From Bedside to Bench and Back: Advancing our Understanding of the Pathophysiology of Cleft Palate and Implications for the Future

Abstract

Objective: To provide a comprehensive understanding of the pathophysiology of cleft palate (CP) and future perspectives.

Design: Literature review.

Setting: Setting varied across studies by level of care and geographical locations.

Interventions: No interventions were performed.

Main Outcome Measure(s): Primary outcome measures were to summarize our current understanding of palatogenesis in humans and animal models, the pathophysiology of CP, and potential future treatment modalities.

Results: Animal research has provided considerable insight into the pathophysiology, molecular and cellular mechanisms of CP that have allowed for the development of novel treatment strategies. However, much work has yet to be done to connect our mouse model investigations and discoveries to CP in humans. The success of innovative strategies for tissue regeneration in mice provides promise for an exciting new avenue for improved and more targeted management of cleft care with precision medicine in patients. However, significant barriers to clinical translation remain. Among the most notable challenges include the differences in some aspects of palatogenesis and tissue repair between mice and humans, suggesting that potential therapies that have worked in animal models may not provide similar benefits to humans.

Conclusions: Increased translation of pathophysiological and tissue regeneration studies to clinical trials will bridge a wide gap in knowledge between animal models and human disease. By enhancing interaction between basic scientists and clinicians, and employing our animal

model findings of disease mechanisms in concert with what we glean in the clinic, we can generate a more targeted and improved treatment algorithm for patients with CP.

Key words: Cleft palate, animal models, pathophysiology, genetic mutations in humans, molecular mechanism

Introduction

Cleft palate (CP) is a type of orofacial cleft that presents in approximately 1 to 25 in 10,000 live births worldwide depending on geographic location[1]. The etiology of CP is complex and multifactorial, influenced both by genetics and environmental factors[2]. People born with CP suffer from disruption of a variety of essential physiological functions, including swallowing, speech, feeding, and respiration[3,4]. Consequently, they often require multiple major surgeries throughout childhood; however, even with surgical intervention, they can experience persistent comorbidities and complications, including hearing loss and postoperative fistulae, that have detrimental effects on learning and development, and often necessitate long-term monitoring and revision surgeries[5,6]. In addition, they may have persistent speech and communication deficits that can impact social development and quality of life[7].

Revision rates for cleft lip and/or palate (CLP) repair range from 9.6-57.4%[8,9], contributing to a substantial emotional and financial burden for patients and their families. Given both the relatively high prevalence of orofacial clefting overall and the high rate of reoperation associated with this condition, numerous animal model studies have been conducted to improve understanding of its underlying pathophysiology, as well as to explore future alternative treatment paradigms[10,11]. Interestingly, however, there is more knowledge about the genes implicated in mouse CP models than those confirmed in humans[12].

In this review, we summarize current knowledge of the pathophysiology behind CP and the mouse models used to recapitulate the human disease. Furthermore, we hope

to highlight research at the forefront of novel treatment developments for patients with CP. In doing so, we provide insight on how we can better translate the mechanistic knowledge gained from these studies to clinical practice and ultimately improve outcomes for patients.

1. Anatomy, embryonic development, etiology and diagnosis of cleft palate in humans

Anatomy

In order to describe the pathogenesis of cleft palate, we must first appreciate the normal anatomy and development of the hard and soft palate. The palate consists of primary and secondary portions. The primary palate consists of the triangular hard palate area anterior to the incisive foramen, including a portion of the alveolar ridge, while the secondary palate includes the rest of the hard palate (HP) and the entirety of the soft palate (SP)[13]. The HP is necessary to provide separation between the oral and nasal cavities, and it forms following the fusion of the maxilla and the palatine bones. The SP is crucial for normal speech and feeding; it closes the nasal airway during swallowing and during some speech sounds, and consists of fibromuscular tissue in the most posterior third of the roof in the mouth between the oropharynx and nasopharynx[14]. The muscular components of the human SP include the palatoglossus, palatopharyngeus, levator veli palatini, tensor veli palatini, and musculus uvulae[14]. The palatoglossus and palatopharyngeus muscles work to depress the soft palate and pull the lateral pharyngeal walls inwards, the tensor veli palatini serves to tense and elevate the soft palate and open the eustachian tube, the levator veli palatini works in conjunction with the tensor to elevate the soft palate and also opens the eustachian tube, and the musculus uvulae moves

the uvula anteriorly and superiorly[14]. With the exception of the tensor veli palatini, which is innervated by the mandibular branch of the trigeminal nerve, all other SP muscles are innervated by the vagus nerve via the pharyngeal plexus[10]. Sensory innervation to the HP and SP is provided by the nasopalatine, greater and lesser palatine nerves, which are all branches of the second (maxillary) division of the trigeminal nerve.

Embryology/palatogenesis

Development of the external face initiates with the appearance of five facial prominences: a central frontonasal prominence, the bilateral maxillary prominences, and the bilateral mandibular prominences (Figure 1). These prominences develop into the primary palate, the upper jaw, the secondary palate, and the lower jaw, respectively,[15] ~~Each facial prominence initially begins to migrate from the dorsal region of the anterior neural tube towards the facial region on day 22 of embryonic development. These prominences develop~~ from tissues within the frontonasal prominence and the first pharyngeal arch. The tissues of the frontonasal prominence give rise to the two medial nasal prominences and lateral nasal prominences, while the tissues of the first pharyngeal arch give rise to the mandibular and maxillary prominences.

