Chapter 7

A Review of Orofacial Clefting and Current Genetic Mouse Models

Aram J. Keteyian and Yuji Mishina

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67052

Abstract

The prevalence of orofacial clefts (OFCs) is nearly 10.2 per 10,000 births in the United States and 9.9 per 10,000 births worldwide. OFCs occur as a result of a break (nonfusion) of orofacial structures during development. This can occur due to a variety of reasons;prenatal exposure to many drugs and environmental factors as well as genetic factors which are implicated in the development of OFCs. While approximately 15 types of clefts have been identified, there are at least four distinct classifications of OFCs. These include complete cleft palate with cleft lip; cleft of the anterior palate, which may/may not involve cleft lip; cleft of the posterior palate; and submucosal cleft. A number of candidate genes have been identified, including transforming growth factor beta (TGFβ) and homeobox genes (e.g., *MSX1*), among many others. What follows is a review of mouse models currently used in research and the classification of their overall contribution to known OFCs.

Keywords: orofacial, cleft lip, cleft palate, genomic, genetics, TGFβ, MSX1, knockout mice, craniofacial, molecular, palatogenesis

1. Introduction

The focus of this chapter is to review a comprehensive list of the genes with known involvement in generating cleft lip with (or without) cleft palate (CL/P) or cleft palate (CP) in mice. Additionally, the associated knockout (KO) and conditional knockout (cKO) models are discussed. Most of the research models currently in use focus on complete CP, and thus not as much is known of the other CP phenotypes. In particular, identifying specific risk genes for CL/P is made simpler when genomic sequencing is done, and clefting associated with syndromes (syndromic) has identified single genetic loci that are involved with abnormalities in palatogenesis. Current mouse models involve a somewhat surprisingly vast array of genes, however, including *Wnt*, *Msx1*/2, *Tbx*, *Pax9*, *Irf6*, *Tgfb*, and *Fgf*. Further elucidation and

categorization of these gene families and their associated defects—whether syndromic or non-syndromic—can aid us in further clarifying the molecular mechanisms underlying orofacial clefting and potentially lead us to targeted, more efficient treatments.

We currently utilize four distinct classifications for OFCs: complete cleft palate with cleft lip; cleft of the anterior palate, which may/may not involve cleft lip; cleft of the posterior palate; and submucosal cleft. Subdivided among these four classifications of OFCs are six categories of developmental defects that have been shown to result in cleft palate in KO or cKO mice. The numerous variants of CL/P can generally be found to fit within one of the following categories: [1]

1. Palatal shelf formation failure
2. Abnormal fusion of palatal shelves
3. Delayed/failed elevation of the palatal shelves
4. Failure of palatal shelf development post-elevation
5. Persistence of medial-edge epithelial cells
6. Secondary defect

Each of the known KO/cKO mice mentioned is bred such that the gene missing is one already known to play a role in the development of CL/P. Implicit within these categories are the KO genes known to lead to each particular type of defect, each of which will be outlined as we move through this chapter.

As we look into the future, OFCs need to be classified with more definitive nomenclature. Currently, we use arbitrary terms to define very broadly into which category these congenital malformations fall, i.e., syndromic versus non-syndromic. As studies are broadened to include a wider array of genetic variants and their regulatory regions, more risk genes for CL/P and CP will surely be identified. As a result, more specific phenotypic classifications will emerge as well. The etiology of OFCs is complex, and the presentation is wide ranging; it is important that we continue to use precise genetic mouse models in order to carefully define a given phenotype before reclassifying human cases. The models mentioned in this chapter and those developed in the future are critical to a more sophisticated understanding of OFC anomalies and etiologic variants. Their development and utilization will ideally lead to a greater breadth and depth of treatment intervention options for patients.

2. Current mouse models utilized for elucidation of molecular mechanisms involved in orofacial clefting

As alluded to previously, a great breadth of genes plays critical roles in palatogenesis. Upon further analysis, a subset of gene families and signaling pathways have emerged as containing the most significant molecules related to normal development of the palate. Of note are the following: transforming growth factor beta (TGFβ), hedgehog, Wnt, fibroblast growth factor (FGF), and the mitogen-activated protein kinase (MAPK) signaling pathway. Each signaling pathway has an expansive list of genes with known involvement in palatogenesis (**Table 1**).

http://dx.doi.org/10.5772/67052

Gene	Syndromic/non-syndromic	Orofacial phenotype
Acvr1/Alk2	Submucosal cleft/fibrodysplasia ossificans progressiva	Und
Acvr2a	Und	Und
Akap8/Akap95	Und	Und
Alx1	Frontonasal dysplasia 3	CL/P
Alx3	Frontonasal dysplasia 1	CL/P
Alx4	Frontonasal dysplasia 2, parietal foramina 2, craniosynostosis 5	Cleft alae nasi
Anp32b	Und	Und
Apaf1	Und	Und
Arid5	Und	Und
Asxl1	Bohring-Opitz syndrome; myelodysplastic syndrome, somatic	CL/P
B9d1	Meckel syndrome 9	Und
Barx1	Und	Und
Bmp4	Microphthalmia, syndromic 6	CL/P
Bmp7	Und	Und
Bmpr1a/Alk3	Juvenile polyposis syndrome	CP
Cask	FG syndrome 4, mental retardation, and microcephaly with pontine and cerebellar hypoplasia	CL/P
Cdc42	Und	CL/P
Cdkn1c/p57kip2	Beckwith-Wiedemann syndrome, IMAGe syndrome	CL/P
Ceacam1	Und	Und
Chd7	CHARGE syndrome	CL/P
Chrd	Und	CL
Chuk/Ikk1/Tcf16	Cocoon syndrome	Und
Cited2	Atrial septal defect 8, ventricular septal defect 2	Und
Col2a1	Achondrogenesis, type II; Stickler syndrome, type I; Kniest dysplasia	CL/P
Crebbp/Cbp	Rubinstein-Taybi syndrome	Und
Crk	Und	Und
Ctgf	Und	Und
Ctnnb1	Mental retardation, autosomal dominant 19	Und
Cyp26B1	Craniosynostosis with radiohumeral fusions and other skeletal and craniofacial anomalies	Und
Cyp51	Und	Und
Dhcr7	Smith-Lemli-Opitz syndrome	CL/P

