch fibers are more prone to carry out anaerobic metabolism. As a result, slow twitch muscles fibers usually require more blood supply and appear red. Normal LVP muscle contains predominantly slow fibers, while LVP in the cleft palate shows a higher proportion of fast fibers and a reduced capillary supply (Lindman et al., 2001). This increased proportion of fast fibers explain why the LVP may easily become fatigued during speech in cleft patients (Hanes et al., 2007). Fast fibers are also more prone to contraction-induced injury (Rader et al., 2007).

Unlike skeletal muscles of the limb, the muscles in the soft palate do not regenerate as readily. Most studies on muscle regeneration are done in the limb muscles instead of the head muscle. Satellite cells, known as the primary muscle stem cells, are responsible for muscle growth and repair after birth (Mauro, 1961). Previously, the difference in cell lineage and origin between the bones of the head and the rest of the skeleton was discussed. Similarly, the muscles in the head present a difference in origin compared to the muscles of the trunk and limbs. The muscles of the head are derived from the branchial arches (branchiomeric muscles), while the rest of the skeletal muscles in the body are derived from the somites (Christ et al., 1995; Noden et al., 2006). One study done on the masseter muscle found a reduced number of satellite cells in the masseter muscle, another example of branchiomeric muscle similar to the muscles of the soft palate (Ono et al., 2010). Furthermore, satellite cells are found to be significantly less numerous in fast fibers than slow fibers, another factor causing a decrease in satellite cell number in cleft palate muscles (Gibson et al., 1982).

One of the three reasons attributing to less desirable outcomes after surgical repair of the soft palate is the intrinsically low regenerative properties of the muscles. It has been suggested that isolated satellite cells may be transplanted into the soft palate muscles for improved regeneration (Carvajal Monroy et al., 2012). Transplanted satellite cells have demonstrated success in stimulating muscle tissue to proliferate and renew in patients with diseases such as Muscular Dystrophy (Motohashi et al., 2014). However, transplanting satellite cells and myoblasts is not without limitations. After isolation, satellite cells may lose their regenerative capacity in culture; they only demonstrate limited survival rates after injection; and their ability to migrate to injury site is also limited (Ferrari et al., 1998).

Alternative to the transplantation approach, the preexisting satellite cells in the soft palate muscle may be stimulated and recruited by growth factors to proliferate and differentiate. Examples of relevant growth factors include insulin-like growth factor 1 (IGF-1) and FGF-2 (Carvajal Monroy et al., 2012). To combat diffusion and enzymatic inactivation, these growth factor may not simply be injected. They are the most effective when incorporated into biodegradable scaffolds for a more controlled release (Whitaker et al., 2001). For soft palate muscle regeneration, the use of growth factors with the appropriate delivery system is more likely to yield desirable results than cell-based therapy (Carvajal Monroy et al., 2012).

Disorganization of the muscle fibers is the second major factor causing nonfunctional soft palate muscle post-surgery. Both scaffolding and surface topography may help achieve better alignment of the soft palate muscles after repair. Surface topography is a method in tissue engineering used to align skeletal muscle fibers. It usually involves a polymer chip with linearly aligned microgrooves that guide myoblast to form unidirectionally aligned juxtaposed myotubes (Zhao et al., 2009). Other techniques to control cellular alignment includes electrospinning,

photolithography, and electron mean lithography (Carvajal Monroy et al., 2012). Growth factors may be printed on sub-micron polystyrene fibers that guides the direction of myoblast activity at the same time (Ker et al., 2011). One challenge with using biodegradable materials and scaffolding is making sure that the material degrades before significant growth of the patient occurs, since reconstructive surgery is usually performed in children (Carvajal Monroy et al., 2012).

Lastly, fibrosis that occurs post-surgery may impair muscle function. During regeneration, the extracellular matrix (ECM) expresses transforming growth factor-beta 1 (TGF-1). Myoblasts that expresses the TGF- 1 gene can differentiate into myofibroblast cells that stimulate scar formation (Li et al., 2004). Naturally, the solution proposed is to inhibit the activity of TGF- 1. Examples include decorin, a member of the small leucine-rich proteoglycan family that reduces TGF- 1 and its synergist, myostatin (Zhu et al., 2007). Further research in other signaling molecules such as nitric oxide is required. Nitric oxide was been shown to down regulate TGF- 1 activity, but its role in muscle regeneration remains unclear (Filippin et al., 2011).