The primary and secondary palates develop separately during palatogenesis, then fuse to form the definitive palate[16] (Figure 2). The primary palate forms following the merging of the maxillary and medial nasal prominences, while the secondary palate forms from two palatal prominences[16,17]. During primary palate development, there is significant growth of the maxillary prominences, leading to contact with the lateral nasal prominences and the merged medial nasal prominence. The fusion of the frontonasal and maxillary prominences allows for the development of the palate, which provides

separation of the oral and nasal cavities[16]. This relies on coordinated growth of the prominences and successful apoptosis of remaining epithelium between them[18]. Fusion of the secondary palate occurs through organized growth and apoptosis along the medial aspects of the palatal shelves[18]. Prior to assuming their final position, the palatal shelves grow inferiorly, which allows the mandible to develop anteriorly and for the tongue to depress into the mouth. The growth of the palatal shelves then switches from a vertical to a horizontal direction, in which the palatal shelves grow towards each other until palatal fusion is complete in week twelve[19]. Normal palatal fusion begins with the medial edge epithelium and subsequently progresses in both anterior and posterior directions from the initial fusion point. Different types of clefting result from failure of some of the facial primordia to fuse[17]. Generally speaking, clefts of the primary palate occur following disrupted fusion of the medial nasal prominences and maxillary prominences in the sixth week of gestation, whereas disrupted elevation or fusion of the palatal shelves between the ninth and twelfth week of gestation can result in a cleft of the secondary palate[17]. Although this difference in timing between the development of the primary and secondary palates can yield either CPO or CL/P, concomitant clefting of the lip and palate is the most common form of clefting[1].

Genetic risk factors for non-syndromic and syndromic CP

CP can present in isolation or occur alongside other malformations as part of a syndrome. Genes found to be causal in non-syndromic CP include *BMP4, FGF8, FGFR2, FOXE1, IRF6, MSX1, PDGFC, SATB2, SUMO1,* and *TBX22*[10,11]. Of these, a mouse model of CP with the corresponding genetic mutation has been developed for all but *FGF8*[12]. Environmental factors play a large role in non-syndromic CP in humans,

with risks imputed to alcohol use, advanced maternal age, drug exposures (e.g. corticosteroids), cigarette smoking, and nutritional deficiencies[20,21]. Clefting presents as part of a syndrome in around 55% of CP patients, yet less than 15% of CL/P patients are considered syndromic.[22] In a more recent epidemiological study of over 3,800 cases of CP, 54.8% of cases of reported CP were isolated without other anomalies, 18% were associated with multiple congenital anomalies, and 27.2% were in recognized conditions such as chromosomal, monogenic, and environmental syndromes[23]. It is interesting to note that the prevalence does vary by geographical region and environmental factors, with the highest rates of oral clefts occurring in Japan and the fewest in South Africa [24]. Many more genes have been associated with syndromes including CP than non-syndromic forms, and some genes implicated in non-syndromic CP can also contribute to CP as part of a syndromic phenotype. Human gene mutations implicated in syndromes with CP phenotype that have been successfully modeled to generate CP in mice include mutations in *CHD7, COL2A1, DHCR7, EFNB1, FGFR1, FGFR2, FOXC2, FOXE1, GLI3, IRF6, KCNJ2, P63 (TP63), SOX9, TBX1, TBX22, TCOF1, TGFBR1,* and *TGFBR2* (Table 1). Numerous additional human and mouse mutations can cause CP; however, the phenotypes in humans are not seen in mice or *vice versa*[10,11]. In Section 2, we discuss the syndromes that mouse models have been able to recapitulate.

Diagnosis

Due to the multiple stages during which palatogenesis can be affected, CP is phenotypically diverse and complex to classify[25]. As such, classification has evolved over the past century. Davis and Ritchie (1922), Brophy (1923), Veau (1931), Fogh-Anderson (1943), Kernahan and Stark (1958), Harkins et al. (1962), Broadbent et al. (1968), Spina

(1973), and others all provide different classifications of CP[26]. Although significant variation in classification/billing systems exists between craniofacial specialists, frequently used systems include the International Statistical Classification of Diseases and Related Health Problems (ICD-10), LAHSHAL, and Veau systems[18]. It is important to note that the ICD-10 system was designed for recording and coding purposes, not for classification[18]. As such, the ICD-10 system does not have a means of classifying clefts by their laterality and has no code for clefts of the alveolus. Veau's system identifies patients as having soft palate cleft only, soft and hard palate cleft, unilateral cleft lip and palate, or bilateral cleft lip and palate[27].

The "LAHSHAL" classification method, developed in 1989, has become a recommended system due to its comprehensiveness, global usage rate, convenience, and complementarity with the ICD-10 system[18]. LAHSHAL is a palindromic acronym that illustrates the anatomic site and severity of clefting, depending on how it is written.[15] The letters refer to the lip (L), alveolus (A), hard palate (H), and soft palate (S), with the first three letters ("LAH") referring to the right side of the mouth and the final three letters ("HAL") referring to the left side of the mouth[15]. When written in uppercase, a letter indicates a complete cleft, whereas a lowercase letter indicates an incomplete cleft[15]. For example, LAHS••• would indicate unilateral complete cleft lip, alveolus, and palate on the right side, while •••SHal would indicate an incomplete cleft lip, incomplete cleft alveolus, and complete cleft palate on the left side. Clinicians also frequently refer to clefts as either "V-shaped" (narrow) or "U-shaped" (wide) clefts; "V-shaped" clefts generally represent primary malformations, while "U-shaped" clefts are often suggestive of interfered palatal closure due to the tongue.[18]