Gene	Syndromic/non-syndromic	Orofacial phenotype
Dhrs3	Und	Und
Dicer1	Rhabdomyosarcoma, embryonal, 2; goiter, multinodular 1; pleuropulmonary blastoma	Und
Dlg1/Dlgh/Sap97	Und	Und
Dlx1	Und	Und
Dlx2	Und	Und
Dlx5	Split-hand/foot malformation 1 with sensorineural hearing loss	CL/P
Dph1/Ovca1	Und	Und
Edn1	Auriculocondylar syndrome 3	CL/P
Efna5	Und	Und
Efnb1	Craniofrontonasal dysplasia	CL/P
Efnb2	Und	Und
Egfr	Und	Und
Eya1	Branchiootic syndrome 1; branchiootorenal syndrome 1, with or without cataracts; anterior segment anomalies with or without cataract	CL/P
Fgf10	Aplasia of lachrymal and salivary glands	Und
Fgf18	Und	Und
Fgf9	Und	Und
Fgfr1	Non-syndromic cleft lip/palate, Hartsfield syndrome, hypogonadotropic hypogonadism 2, Pfeiffer syndrome	CL/P
Fgfr2	Apert Syndrome	CL/P
Foxc2/Mfh1	Lymphedema-distichiasis syndrome	CL/P
Foxd3	Und	Und
Foxe1/Titf2/Fkhl15	Bamforth-Lazarus syndrome	CL/P
Foxf2	Und	Und
Fst	Und	Und
Fuz	Neural tube defects	Und
Fzd2	Und	Und
Gab1	Und	Und
Gabrb3	Epilepsy, childhood absence, susceptibility to, 5	CL/P
Gad/Gad67	Cerebral palsy, spastic quadriplegic, 1	CL/P
Gbr2	Und	Und
Gbx2	Und	Und
Gdf11/Bmp11	Und	Und

Gene	Syndromic/non-syndromic	Orofacial phenotype
Glce	Und	Und
Glg1	Und	Und
Gli2	Culler-Jones syndrome, holoprosencephaly-9	CL/P
Gli3	Greig cephalopolysyndactyly	CL/P
Gpr124	Und	Und
Grb2	Und	Und
Gsc	Short stature, auditory canal atresia, mandibular hypoplasia, skeletal abnormalities	Und
Gsk3b	Und	Und
Hand2/dHand	Und	Und
Hic1	Und	Und
Hoxa2	Microtia with or without hearing impairment	Und
Hs2st1	Und	Und
Hspb11/Ift25	Und	Und
Hspg2	Dyssegmental dysplasia, Schwartz-Jampel syndrome, type 1	Und
Ilk	Und	Und
Impad1/Jaws	Chondrodysplasia with joint dislocations, GRAPP type	CL/P
Inhba	Und	Und
Inpp5e	Mental retardation, truncal obesity, retinal dystrophy, and micropenis	Und
Irf6	Van der Woude syndrome, orofacial cleft 6, popliteal pterygium syndrome 1	CL/P
Itgb1	Und	Und
Itgb8	Und	Und
Jag1	Alagille syndrome	Und
Jag2	Und	Und
Jmjd6/Ptdsr	Und	Und
Kat6a/Moz/Myst3	Und	Und
Kcnj2	Andersen syndrome, atrial fibrillation, familial, 9; short QT syndrome 3	CL/P
Kif3a	Und	Und
Lhx7	Und	Und
Lhx8	Und	Und
Lrp6	Und	Und

Gene	Syndromic/non-syndromic	Orofacial phenotype
Luzp1	Und	Und
Map3k7/Tak1	Und	Und
Mef2c	Chromosome 5q14.3 deletion syndrome, mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations	Und
Meox2	Und	Und
Mn1	Meningioma	Und
Mnt	Und	Und
Msx1	Ectodermal dysplasia 3, Witkop-type Orofacial cleft 5	CL/P
Msx2	Craniosynostosis, type 2; parietal foramina 1, parietal foramina with cleidocranial dysplasia	CL/P
Nabp2/Obfc2b/hSSB1	Und	Und
Nprl3	Und	Und
Ofd1	Joubert syndrome 10, oral-facial-digital syndrome I, Simpson-Golabi-Behmel syndrome, type 2	CL/P
Osr2	Und	CL/P
Pak1ip1	Und	Und
Pax9	Tooth agenesis, selective, 3	Und
Pbx1	Leukemia, acute pre-B-cell	Und
Pdgfc	Und	CL/P
Pdgfra	Gastrointestinal stromal tumor, somatic	CL/P
Pds5a	Und	Und
Pdss2	Coenzyme Q10 deficiency, primary, 3	Und
Phc1/Rae28	Und	Und
Piga	Multiple congenital anomalies-hypotonia-seizures syndrome 2; paroxysmal nocturnal hemoglobinuria, somatic	Und
Pitx1	Clubfoot, congenital, with or without deficiency of long bones and/or mirror-image polydactyly, Liebenberg syndrome	CL/P
Pitx2	Axenfeld-Rieger syndrome, type 1; iridogoniodysgenesis, type 2; Peters anomaly	Und
Pkdcc/Vlk	Und	Und
Pnn	Und	Und
Prdm16	Cardiomyopathy, dilated, 1LL; left ventricular noncompaction 8	
Prickle1	Epilepsy, progressive myoclonic	Und
Prrx1/Prx1/Mhox	Agnathia-otocephaly complex	CL/P
Ptch1/Ptc1	Basal cell nevus syndrome (Gorlin syndrome)	CL/P