In conclusion, improved muscle regeneration in the soft palate leads to better surgical outcome in repairing the cleft soft palate. In cleft palate patients, the soft palate muscles do not regenerate as readily, which calls for growth factors in suitable delivery systems such as a polymer scaffolding. The scaffolds may also offer architecture and guidance for myoblast activity, making sure that the new muscle fibers generated are properly aligned. Scar tissue, also known as fibrosis formation may be avoided using factors such as decorin.

Conclusions and future directions

Orofacial clefting is one of the most common birth defects, and a team of specialists is usually required to treat one patient. All cleft palate cases may divided into three categories: isolated cleft lip and/or alveolus; isolated cleft palate; and combined cleft lip, alveolus, and palate. Each of the three main categories may be further divided based on the severity of the clefts as complete or incomplete, and unilateral or bilateral. There are many possible causes for the cleft palate, including genetic and environmental factors. It could either occur as the only birth defect (non-syndromic) or as a part of a syndrome such as the DiGeorge syndrome.

The human palate is divided into the hard (anterior) and the soft (posterior) palate, with the incisive foramen further dividing the hard palate into the primary (anterior) and the secondary (posterior) palate. The palate develops between the 4th and the 12th to 13th weeks after conception in the human embryo. In normal development, five pairs of bilaterally symmetric protrusions (branchial arches) extends laterally to approach the midline on the ventral side. Cells in facial development are largely neural crest cells, with some others from lineages such as the mesenchyme, mesoderm, ectoderm, and endoderm, depending on their physical location. As the branchial arches from each side fuse into one, the migrating neural crest cells undergo the epithelial-to-mesenchymal transition (EMT), one of the most important events in facial development. The neural crest cells receive signals from surrounding epithelial tissues, the fibroblast growth factor (FGF), bone morphogenetic protein (BMP) families, sonic hedgehog (SHH), and components of the endothelial signaling pathway. Genes such as the Tbx1 gene, homeobox transcription factors (MSX1, MSX2), NOGGIN (NOG), Nectin1 (PVRL1 gene) are known to influence cellular changes that allows the branchial arches to fuse and transform into one, confluent mesenchymal cell layer with structural integrity. The cleft palate may also occur

due to environmental teratogens and nutritional deficiencies and excesses. Oxidative stress, retinoic acid, and environmental toxins such as TCDD has been shown to cause cleft palate in animal models.

The extracellular matrix (ECM) plays an important role in cell regulation both in the embryo and maintaining homeostasis after birth. Cell-cell communication are achieved through juxtracrine, autocrine, paracrine, and endocrine signaling, soluble factors carried to cell receptors via the ECM. Research about ECM proteins, signaling molecules, and cellular pathways not only revels causes for CLP, but also offers possibilities in tissue engineering and regeneration as novel methods of cleft palate repair. Secretomes are a board category of membrane protein that interacts with and secrets molecules into the ECM. Inside secretomes are matrisomes. Matrisome-associated proteins include ECM-related proteins, soluble factors, and ECM regulators (enzymes). The ECM creates space for cell migration during palatogenesis through manipulating each ECM components to appropriate levels. Local mediators, peptides, growth factors, and microRNAs work concurrently to arrange cell patterns and induce differentiation during palatogenesis.

Remodeling of the ECM during palatogenesis involves many genes and enzymes, many of which belong in the metzincin family of metalloproteinases. The ECM remains a dynamic environment through two opposing forces of building up and breaking down. Enzymes such as Matrixins (MMPs) are known to degrade ECM components, while the tissue inhibitors of metalloproteinases (TIMPs) are known to increase rigidity of the ECM by inhibiting the MMPs. ECM components in both normal development and pathological conditions may be studied through novel proteomics methods involving digesting the proteins into peptides before analyzing using mass spectroscopy.