Other frequent, mild phenotypes seen in cleft patients include a split (bifid) uvula, a blue tint to the soft palate tissue (zona pellucida), and midline furrowing of the palate. A submucosal cleft describes the phenotype when the mucous membranes of the palate remain intact, but the soft palate muscles are not fully formed. These palatal abnormalities may not interfere with essential functions so in many cases surgical repair is not indicated, and some individuals with these phenotypes may remain undiagnosed.[28]

Prenatal diagnosis of CLP via transabdominal ultrasound performed during the second trimester of pregnancy has become increasingly common as imaging technology has improved. Upon visualization of the fetal face, prenatal ultrasound has 88% sensitivity in detecting the more outwardly visible cleft lip but 0-1.4% sensitivity in diagnosing CP[29]. However, imaging of the intact SP and uvula often yields an "equals sign" during an ultrasound, with its absence suggesting CP[30]. Recent advances, including the use of three dimensional prenatal ultrasounds and advanced software applications has allowed for improved examination of the fetal palate. This advanced technology has created new techniques to improve the diagnosis of CP. This includes the "3D-reverse face," the "flipped face," the Faure technique and "angle insonation", the "oblique face," and the "retronasal triangle" views[31,32]. The oblique view is particularly promising when the secondary palate is involved, in which 100% of cases with hard palate involvement are noticed. The ultrasound targeted fetal magnetic resonance imaging (MRI) has also shown a potential use in the diagnosis of isolated clefting of the soft and hard palate, with a 96% positive predictive value reported[32]. Finally, the surface rendered oro-palatal (SROP) view allows for visualization of the superior lip, alveolar ridge, and secondary palate. The surface-rendered representation aspect can be easier for parents to

comprehend which may allow for more effective communication with parents and the rest of the oro-facial team[33]. As prenatal testing becomes more widespread and our understanding of the genetics of CP deepens through human and animal studies, there is strong potential to further improve prenatal diagnosis in our effort to optimize care for patients with CP.

2. Molecular mechanisms of cleft palate and transgenic mouse models

In mice, the palate originates from the primary and secondary palate primordia; the former ultimately gives rise to a portion of the HP, while the latter develops into the remaining HP and SP. Palatogenesis begins at E11.5 and consists of a sequence of events ultimately resulting in palatal fusion. First, there is palatal shelf growth, followed by elevation and fusion of the shelves at the midline, which is largely dependent on palatal shelf contact and adhesion. Lastly, there is ablation of the midline epithelial seam, allowing for complete fusion by E15.5-16.5[34] (Figure 3).

Medial edge epithelial cells and their role during palatal fusion

Medial edge epithelial (MEE) cells have been identified as integral to palatogenesis, as their apoptosis and/or migration ultimately allows for completion of palatal fusion[35–37]. The MEE is multilayered, composed of periderm and basal epithelial cells, which in combination with the mesenchyme ultimately constitute the palatal shelves. Interestingly, the timing of MEE apoptosis has been demonstrated to be contingent upon the location of MEEs and to be instigated via contact with the anterior palatal shelf[38]. Certain signaling molecules, including TGF-B and retinoic acid, dictate this process[34]. In particular, of TGFB3 expression occurs in medial edge epithelial cells

prior to palate shelf adhesion and subsequently promotes palatal shelf fusion. Accordingly, while able to demonstrate proper orientation and growth, Tgfb3$^{-/-}$ embryos exhibit poor palate shelf adherence and persistence of a peridermal layer[39–41]. Further, mouse model investigation has identified that activation of *Irf6* in the basal layer of the MEE is indispensable for subsequent Tgfb3-mediated palatal fusion[42]. In addition, downstream activation of IRF6 in the TGFB-Smad4 signaling pathway leads to abruption of p63-mediated proliferation of midline epithelial seam cells and subsequently allows their breakdown[41,42]. During this palatal shelf fusion process, epithelial cell extrusion and epithelial displacement further allows for a more continuous fusion to occur[43].

Kim et al. (2015) used mouse palatal explants to demonstrate the role of MEE cells during fusion. This study tracked epithelial cells using *K14-Cre* mediated lineage tracing and followed their movements to the oral or nasal direction at the time of fusion. In doing so, live imaging revealed that these cells generate cellular bridges to form a transient epithelial seam between juxtaposing shelves[44]. Peridermal cells were also identified within the seam[43]. Thus, the role of EMT (epithelial to mesenchyme transition) in palatal fusion remains to be further investigated.

A more recent study by this same research group provided additional clarity on MEE migration patterns and behaviors. Again using live imaging, they found that in migratory epithelial cells, there was co-expression of both basal epithelial and periderm markers. Thus, these experiments revealed intermediate differentiation states of these cells during migration toward the oral and nasal surfaces, subsequently suggesting that migration and differentiation occur simultaneously[44].

Cranial neural crest cells and their contribution to palatogenesis (bone and muscle development)

Cranial neural crest cells (CNCCs) have been shown to be integral to craniofacial development, serving as the precursors to a multitude of different cell types. In contrast with the hard tissue of the body, which are mesoderm-derived, hard tissues of the skull including frontal bone, facial bones and cartilage, and dental tissues (except enamel), are derived from these diverse CNCCs. It is important to recognize that while many of the craniofacial hard tissues are derived from CNCCs, others, such as the parietal, occipital and other bones at the cranial base are mesoderm derived. Induction of precursor CNCCs is followed by epithelial-to-mesenchymal transition (EMT) and then their migration along the side of the neural tube as these CNCCs undergo subsequent proliferation and differentiation influenced by the surrounding environmental signals to give rise to a diverse array of tissues during craniofacial development[34].