Gene	Syndromic/non-syndromic	Orofacial phenotype
Pygo2	Und	Und
Rad23b	Und	Und
Rax	Microphthalmia, isolated 3	Und
Recql4	Baller-Gerold syndrome, RAPADILINO syndrome, Rothmund-Thomson syndrome	CL/P
Ror2	Robinow syndrome, autosomal recessive	CL/P
Rspo2	Und	Und
Runx2	Cleidocranial dysplasia	CL/P
Ryk	Und	Und
Ryr1	Central core disease, King-Denborough syndrome, minicore myopathy with external ophthalmoplegia	Und
Sall3	Und	Und
Satb2	Glass syndrome	CL/P
Sc5d/Sc5dl	Und	Und
Schip1	Und	Und
Sdccag8	Bardet-Biedl syndrome 16, Senior-Loken syndrome 7	Und
Serpinh/Hsp47	Osteogenesis imperfecta, type X	Und
Shh	Holoprosencephaly-3	CL/P
Shox2	Und	Und
Sim2	Und	Und
Slc32a1/Viaat	Und	Und
Smad4	Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome	Und
Smad7	Und	Und
Smo/Smoh	Basal cell carcinoma, somatic	Und
Smoc	Microphthalmia with limb abnormalities	CL/P
Snai2	Piebaldism	Und
Sox11	Mental retardation, autosomal dominant, 27	Und
Sox5	Und	Und
Sox9	Acampomelic campomelic dysplasia	CL/P
Sp8	Und	Und
Spry1	Und	Und
Spry2	Und	Und
Sumo1	Orofacial cleft 10	CL/P
Tbx1	DiGeorge syndrome	CL/P

Gene	Syndromic/non-syndromic	Orofacial phenotype
Tbx2	Und	Und
Tbx22	Cleft palate with ankyloglossia	CL/P
Tcof1	Treacher-Collins syndrome	CL/P
Tctn2	Meckel syndrome 8	CL/P
Tgfb2	Loeys-Dietz syndrome, type 4	CL/P
Tgfb3	Arrhythmogenic right ventricular dysplasia 1	CL/P
Tgfbr1/Alk5	Loeys-Dietz syndrome, type 1	CL/P
Tgfbr2	Loeys-Dietz syndrome, type 2	CL/P
Trp63/Tp63	Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome 3; orofacial cleft 8, Hay-Wells syndrome, limb-mammary syndrome	CL/P
Tshz1	Aural atresia, congenital	Und
Ugdh	Und	Und
Vax1	Microphthalmia, syndromic 11	CL/P
Vegfa	Und	Und
Wdpcp	Und	Und
Whsc1	Und	Und
Wls/Gpr177	Und	Und
Wnt5a	Robinow syndrome, autosomal dominant	CL/P
Wnt9b	Und	Und
Zeb1	Corneal dystrophy	Und
Zic3	Congenital heart defects, non-syndromic; heterotaxy, visceral, 1; VACTERL association	CL/P
Zpf640/Mzf6d	Und	Und

Genes highlighted here are specifically mentioned in the pathways discussed in this chapter and listed separately in **Tables 2**–**7**. Phenotypes included are derived from the Online Mendelian Inheritance in Man (OMIM).

Table 1. Summary of genes with known involvement in the etiology of orofacial abnormalities in mice.

Upon cross-referencing the KO mice available through the Jackson Laboratory (http://www.informatics.jax.org/diseasePortal) and performing a literature search on PubMed, Web of Science, and similar scholarly databases, we can provide an accurate account of all currently available mouse models with phenotypes concurrent with our understanding of CL/P. Furthermore, physicians and researchers alike are searching for a coalescence of treatment strategies, including gene therapy, to replace our current therapeutic approaches that consist mainly of a lifetime persistence of surgeries with less than consistent results due, in part, to non-standardization of procedures. What follows is an in-depth look, in order of current dominance in the landscape of research, at the mouse models currently being used to study the etiologic determinants of orofacial clefting.

2.1. TGF beta (TGFβ) signaling pathway

A number of genes from the TGF beta (TGFβ) signaling pathway that play a role in palatogenesis in mice are many (**Table 2**). Members of this "superfamily" play an important role in the development of Meckel's cartilage and the mandible— thus, alteration or inactivation of particular members can lead to cleft palate [2]. TGFβ receptors are dimeric and consist of two types—type I and type II—of receptors with serine/threonine kinase activation. Once activated, these receptors function in such a way that SMAD transcription factors are phosphorylated, and through a cascade, eventually these SMADs make it into the nucleus where they function to modulate the transcription of particular subsets of genes [3]. The SMADs can either activate or repress the gene to which they bind. As such, a combination of dimeric receptors and ligands can result in any number of outcomes for a cell. In particular, TGFβ is