The most popular method in cleft palate repair and reconstruction to date is one or multiple autogenous bone grafts (first generation of palatal reconstruction). Compared to other bones in the body, craniofacial bones are different in cell lineage, as they are derived from neural crest cells that later transitioned into mesenchymal cells in embryo. Bone in the body are derived from somites. Furthermore, craniofacial bones are mostly flat bones that underwent intramembranous ossification as opposed to long bones that underwent endochondral ossification, such as the femur. For this reason, flat bones such as the anterior iliac crest are often used as donor sites for cleft palate repair. Some of the biggest challenges with autogenous bone grafts re the limited bone volume and donor site morbidity. Post-surgical complications may include chronic pain of the donor site or even the loss of the graft due to inflammation, bone resorption, and the development of oronasal fistulas.

To better patient outcome, the second generation of palatal construction utilizes biomaterials and growth factors. Osteoconductive biomaterials such as the hydroxyapatite are used in conjunction with growth factors such as the bone morphogenetic protein (BMPs). However, this method and those similar to it did not prove to be significantly more effective than traditional autogenous bone grafts. The third generation of palatal reconstruction arose with the 3D cell culture techniques. Cell aggregates, spheroid, and organoids closely mimics real cell morphology, physiology, and pathology.

Tissue-engineering in palatal reconstruction may be divided into the “top-down” and the “bottom-up” approach. The “top-down” approach takes place *in vitro* and involves producing functional tissue using proliferating cells within scaffolding biomaterials. The “bottom-up” approach takes place *in vivo* and produces modular tissue units (spheroids) from adult stem cells

that are responsible for synthesizing their own ECM. Bone bioengineering research is still largely in its infancy stage, with little to no clinical data from human trials.

Many different types of stem cells may be used for palatal reconstruction, including bone-marrow stem cells (BMSCs), mesenchymal cell cells (MSCs), gingival mesenchymal stem/progenitor cells (GMSCs), adipose-derived mesenchymal cells (AMCs) and stem cell located in dental tissues: dental follicle progenitor stem cells (DFPSCs), stem cells from apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs), dental pulp stem cells (DPSCs), and stem cells from exfoliated deciduous teeth (SHEDs). Because the exfoliation of SHEDs is a natural part of development, they are much more easily obtainable than autogenous bone grafts and some other sources of stem cells. Both SHEDs and DPSCs allow cell sheets and 3D spheroid cultures, showing their promising future in many different areas of regenerative medicine. However, more research is still required before stem cell therapy becomes widely and routinely used in palatal reconstruction.

Recent advancements in biotechnology also open new possibilities in craniofacial reconstruction. Computer-aided design (CAD) is widely used in many areas of dentistry including facial reconstruction. CAD provides more accurate measurements and planning that is required to reconstruct complex structures such as the face. Three-dimensional printing made it possible to fabricate biomimetic scaffolds of various materials, to print cells directly as aggregates, or biomaterials with cells. Tissue-organ printer (ITOP) allows tissues of any size to be printed, different from the previous 3D printers that failed to provide enough structural support to print tissue that are large enough to be clinically relevant. Similarly to stem cell-based therapy, the ITOP technology still needs time in the research lab before entering the operating room.

Lastly, the muscles of the soft palate sometimes may not obtain full function after cleft palate repair due to the intrinsically limited regenerative properties of the muscles, the disrupted organization of the muscle fibers due to the abnormal insertion in a cleft palate, and scar tissue formation. Biomaterials and scaffolding may be used to deliver satellite cells to the soft palate muscles for improved regeneration. Both scaffolding and surface topography have been suggested to improve muscle fiber organization and alignment after cleft palate repair. To reduce fibrosis, signaling molecules such as decorin and nitric oxide could be used to down-regulate the activity of TGF- 1, though their roles in muscle regeneration needs to be further investigated before clinical use.

As one of the most common birth defects, cleft palate and lip has been studied extensively. Genes, growth factors, and ECM involvement with both normal and pathological palatogenesis have been studied in various animal models. Numerous syndromes involving orofacial clefting have been identified. Previous knowledge about orofacial clefting has led to better surgical outcomes and improved treatments. However, because 3D printing and bioengineering are recent developments, more research is needed before they could be used as ways to repair orofacial clefting. As 3D printing and bioengineering technology matures, clinical trials might become available in humans, an exciting new direction toward not only palatal reconstruction, but regenerative medicine in general.

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