Specifically, in regard to craniofacial development, CNCCs migrate ventrolaterally and, adjacent to the endoderm, mesoderm, and ectoderm, the CNCCs play an important role in forming the frontonasal prominence and the pharyngeal arches. CNCCs originating from rostral rhombomeres of the hindbrain region ultimately give rise to the maxillary and mandibular prominences[45–48]. These sequential events involve regulation by growth factors and transcription factors, ultimately leading to CNCC differentiation into bone, cartilage, tooth, and cranial nerve ganglia. Importantly, research has demonstrated that CNCCs remain undifferentiated while migrating, instead only

differentiating once they have reached their final destination and receive tissue-specific signals that provide cues to allow for proper cellular differentiation, and forming specific organs with the proper shape and size[49–52].

Palatogenesis is highly contingent on CNCCs, as the various palatal entities are comprised of a combination of CNC-derived ecto-mesenchyme and pharyngeal ectoderm. Using the *Wnt1-Cre/R26R*, mouse model, studies have provided crucial information in regard to the movement and activity of CNCCs throughout embryogenesis[53,54]. Numerous mutations that lead to CNCC-derived palatal mesenchyme dysfunction and dysregulation have been associated with failure of palate elevation and subsequent cleft palate development in mice, including those in *Pax9, Pitx1,* and *Osr2*[55–57]. Similarly, mutations and/or inactivations of *Msx1, Lhx8*, and *Tgfbr2* in CNCC cells beget a failure of palatal fusion following elevation[53,58–60]. These studies collectively highlight the central role of CNCCs in palate development.

Genetic and epigenetic regulation of palatogenesis

Studies have demonstrated the importance of epigenetic regulation in palatogenesis and palate mesenchyme formation. Processes such as abnormal DNA methylation in mouse models can lead to orofacial clefting[61,62]. DNA methylation is vital to the establishment and maintenance of chromatin structures and subsequently to transcription of genes throughout cellular developmental processes[63]. Thus, interruption of appropriate DNA methylation in genomic regions that influence palatogenesis can lead to disruption of cellular growth, proliferation, tissue formation, and subsequent development of a CP phenotype. An example of this is with A/strain mice, in which if a

promotor region is hypomethylated, this confers abnormal gene transcription that precludes its usual function[64]. As within gene inheritance, studies have demonstrated that epigenetic states dictated by distinct DNA methylation can also be passed onto offspring and thus CP abnormalities can be conserved across generations [65,66].

It has been shown that loss of *Kdmb6b*, an H3K27me3 demethylase, in CNCCs leads to complete cleft palate and soft palate muscle defects as well. The mechanism behind this phenotype involves activation of P53 and subsequent defects in cell proliferation and differentiation[67]. Similarly, both methyltransferase *Kmt2a* and demethylase *Kdm6a* have been found to be integral to both neural crest-mediated craniofacial development and cardiac development[68,69]. Further, ablation of *Arid1a*, a subunit of the SWI/SNF chromatin remodeling complex, in CNCCs has been shown to cause craniofacial abnormalities and lethality [70].

In humans, select case studies have identified the role of epigenetic regulation in the development of cleft palate. For example, mutations in *SATB2*, a chromatin-remodeling factor, as well as alternations in DNA methylation, can result in failure of palatogenesis and subsequent cleft palate development[71–73]. In addition, distinct DNA methylation profiles have been identified in various types of orofacial clefts, both in genes known to cause CP and also in novel regions[74]. Better understanding of epigenetics and biomarkers in CP subtypes will help better elucidate its complex and multifaceted etiology.

Transgenic mouse models

CP in mice is similar to that in humans, allowing mouse models to be harnessed in order to study both the pathophysiology of the disease and potential curative treatments[34]. Below we highlight certain genes and pathways that have been used to recapitulate the human phenotype in mouse models.

Fibroblast growth factor receptor (FGF) signaling pathway

Mutations to FGF receptors, *FGFR1* and *FGFR2*, are implicated in a host of craniofacial syndromes associated with orofacial clefting[75]. In humans, mutations of *FGFR1* can lead to Kallman syndrome, characterized by CLP, anosmia, and hypogonadotropic hypogonadism[76,77], or to non-syndromic CLP or CP[78,79]. *FGFR2* mutations similarly can be found in both non-syndromic CLP or CP[78,79] or as part of Apert syndrome, which is characterized by craniosynostosis, syndactyly, and incomplete penetrance of CP[80]. Consistent with human studies, in mice, mutation of *Fgfr1* leads to CP due to failure of palatal shelf elevation, as well as craniofacial bone defects[81]. Two mouse models with conditional inactivation of *Fgfr2* in mesenchymal cells have been generated to successfully recapitulate the CP phenotype: *Fgfr2b*[60,82] and *Fgfr2c* (gain-of-function)[83]. In addition to CP, the *Fgfr2b* mutants demonstrate altered proliferation in the palatal shelves; tooth, salivary gland, limb, skin, and pituitary anomalies; fusion of joints; trachea and lung defects; absence of incisors; and a thin mandible. Given that similar skeletal defects appear in *Msx1*$^{-/-}$ mutants[58], it has been suggested that Fgf signaling may be highly connected to the critical role that Msx1 plays in epithelial-mesenchymal interactions during craniofacial skeletal development[60,82]. The *Fgfr2c* gain-of-function mouse was developed to generate an animal model of Crouzon and Pfeiffer syndromes. While these syndromes are not known to cause CP, they cause a variety of craniofacial

anomalies[84]. Mice harboring this mutation have an increase of osteoblast progenitor cells but no change in number of osteoclasts, identifying *Fgfr2*'s role in regulating craniofacial osteogenesis[83].