Gene	Syndromic/non-syndromic	Orofacial phenotype
Acvr1/Alk2	Submucosal cleft/fibrodysplasia ossificans progressiva	Und
Acvr2a	Und	Und
Bmp4	Microphthalmia, syndromic 6	CL/P
Bmpr1a/Alk3	Juvenile polyposis syndrome,	CP
Chrd	Und	CL
Cited2	Atrial septal defect 8, ventricular septal defect 2	Und
Foxc2/Mfh1	Lymphedema-distichiasis syndrome	CL/P
Foxd3	Und	Und
Foxe1/Titf2/Fkhl15	Bamforth-Lazarus syndrome	CL/P
Foxf2	Und	Und
Fst	Und	Und
Gdf11/Bmp11	Und	Und
Inhba	Und	Und
Map3k7/ Tak1	Und	Und
Smad4	Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome	Und
Smad7	Und	Und
Tgfb2	Loeys-Dietz syndrome, type 4	CL/P
Tgfb3	Arrhythmogenic right ventricular dysplasia 1	CL/P
Tgfbr1/Alk5	Loeys-Dietz syndrome, type 1	CL/P
Tgfbr2	Loeys-Dietz syndrome, type 2	CL/P

Table 2. TGF beta/BMP signaling pathway.

involved in several critical functions that take place during embryogenesis, including proliferation, apoptosis, and cell differentiation.

Also, critical to normal development of the palate is the temporal and spatial distribution of the members of the TGFβ signaling pathway. The importance of this timing aspect may be that these structures, similar to morphogens, inducing specific tissue formation at identifiable time points in development [4]. This information can be used in the development of novel treatment strategies in humans with known gene mutations or deficiencies.

Typically, TGFβ receptor activation recruits and phosphorylates SMAD2 and SMAD3 at the carboxyl terminus via TGFβ receptor I. This method of signaling is generally what is meant by the term SMAD-dependent TGFβ signaling. However, TGFβ signaling can occur in lieu of SMAD activation via phosphorylation—pathways known to be activated in this manner include MAPK pathways (i.e., ERK, NJK, and p38) [5]. Inherently, this creates a purported "balance" between the levels of SMAD-dependent and SMAD-independent TGFβ signaling that exists through the development of normal palatogenesis. When we discuss the SMAD-independent pathways, it has been proposed that these are the result of posttranslational modifications which occur to either of the two types of TGFβ receptors. These mechanisms and their subsequent cascades are under current investigation and not yet entirely known [5].

Distinct members of the TGFβ superfamily, utilizing a separate series of SMAD proteins (SMAD1/5/9), are the bone morphogenetic proteins (BMPs). There are a number of BMP ligands known and two distinct receptor types—type I and type II. As mentioned, there appears to be a temporal and spatial distribution of this family, which is critical for the function of BMPs, which are very well researched with regard to palatogenesis. In particular, *Bmp4* cKO mice show clefting of the lip, both uni- and bilaterally [6]. Understandably, BMP receptors play a role in orofacial clefting as well; in addition, there is a distinct involvement in tooth morphogenesis for BMP receptors, notably *Bmpr1a* [7]. This molecule and its related receptors have an essentially unparalleled significance in the etiologic pathogenesis of CL/P. *Bmpr1a* cKO embryos, while also showing tooth morphology defects, die from orofacial clefting [6, 7].

2.2. Hedgehog signaling pathway

When one first thinks of SHH, it is likely that we recall the molecule's importance in left-right patterning of the embryo, dorsal-ventral establishment of the neural tube, and brain development, among other functions. Intrinsic properties of these morphogenic functions include signaling for cell proliferation and survival. The alteration of these properties can lead SHH receptors and/or ligands to function abnormally, thus, in some cases, altering the patterning of cranial neural crest cells during embryonic development. Modulation of the molecules involved in hedgehog signaling has been shown to present with CL/P phenotype in mice.

The full breadth of hedgehog signaling molecules with known involvement in orofacial clefting in mice spans several other pathways (**Table 3**). A notable characteristic of the mechanism of action for *Shh* can be observed in nasal epithelium of mice where *Shh* is reported absent. These mice develop cleft palate, while mice with overexpressed *Shh* are shown to express failure of growth of the maxillary processes and thus no fusion; this leads to cleft palate and several missing bones within the nasal process [8].

Gene	Syndromic/non-syndromic	Phenotypes
Gli2	Culler-Jones syndrome, holoprosencephaly-9	CL/P
Gli3	Greig cephalopolysyndactyly	CL/P
Ptch1/*Ptc1*	Basal cell nevus syndrome (Gorlin syndrome)	CL/P
Shh	Holoprosencephaly-3	CL/P
Smo/*Smoh*	Basal cell carcinoma, somatic	Und

Table 3. Hedgehog signaling pathway.

Another notable molecule involved in the hedgehog signaling pathway is *Ptch1*, a transcriptional target of *Shh* as well, which displays a gradient mimicking that of *Shh* in the palatal shelves during early palatogenesis, at E13.5 [8]. Similarly, the palatal mesenchyme adjacent to the medial-edge epithelium (MEE) present in the nasal epithelium expressed *Smo* in significant amounts [9]. In each case with the hedgehog signaling molecules, there is expression in the palatal mesenchyme, with the highest level of expression for most molecules adjacent to the palatal oral epithelium [9]. The awareness of this spatial and temporal expression provides a niche for the insertion or potential innervation of gene products given therapeutically. The effects of an abnormal amount of SHH signaling are palpable. Restoration of the proper balance of SHH signaling throughout development may play a role in treatment options in the near future, and delivery methods are currently underway to target particular areas of known involvement in CL/P.