Transforming growth factor β (TGF-β) signaling pathways

Elevated TGF-β signaling has been implicated in craniofacial defects including Loeys-Dietz syndrome[85]. Mutation of *TGFBR1* or *TBFBR2* can lead to cardiovascular, skeletal, craniofacial, and cognitive developmental defects, as well as bifid uvula and/or CP[86]. In mice, numerous models have been generated to recapitulate these defects and demonstrate the importance of *Tgfbr1* and *Tgfbr2* in both neural crest and epithelial cells in palatal shelf development, including mice with conditional deletion of *Tgfbr1* in epithelial tissue, neural crest tissues[87], and *Tgfb3*-expressing cells[88]. Similarly, mutant mice with conditional deletion of *Tgfbr2* in neural crest cells[53] and palatal epithelial cells[89] have also been generated. These models revealed that loss of *Tgfbr2* in epithelial cells leads to the failure of primary palate fusion with the secondary palate[89]. Loss of *Tgfbr2* in cranial neural crest cells leads to a cell proliferation defect, rather than a migration defect[53]. Furthermore, loss of *Tgfbr2* leads to elevated TGF-β2 expression, activation of noncanonical TGF-β signaling, and craniofacial malformations, making this animal model highly relevant for human disease[41]. In contrast, deleting the TGF-β type I receptor gene *Tgfbr1* both in embryonic ectodermal and neural crest lineages leads to a more severe facial cleft than is seen in *Tgfbr2* mutants, providing evidence that *Tgfbr1*'s influence on craniofacial morphogenesis may be independent of its usual binding with the TGF-β type II receptor[87].

Hedgehog (Hh) signaling pathway

Pallister-Hall Syndrome is a lethal condition caused by mutation in *GLI3*, a proposed inhibitory component of the sonic hedgehog pathway (Shh)[90]. Patients can have a range of pathologies, including hypothalamic hamartoma, bifid epiglottis, polydactyly, anal atresia, and oral anomalies, including CP[91]. *Gli3*^{xtJ} mutant mice exhibit sternal and craniofacial skeletal anomalies as well as delayed elevation of the palatal shelves. Similar to the human condition, loss of *Gli3* is predicted to cause defective Indian hedgehog (Ihh) and/or Shh signaling, subsequently impairing skeletal development[92]. Accordingly, impaired Shh signaling in the palatal shelf epithelium has also been shown to generate mutants with a wide CP.[93] *Smo* has been demonstrated to be expressed widely in the palatal mesenchyme during development, particularly at E13.5-14. Ablation of *Smo* in neural crest cells leads to CP disruption of growth and morphogenesis of the palatal shelves preceding elevation and fusion. As a consequence, there is severe interruption of the overall craniofacial skeleton, and tongue as well as lower incisor agenesis[94]. Ihh signaling is also implicated in palatogenesis as it has been found to be expressed widely in the developing palatal bones, with *Ihh* mutants exhibiting impaired secondary palate ossification[95].

Wnt/β-catenin signaling pathway

β-catenin is an essential component of the Wnt signaling pathways. Canonical Wnt/β-catenin signaling is integral to craniofacial development[96], particularly through its effects on neural crest cell differentiation and survival[97]. Inactivation of a key co-receptor of the Wnt/β-catenin signaling pathway, *Lrp6*, elicits severe craniofacial anomalies,

including CP and CL/P, as a consequence of defective fusion of both the palate and the upper and lower lips[98]. Inhibition of *Lrp6*-mediated Wnt signaling pathways further illustrates its role in normal lip development. *Lrp6* mutants demonstrate reduced expression of *Msx1* and *Msx2*, genes critical for the proper outgrowth of orofacial primordia, particularly at the site of palatal fusion[99].

Functional significance of transcription factors and others in regulating palatogenesis

Factors involved in DNA transcription play a crucial role in palatogenesis, including those encoded by *TBX22, TBX1, FOXC2, FOXE1, IRF6, CHD7, P63, SOX9,* as discussed below. In addition, genes involved in metabolic pathways (*DHCR7),* potassium channel modulators (*KCNJ2)*, and ribosome biogenesis factors (*TCOF1*) have also been implicated in CP.

In humans, deletion of 22q11.2 including *TBX1* can lead to DiGeorge/velocardiofacial syndrome, a primary immunodeficiency syndrome with the additional features of CP, heart defects, hypocalcemia, and hypoplasia of the thymus and parathyroid glands[100]. *Tbx1*$^{-/-}$ mice similarly present with CP and developmental anomalies that resemble DiGeorge syndrome[101], providing strong evidence for *TBX1* as a key candidate gene in the etiology of this syndrome.

TBX22 mutation in humans leads to X-linked non-syndromic CP and ankyloglossia[102]; similar to humans, *Tbx22*$^{-/-}$ mice display a phenotype consisting of ankyloglossia, choanal atresia, and a submucous cleft due to decreased posterior palatal bone formation[103]. Tbx1 is expressed in the pharyngeal endoderm and pharyngeal arch muscle progenitors[104], and subsequently impacts osteogenic patterning of the palate[103].

FOXC2 mutation in humans generates a phenotype of distichiasis, lymphedema, and CP[105]. Haploinsufficient *Foxc2* mice have an abnormal lymphatic system and distichiasis[106], whereas homozygous mutants display craniofacial and skeletal abnormalities, including 100% penetrance of clefting of the secondary palate[107].

TTF-2 mutations in humans can lead to non-syndromic CLP[108] or Bamfort-Lazarus syndrome, with CP, thyroid agenesis, and choanal atresia as presenting phenotypic features[109]. Null mutation of this gene in a mouse leads to thyroid dysgenesis and CP; however, choanal atresia is absent[110]. The defunct palatogenesis seen in these mice is postulated to be due to the loss of TTF-2 expression in the craniopharyngeal endoderm.

Mutations in *IRF6* have been implicated in both syndromic (Van der Woude syndrome)[111] and non-syndromic CLP or CP[112], along with many other developmental anomalies. Various mouse models have been generated and successfully recapitulated the CP phenotype seen in the human condition, including $Irf6^{R84C/R84C}$ (missense mutation)[113], $Irf6^{+/R84C}; Sfn^{+/Er}$ compound mutants[113], and $Irf6^{gtl/gtl}$ (null allele)[114]. All three mutant models have abnormal intraoral adhesions, as well as adhesions of various other body parts. The pathophysiology underlying these Irf6-mediated defects is disrupted keratinocyte proliferation and differentiation and intraoral adhesions that prevent the palate from elevating[113,114]. The inability of the palate to elevate may be due to a primary defect in elevation or secondary to abnormal craniofacial features caused by constriction of the skin and intraoral adhesions.[113,114]

CHD7 mutations have been identified as contributing to both non-syndromic CLP[115] and CHARGE syndrome, characterized by coloboma of the iris, heart anomalies, choanal atresia, retarded growth, genitourinary problems, hypogonadism, and ear deformities[116]. Whirligig mice with *Chd7* heterozygous mutation have CP and features of CHARGE syndrome with around 35% phenotype penetrance. While these mutants have eye abnormalities, colobomas are not seen. It is postulated that the underlying cause of abnormal organ development in these mutants is secondary to abnormal epithelial-mesenchymal interactions given its widespread expression in both tissue layers[117].

P63 (TP63) mutation is present in a host of clefting syndromes, as well as non-syndromic CLP. P63 is a transcription factor of p53 with important roles in epithelial proliferation and differentiation[118]. *P63*$^{-/-}$ mice exhibit limb abnormalities, ectodermal organ anomalies, and a truncated maxilla and palate; however, the presence of a specific CP phenotype is unclear, and the mice die shortly after birth[119]. The role of P63 in the pathophysiology of craniofacial and skeletal defects is a consequence of loss of ectodermal-mesenchymal interactions.

SOX9 mutation has been implicated in Pierre Robin Sequence, which presents with retrognathia, glossoptosis, and obstructive sleep apnea[120], as well as in a severe skeletal dysmorphology syndrome known as campomelic dysplasia[121]. Chromosomal abnormalities near the *SOX9* locus lead to its dysregulation and subsequently impair the SOX9 protein's ability to coordinate craniofacial development. *Sox9*$^{+/-}$ mice exhibit CP and craniofacial abnormalities, and investigation identified that loss of *Sox9* prevents proper chondrocyte differentiation[121]. Inactivation of *Sox9* in mouse neural crest cells results in an absence of endochondral craniofacial skeletal components, CP, a short

dome-shaped skull, and a shortened mandible and snout, providing further evidence of *Sox9*'s important role in cartilage pathways and development[122]. In addition, overexpression of Sox9 can also lead to cleft palate secondary to delayed endochondral bone formation[123].

KCNJ2 mutation in humans has been found to generate a CP phenotype in some individuals[124]. Further, reduction of *KCNJ2* expression has also been identified in patients with Pierre Robin Sequence[120]. *Kir2.1*$^{-/-}$ mice, which are encoded by *KCNJ2,* have 100% penetrance of CP, with no other gross abnormalities; in contrast, heterozygous mutants never demonstrate this defect[125]. The pathophysiology of clefting in this model involves abnormal proliferation of the palatal shelf mesenchyme, as well as abnormal BMP signaling[126].

Mutation in *DHCR7* causes Smith–Lemli–Opitz syndrome (SLOS), a congenital disorder of cholesterol metabolism that presents with CP, among other abnormalities[127]. A null mutation of the mouse homolog (*Dhcr7*$^{-/-}$) recapitulates features of SLOS including CP as well as an enlarged bladder and immature lungs, leading to early death. Interestingly, the role of *Dhcr7* in abnormal palatogenesis is postulated to be secondary to abnormal folate metabolism, as the folate receptor is particularly susceptible to lipid composition alterations[128]. Accordingly, it is well established that folate deficiency is associated with increased risk of orofacial clefting[129].

TCOF1 mutation in humans leads to Treacher-Collins syndrome, characterized by mandibular and maxillary hypoplasia, ear abnormalities, and CP[130]. *TCOF1,* while not a transcription factor, is a nuclear phosphoprotein involved in ribosomal DNA gene

transcription[131]. Haploinsufficiency of *Tcof* in a mouse model recapitulates the craniofacial abnormalities, including CP[132]. The mechanism behind the craniofacial anomalies seen in *Tcof1*$^{+/-}$ mice involves abnormal cranial neural crest cell proliferation mediated through p53 stabilization[133].