2.3. Wnt signaling pathway

The Wnt signaling pathway plays another exceptional role in craniofacial morphogenesis in mice (**Table 4**). There are 19 known Wnt proteins found in humans, with combinations of differing ligands and receptors allowing for a mixture of modulatory effects from similar molecules. Between the receptors available, there exist three distinct pathways: the β-catenin-dependent (canonical), β-catenin-independent planar cell polarity (PCP), and β-catenin-independent Ca^{2+} pathways. β-Catenin is a transcription factor that, when Wnt ligands are present, will persist and

Gene	Syndromic/non-syndromic	Phenotypes
Ctnnb1	Mental retardation, autosomal dominant 19	Und
Edn1	Auriculocondylar syndrome 3	CL/P
Fzd2	Und	Und
Gsk3b	Und	Und
Lrp6	Und	Und
Prickle1	Epilepsy, progressive myoclonic	Und
Wnt5a	Robinow syndrome, autosomal dominant	CL/P
Wnt9b	Und	Und

Table 4. Wnt signaling pathway.

translocate into the nucleus; the factor is otherwise degraded [7]. The Wnt pathway is involved in a variety of embryogenic and developmental events, similar to the SHH pathway. In terms of craniofacial development, we see a critical role for the Wnt signaling pathway when we observe the generation, migration, proliferation, and survival of cranial neural crest cells [10].

A notable Wnt ligand involved in canonical signaling is *Wnt9b*. Expressed between the facial processes, alterations in signaling of this molecule have shown to express clefting in mice. Additionally, *Wnt9b* null mice have a distinctly shorter nasal process and shortened maxillary processes, a direct link to bilateral CLP [11]. This expression is apparent with FGF molecules, one of the many molecules involved with and expressively determined by Wnt signaling. A deletion of either the epithelium in which *Wnt9b* is found or a KO of the ligand (gene product) itself results in a similar cleft lip phenotype [11].

While the plethora of numerous other Wnt signaling targets and mediators exist, a receptor of particular interest and importance currently is *Lrp6*. This receptor functions in the canonical Wnt pathway as well and contains members of the Frizzled family as well as a co-receptor, which can be low-density lipoprotein receptor-related protein 6 (LRP6). Research has shown that *Lrp6* null mice demonstrate bilateral clefting of the lip as well and cleft palate and midline clefting of the mandible [12]. These mice also express defects in the neural tube, eye, and brain among others. The orofacial clefting defects were observed at E13.5 in these *Lrp6* null mice, with full penetrance of CLP and mandibular defects [12]. Again, we see a pattern that current research has established wherein a spatial and temporal time table has been created. This knowledge, as it continues to expand with further genomic testing and mouse model availability, should prove highly useful in the development of novel therapies.

2.4. FGF signaling pathway

While it has already been briefly discussed, one can see that the FGF signaling pathway also expands across several currently known molecular cascades. In humans and in mice, mutations resulting in dysfunction of the FGF signaling pathway are known to result in a variety of craniofacial abnormalities and syndromes—one proponent of which is orofacial clefting. An important role of FGF signaling is seen in the induction of the neural crest while being widely expressed in epithelial-mesenchymal interactions elsewhere. Particularly in the facial primordia, FGF signaling is absolutely critical in the proper development and formation of the palate as it is present in both endochondral (i.e., Meckel's cartilage) and intramembranous bones [13]. When we consider palatogenesis, FGF molecules have been shown to be involved in multiple stages—from palatal shelf elevation to fusion of MEE. KO mice have played a key role in our understanding of the function of various FGFs and their relation to orofacial clefting.

There are 23 distinct FGF ligands known and four receptors to which they bind. Alternative splicing generates several receptor variants which allows for multiple binding combinations and, thus, different functionalities temporally during embryogenesis. Various receptors are located in the epithelium and mesenchyme throughout the embryo, and research has elucidated many roles that these molecules play; for our interest, much emphasis has been placed on suture fusion (craniosynostosis) and palatogenesis.

Mutations in FGF receptors have been shown to present with a variety of midfacial syndromes in mice as well (**Table 5**). For example, in humans, gain-of-function mutations in *FGFR2* and *FGFR3* have been consistently observed in individuals with Crouzon syndrome—a genetic disorder that includes craniosynostosis in its list of defects associated with the syndrome. More relevant here, however, is that a KO mouse model in which the *Fgfr1* receptors are missing in the cranial neural crest (CNC) cells directly results in CLP due to failures in the proliferation and migration of said cells [14]. Likewise, research has shown that ectopic activation of *Fgf8* results in increased proliferation and a failure of the palatal shelves to elevate properly [15]. This is exceptionally interesting in that it is a rare case in which an increase in cell proliferative activity has resulted in CP; in many cases, CP is the result of an obvious decrease in the amount of cell proliferation. In the case of *Fgf8* activation, the palatal shelves were still unable to elevate in a normal manner, and thus the palatal morphology was altered, and a CP phenotype was observed.

Gene	Syndromic/non-syndromic	Phenotypes
Fgf10	Aplasia of lachrymal and salivary glands	Und
Fgf18	Und	Und
Fgf9	Und	Und
Fgfr1	Non-syndromic cleft lip/palate, Hartsfield syndrome, hypogonadotropic hypogonadism 2, Pfeiffer syndrome	CL/P
Fgfr2	Apert syndrome	CL/P
Gbr2	Und	Und
Spry1	Und	Und
Spry2	Und	Und

Table 5. FGF signaling pathway.