While many of the aforementioned mutant mouse models recapitulate the CP phenotypes seen in humans, there are caveats to these models and myriad differences between the human and mouse conditions. Often, only homozygous mutant mice have an abnormal phenotype; in contrast, humans harboring a corresponding heterozygous mutation may present with a cleft. Two examples of this are *TBX/Tbx1* and *MSX/Msx1*[118]. It has been postulated that this phenomenon could be a result of the differences in the genetic and environmental backgrounds between mice and humans, or due to the much more rapid time frame of upper lip and primary palatogenesis in mice compared to humans. In addition, there are numerous mouse models described in which the mutants exhibit CP, while the corresponding mutations in humans do not lead to a CP phenotype, as with *FGFR3, PVRL2, MSX2,* and *ESR1*, among others. Finally, there is a paucity of data on environmental factors that affect palatogenesis, and how these may interplay with the genetic aberrations in regulating palatogenesis still needs further investigation. Clearly, animal models have significantly enhanced our understanding of the molecular and cellular mechanisms of CP development. It is crucial to enhance the connection between basic scientific studies of CP and their clinical implications. Our effort in bridging the gap between basic science and clinical studies will pay dividends towards improved understanding and potential innovative treatment for CP.

3. Tissue engineering and clinical translation in treating cleft palate

While much animal research has been conducted to reveal the key mechanisms and pathways involved in the pathogenesis of CP, there is still a large gap in the translation of this knowledge into clinical practice. A handful of pioneering studies, however, have shown promise for bridging this gap and improving care for CP patients.

Studies using a *Wnt1-Cre-Tgfbr2*$^{fl/fl}$ mouse model have been conducted that provide critical information in regard to the role of TGF-β signaling in palate formation. Iwata et al. (2012) demonstrated that TGFβ2 activates a SMAD-independent TGF-β signaling pathway in the absence of *Tgfbr2*, which adversely impacts the proliferation of cranial neural crest cells during palatogenesis. With this knowledge, they treated *Wnt1-Cre-Tgfbr2*$^{fl/fl}$ palatal explants with a TGFβ2 neutralizing antibody, which restored palatal mesenchymal cell proliferation[134]. These results were corroborated by further genetic rescue experiments demonstrating that the majority of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre*;*Tgfb2*$^{+/-}$ mice with reduced TGFβ2 ligand expression levels had normal palates. The findings of this study thus not only highlight the importance of TGF-β signaling in craniofacial development but also demonstrate the promise of TGF-β modulation as a potential novel preventive therapy for craniofacial malformations[85].

A second study using mice with a deletion of *Tgfbr2* in neural crest cells demonstrated that these *Tgfbr2* mutants have abnormal lipid accumulation in palatal mesenchymal cells and subsequently reduced lipolysis. Treatment with p38 mitogen-activated protein kinase (MAPK) inhibitor or a clinically-approved drug known to inhibit p38-MAPK successfully restored normal lipid metabolism, cell proliferation of palatal mesenchymal cells, and ultimately rescued palatal development in 44% of treated *Tgfbr2* mutant mice[135].

Core binding factor B (Cbfb) is a cofactor of Runx1, a gene well-established as integral for anterior palatal fusion. Sarper et al. used *Cbfb* mutants to demonstrate that Cbfb is integral to palatogenesis as its mutation leads to failed fusion of the anterior epithelium. These mutants have abnormal TGFB3 expression and Stat3 phosphorylation. Delivery of TGFB3 *in vitro* has been demonstrated to rescue normal palate fusion. In addition, *in vivo* delivery of folic acid in *Cbfb* mutants can further rescue an abnormal CP phenotype via activation of the Stat3 pathway at a rate of 67%[136].

Using a *Pax9*$^{-/-}$ mouse model, Jia et al. employed various small-molecule Wnt agonists (Dickkopf (Dkk) inhibitors) to rescue the CP phenotype. Intravenous delivery of these molecules into pregnant *Pax9*$^{-/-}$ mice allowed for restoration of cell proliferation and osteogenesis *in utero*. Accordingly, the palatal shelves exhibited appropriate growth and fusion, providing evidence that upstream manipulation of the Pax-dependent Wnt signaling pathway can potentially rescue abnormal palatogenesis[137].

Similarly, Li et al. used small molecular inhibition of Dkk activity *in utero* in *Pax9* embryos to partially rescue deficient secondary palatogenesis typically seen in these mutants. Moreover, they demonstrated that inactivation of *Wise*, which is involved in palate development by encoding a canonical Wnt antagonist, can lead to the restoration of normal palate morphogenesis in *Pax9* mutants. Lastly, this study identified that this rescue is partially mediated by hyaluronic acid, which has been shown to be indispensable in palate shelf elevation. While *Pax9* mutants exhibited impaired elevation and reorientation of the palatal shelves secondary to decreased hyaluronic acid, inactivation of *Wise* in these mutants allowed for proper palatal elevation and

reorientation in 80% of mice, coupled with restored accumulation of mesenchymal hyaluronic acid.[138]

Msx1 is essential for the expression of Bmp4, Bmp2, and Shh during palatogenesis. In particular, *Msx1*$^{-/-}$ mice exhibit cell proliferation defects. Zhang et al. used both *in vivo* and *in vitro* analysis to investigate and subsequently rescue these defects. In *Msx1*-deficient mice, driving Bmp4 expression with the *Msx1* promoter rescued the CP phenotype and restored cell proliferation along with Shh and Bmp2 expression[139].

Given these exciting findings, one key question remains: how can these therapies be translated into the clinic? One study directly translated mouse model findings to successfully prenatally rescue the development of X-linked hypohidrotic ectodermal dysplasia (XLHED), caused by a loss-of-function mutation in *EDA*. Patients with XLHED typically present with abnormally developed ectodermal structures, including sweat glands, respiratory glands, teeth, hair, and skin[141]. In a mouse model, *in utero* delivery of a recombinant protein containing the binding site for EDA to the amniotic fluid led to the normal development of *Eda*-deficient mice[142]. This discovery was applied to three individuals prenatally diagnosed with XLHED. The same protein administered in the mouse model was delivered to the twins intra-amniotically and successfully and completely rescued the twins from the condition[143].

Precision therapies for diseases with predictable Mendelian inheritance, including those that replace enzymes and proteins or influence the expression of genes implicated in disease pathogenesis, have demonstrated success in mitigating disease development.

Conditions that have been proven amenable to this technique include cystic fibrosis, spinal muscular atrophy, and lysosomal storage disorders[144]. However, unlike these diseases, CP exhibits significant genetic heterogeneity and many of the inheritance patterns and disease-causing genes remain unknown, presenting a substantial barrier to therapeutic modulation *in utero*[145]. Further, many of the genes implicated in CP are expressed in many tissues, and thus investigation into methods for precise targeting of therapy is warranted. In addition, given the aforementioned challenges of prenatal detection of CP, it is imperative that we establish improved methods of diagnosis to allow for appropriate prenatal counseling and possible therapeutic intervention based on reliable clinical evidence.

Tissue regeneration has also been explored as a modality to improve treatment paradigms. Recently, bone regeneration has been successfully demonstrated through innovative combinations of scaffolds with various seeded cell populations, both in humans and in animal models[146–150]. However, even with successful surgical closure of the CP defect, there can be persistent functional impairment due to improper muscle attachments, insertions, and muscle atrophy[151]. Further, patients with CP may have a smaller population of muscle stem cells (i.e., satellite cells) and those cells may have compromised function, precluding proper muscle regeneration. Additionally, patients can experience muscle fibrosis. While there have been ample studies on strategies to improve bone regeneration, there is comparatively less research on muscle regeneration[152,153]. A select number of biological and synthetic scaffolds have been engineered to allow for muscle regeneration[154–156], but these have yet to be investigated in the context of palatal myogenesis. It has been suggested that a cranial neural crest cell-like niche would be

critical to support proper muscle regeneration[10]. Factors secreted by cranial neural crest-derived mesenchymal cells may be integral to allow for myogenic precursor migration, proliferation, and differentiation in tissue engineering-based and scaffold-mediated cleft repair[134,153,157].

4. Conclusion and future directions

Much work has yet to be done to connect our mouse model investigations and discoveries to the human condition of CP. The success of innovative strategies for tissue regeneration and precision medicine in mice provides promise for an exciting new avenue for improved and more targeted management of cleft care in patients. However, significant barriers to clinical translation remain. Among the most notable challenges include the differences in some aspects of palatogenesis and tissue repair between mice and humans, suggesting that potential therapies that have worked in animal models may not provide the same benefit to humans[151]. In addition, there are concerns related to the timing and safety of *in utero* manipulation of the developing palate, as well as the difficulty of prenatal diagnosis[145].

Epidemiologic factors are also critical to take into consideration when paving the way for the future of cleft repair. Beyond the vast genetic heterogeneity presented in this review, other variables further individualize and complicate treatment. The prevalence of CP varies greatly across the world, with the highest rate of 25.31 per 10,000 live births in British Columbia to the lowest rate of 1.35 per 10,000 live births in Cuba[1]. Further, there is a higher prevalence of clefting in females than in males, and there is variation across ethnic groups with the highest rates in Asians and the lowest in Africans[158]. These

disparities are postulated to involve differences in hormones and facial structure. In addition, maternal factors such as smoking, alcohol consumption, diabetes, corticosteroid use, and obesity increase the risk of having a child with CP[1]. Given these many contributing factors to both the etiology and pathogenesis of CP, efforts should be taken to understand the complete patient and maternal history when developing a treatment strategy. In summary, increased translation of pathophysiological and tissue regeneration studies to clinical trials will bridge a wide gap in knowledge between animal models and human disease. By enhancing interaction between basic scientists and clinicians, and employing our animal model findings of disease mechanisms in concert with what we glean in the clinic, we can generate a more targeted and improved treatment algorithm for patients with CP.

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Figure legends

Figure 1. Schematic drawing representing the development of pharyngeal arches and craniofacial complex. **A** and **B** show the development of pharyngeal arches and tissue components within each pharyngeal arch in four- or five weeks old embryo. **C-F** show the development of the frontonasal, bilateral maxillary, and bilateral mandibular prominences (frontal view) into the upper jaw, and lower jaw, respectively. **G-J** show that development of the primary palate occurs as a consequence of the fusion of the paired medial nasal prominences, forming the intermaxillary segment (axial view). Simultaneously, the secondary palatine processes extend medially from the maxillary prominences, completing palatal shelf fusion.

Figure 2. Schematic representation of human and mouse cleft palate phenotypes. **A** and **D** demonstrate a normal palate, **B** and **E** demonstrate a complete cleft of the hard and soft palate, and **C** and **F** demonstrate a cleft of the soft palate only.

Figure 3. Normal palatogenesis in a mouse. **A** depicts the primordial palate, **B** vertical palatal shelf growth, **C** palatal elevation, **D** palatal fusion, and **E** final palate phenotype.