The FGF signaling pathway has been, and is currently being, extensively studied. Spatial expression of the molecules involved in the pathway has been seen widely throughout the developing mouse embryo, while the temporal expression continues to be expounded upon. Investigations are ongoing to further our knowledge of why characteristically opposing molecular processes (i.e., reduction versus activation of cellular proliferation) may result in the same phenotype. In all, what remains important is that future treatment options are expanding all the time. The more we learn about all the plethora of molecular signals that interact during embryogenesis—which is similar enough between mouse and human—the more physicians and surgeons are able to generate new and better therapies.

2.5. MAPK signaling pathway

The mitogen-activated protein kinase (MAPK) signaling pathway—also known as the ERK pathway—plays a role in craniofacial development of mice as early as E10.5 [16]. MAPK is a protein kinase that functions in conjunction with two others, MAPKKK (e.g., RAF) and MAPKK (e.g., MEK1/2). Upon activation, these effector molecules can act in either the cytosol or the nucleus. Growth factors, including TGFβ, BMPs, and fibroblast growth factor (FGF),

can modulate this same protein kinase cascade, and each of the molecules listed is also known to be involved with development of the palate [17]. Additionally, analysis of the potential spatial representation of active (phosphorylated) ERK1/ERK2 in the palate has resulted in the discovery this pathway persists in both the epithelium and the mesenchyme associated with the developing palatal shelves [17].

Immunohistochemistry using an antibody against an activated form of ERK has shown ERK signaling in the frontonasal process, brachial arches, and extraembryonic ectoderm, among other craniofacial-associated regions [16]. Research has also shown associations between MAPK signaling and growth factor pathway genes that include *Fgf9/10/18*, *Alk5*, and *Itgb1* among others and vary craniofacial clefting and defects in mice, including mandibular osteogenic and tongue abnormalities [17]. The inclusion of the mandible and tongue is important in that it adds to the overall complexity of the defect, thus making treatment options that much more of a priority. Current investigations are ongoing to pinpoint time points and the distribution of MAPK signaling and its numerous molecular effectors during embryogenesis in mice (**Table 6**).

Gene	Syndromic/non-syndromic	Phenotypes
Chuk/Ikk1/Tcf16	Cocoon syndrome	Und
Egfr	Und	Und
Grb2	Und	Und
Pdgfra	Gastrointestinal stromal tumor, somatic	CL/P
Crk	Und	Und
Itgb1	Und	Und

Table 6. MAPK signaling pathway.

2.6. Homeobox proteins

Homeobox proteins and their respective KO/mutant mouse models are used to represent easily observable phenotypes. Some of the most well-studied homeobox genes in mice include *Msx1/2*, *Pax9*, and *Alx1* [1]. The reason for their grouping and relatively well-known actions has to do with the fact that transcription factors encoded by homeobox genes act in a site-specific manner [18]. These gene products exist, segmentally, throughout the body and are palpable during nearly all stages of development. As such, we know that there are Hox homeogenes which control bone patterning in the limb buds; similarly, there are separate homeogenes that are associated with craniofacial development in mice (**Table 7**).

Specifically, research has shown that a human *MSX1* missense mutation can lead to orofacial clefting as well as selective tooth agenesis [19]. Mutations in this gene, as seen in other homeogenes, can lead to dysfunctional protein products that act via transcriptional repression. In the case of *Msx1*, the homeodomain interacts directly with the TATA-binding protein (TBP) and acts directly at the start of transcription by repressing the gene completely to which it translocates. In some scenarios, heterodimers will form between homeodomain proteins, and a balance must persist in which they are co-regulatory.

Gene	Syndromic/non-syndromic	Phenotypes
Alx1	Frontonasal dysplasia 3	CL/P
Alx3	Frontonasal dysplasia 1	CL/P
Alx4	Frontonasal dysplasia 2, parietal foramina 2, craniosynostosis 5	Cleft alae nasi
Barx1	Und	Und
Dlx1	Und	Und
Dlx2	Und	Und
Dlx5	Split-hand/foot malformation 1 with sensorineural hearing loss	CL/P
Gbx2	Und	Und
Gsc	Short stature, auditory canal atresia, mandibular hypoplasia, skeletal abnormalities	Und
Hoxa2	Microtia with or without hearing impairment	Und
Msx1	Ectodermal dysplasia 3, Witkop-type orofacial cleft 5	CL/P
Msx2	Craniosynostosis, type 2; parietal foramina 1, parietal foramina with cleidocranial dysplasia	CL/P
Pax9	Tooth agenesis, selective, 3	Und
Pitx1	Clubfoot, congenital, with or without deficiency of long bones and/or mirror-image polydactyly, Liebenberg syndrome	CL/P
Pitx2	Axenfeld-Rieger syndrome, type 1; iridogoniodysgenesis, type 2; Peters anomaly	Und
Prrx1/*Prx1*/*Mhox*	Agnathia-otocephaly complex	CL/P
Rax	Microphthalmia, isolated 3	Und
Shox2	Und	Und
Vax1	Microphthalmia, syndromic 11	CL/P

Table 7. Homeobox protein signaling pathway.

As a result of these proteins acting within their respective zones (or "sites"), one can assume that there is an overlap with the adjacent homeodomain. Such overlap is observed between *Msx1* and *Msx2* throughout the craniofacial structures during development—including the skull, suture mesenchyme, and teeth [20]. Inherent in their molecular categorization is the idea that we know where, and upon which tissues, these proteins interact. There are a number of homeogenes involved in craniofacial development that modulate palatogenesis and patterning, among a variety of other roles. Due to their known functions during embryogenesis, further research is ongoing regarding the effect of varying homeogene mutations on cell proliferation, survival, and adhesion. The culmination of knowledge that lies within these determinants of normal development will indubitably result in opportunities for the future application of therapeutic modalities.

2.7. Remaining mouse strains exhibiting CL/P phenotype

Here, we have put into one table a list of the genes with a known association, whether syndromic or non-syndromic, to the development of the palate in mouse (**Table 1**). It should be noted that not all genes in this table have shown their identical, cross species phenotype in humans.

2.8. The future of CL/P therapy

A bonafide surgical protocol remains to be standardized for the repair of CL/P. Fortunately, ongoing research concerning therapeutic interventions for this relatively common birth defect has recently begun to delve into new and improved options for repair with, hopefully, more consistent and stable results for patients. The current "golden standard" treatment option for pediatric oral surgeons involves bone grafting, or alveoloplasty, usually from autogenous sites—but this has many complications associated with both the grafting procedure and the agreed-upon effectiveness in reconstructing the palate over time [21]. Postoperative follow-up has shown success rates ranging from 41 to 73%, which is far from standardized, while there also exists the possibility (in 11–23% of patients) of oronasal fistulas, which come with their own brand new set of complications for the patient [22]. In short, the most effective interventions in use today are far from ideal for the patent and result in long-term risk of complications from grafting procedures, disturbance of adjacent craniofacial development, and, over time, a significant financial encumbrance on the patient. Techniques including gene delivery, in vitro engineered tissue transplantation, and regenerative medicine are being probed for efficacy, and some are showing promising results thus far.

An exceptionally exciting modality is the use of stem cells. One method of delivering these cells is via a biocompatible scaffold upon which cells that have been previously harvested were cultured and attached. Materials including collagen, hyaluronic acid, and hydroxyapatite have been utilized in attempts to develop such scaffolds [23–25]. These scaffolds have been engineered as injectable gels, mesh networks, and foams. Ideally, this aids in the procedure being as minimally invasive as possible while also providing maximum benefit and adequate delivery to the area of interest. This therapy can be modified to include signaling molecules and other types of differentiated cells—which preferably have a known clinical outcome and avoid the possibility of rejection and/or disunity with the surrounding host cells—and injected in a similar fashion or applied to previously engineered palates. Currently, autogenous mesenchymal stem cells (MSCs) are regarded as the optimum choice for in vivo osteogenic reconstructions; these can come from umbilical cord blood, Wharton's jelly, and even the patient's own bone marrow [26]. Tissue regenerative-specific repair of CL/P has been demonstrated with some success, and some are now advocating for in depth considering of its potential to replace traditional autogenous grafting procedures [27].

Regarding clinical studies in progress, one group has shown that in vitro differentiated MSCs derived from bone marrow were delivered with platelet-derived growth factor and significant improvement was observed 3 months post-op [28]. Similarly, recombination therapies are being used to induce osteoblastic differentiation with BMPs formed from stem cells, and resulting immunohistological analysis of the bone that formed has shown normal, vital

structure [29]. Finally, platelet-rich plasma (PRP) is being studied with regard to its potential for tissue repair in vivo. A wide variety of growth factors are present in a platelet-rich solution and have been shown to promote angiogenesis and extracellular matrix formation [30]. This intervention has some positive results—it has been shown that PRP can enhance bone regeneration and thus may be a useful alternative to traditional procedures for CL/P patients [31].

A number of prospective therapeutic interventions are currently being investigated, many with exciting outcomes thus far. CL/P etiology is not yet completely understood and is extremely complex. In order to properly apply this research to the human subjects, we must further our research to bridge the gap between an understanding of the signaling pathways, the rescue of the animal phenotype, and the translation of this knowledge into human treatment. As research continues on the pathways mentioned in this chapter, further clinical trials should become available, and treatment outcomes for patients can rapidly and significantly improve. Moving forward, more work is needed to establish a new standard of care and a protocol for various differing types of orofacial clefts, but progress has proceeded rapidly in recent years, and the outlook is bright for the future of care for CL/P patients.

In summary, it remains within animal research where the next steps in the elucidation of potential treatments for CL/P must be made. Understanding the biological, molecular signaling pathways and identifying a broad cause for the clefting phenotype are only the first steps in understanding how to treat it. Now, we need to look toward a greater understanding of the critical downstream events that occur as a result of the KO or cKO models being used; what types of tissue-tissue interactions are changing? What is the scope of the molecular activity being altered as a result of changing the capabilities of one gene? Once more of these questions are answered in animal models, the translation of lab research to the rescue of human phenotypes will become more clear. Until then, it is crucial to continue to identify all that we can in order to bridge the gap between KO/cKO mice, the expansive etiology surrounding their conditions, and the rescue of their control phenotypes.

Author details

Aram J. Keteyian* and Yuji Mishina

*Address all correspondence to: aketeyia@umich.edu

Department of Biologic and Materials Science, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

References

[1] Funato N, Nakamura M, Yanagisawa H. Molecular basis of cleft palates in mice. World J Biol Chem [Internet]. 2015 Aug 26 [cited 2016 Apr 30];6(3):121–138. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4549757&tool=pmcentrez&rendertype=abstract

[2] Lei R, Zhang K, Liu K, Shao X, Ding Z, Wang F, et al. Transferrin receptor facilitates TGF-β and BMP signaling activation to control craniofacial morphogenesis. Cell Death Dis [Internet]. Nature Publishing Group; 2016 Jun 30 [cited 2016 Jul 16];7(6):e2282. Available from: http://www.nature.com/doifinder/10.1038/cddis.2016.170

[3] Iwata J, Parada C, Chai Y. The mechanism of TGF-β signaling during palate development. Oral Dis [Internet]. Blackwell Publishing Ltd; 2011 Nov [cited 2016 Jul 16];17(8):733–744. Available from: http://doi.wiley.com/10.1111/j.1601-0825.2011.01806.x

[4] Dudas M, Kaartinen V. TGF-β superfamily and mouse craniofacial development: interplay of morphogenetic proteins and receptor signaling controls normal formation of the face. Curr Top Dev Biol. 2005;66:65–133.

[5] Kang JS, Liu C, Derynck R. New regulatory mechanisms of TGF-β receptor function. Trends Cell Biol. 2009;19(8):385–394.

[6] Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, et al. Distinct functions for Bmp signaling in lip and palate fusion in mice. Development [Internet]. The Company of Biologists Ltd; 2005 Mar [cited 2016 Jul 16];132(6):1453–1461. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15716346

[7] Suzuki A, Sangani DR, Ansari A, Iwata J. Molecular mechanisms of midfacial developmental defects. Dev Dyn [Internet]. 2016 Mar [cited 2016 Jul 16];245(3):276–293. Available from: http://doi.wiley.com/10.1002/dvdy.24368

[8] Cobourne MT, Xavier GM, Depew M, Hagan L, Sealby J, Webster Z, et al. Sonic hedgehog signalling inhibits palatogenesis and arrests tooth development in a mouse model of the nevoid basal cell carcinoma syndrome. Dev Biol. 2009;331(1):38–49.

[9] Rice R, Connor E, Rice DPC. Expression patterns of Hedgehog signalling pathway members during mouse palate development. Gene Expr Patterns. 2006Vol. 6.

[10] Mani P, Jarrell A, Myers J, Atit R. Visualizing canonical Wnt signaling during mouse craniofacial development. Dev Dyn [Internet]. 2010 Jan [cited 2016 Jul 16];239(1):354–363. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19718763

[11] Jin YR, Han XH, Taketo MM, Yoon JK, Abu-Issa R, Smyth G, et al. Wnt9b-dependent FGF signaling is crucial for outgrowth of the nasal and maxillary processes during upper jaw and lip development. Development [Internet]. Oxford University Press for The Company of Biologists Limited; 2012 May [cited 2016 Jul 16];139(10):1821–1830. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22461561

[12] Song L, Li Y, Wang K, Wang YZ, Molotkov A, Gao L, et al. Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. Development [Internet]. The Company of Biologists Ltd; 2009 Sep [cited 2016 Jul 16];136(18):3161–3171. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19700620

[13] Nie X, Luukko K, Kettunen P. FGF signalling in craniofacial development and developmental disorders. Oral Dis [Internet]. Blackwell Publishing Ltd; 2006 Mar [cited 2016 Jul 18];12(2):102–111. Available from: http://doi.wiley.com/10.1111/j.1601-0825.2005.01176.x

[14] Wang C, Chang JYF, Yang C, Huang Y, Liu J, You P, et al. Type 1 fibroblast growth factor receptor in cranial neural crest cell-derived mesenchyme is required for palatogenesis. J Biol Chem [Internet]. American Society for Biochemistry and Molecular Biology; 2013 Jul 26 [cited 2016 Jul 18];288(30):22174–22183. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M113.463620

[15] Wu W, Gu S, Sun C, He W, Xie X, Li X, et al. Altered FGF signaling pathways impair cell proliferation and elevation of palate shelves. Zhang X, editor. PLoS One [Internet]. Public Library of Science; 2015 Sep 2 [cited 2016 Jul 18];10(9):e0136951. Available from: http://dx.plos.org/10.1371/journal.pone.0136951

[16] Corson LB, Yamanaka Y, Lai KMV, Rossant J. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development [Internet]. The Company of Biologists Ltd; 2003 Oct [cited 2016 Jul 18];130(19):4527–4537. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12925581

[17] Parada C, Han D, Grimaldi A, Sarrión P, Park SS, Pelikan R, et al. Disruption of the ERK/MAPK pathway in neural crest cells as a potential cause of Pierre Robin sequence. Development [Internet]. Oxford University Press for The Company of Biologists Limited; 2015 Nov 1 [cited 2016 Jul 18];142(21):3734–3745. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26395480

[18] Nassif A, Senussi I, Meary F, Loiodice S, Hotton D, Robert B, et al. Msx1 role in craniofacial bone morphogenesis. Bone. 2014;66:96–104.

[19] Vastardis H, Karimbux N, Guthua SW, Seidman JG, Seidman CE. A human MSX1 homeodomain missense mutation causes selective tooth agenesis. Nat Genet [Internet]. Nature Publishing Group; 1996 Aug [cited 2016 Jul 18];13(4):417–421. Available from: http://www.nature.com/doifinder/10.1038/ng0896-417

[20] Alappat S, Zhang ZY, Chen YP. Msx homeobox gene family and craniofacial development. Cell Res [Internet]. Nature Publishing Group; 2003 Dec [cited 2016 Jul 18];13(6):429–442. Available from: http://www.nature.com/doifinder/10.1038/sj.cr.7290185

[21] Arangio P, Marianetti TM, Tedaldi M, Ramieri V, Cascone P. Early secondary alveoloplasty in cleft lip and palate. J Craniofac Surg [Internet]. 2008 Sep [cited 2016 Jul 19];19(5):1364–1369. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18812864

[22] Tavakolinejad S, Ebrahimzadeh Bidskan A, Ashraf H, Hamidi Alamdari D. A glance at methods for cleft palate repair. Iran Red Crescent Med J [Internet]. Kowsar; 2014 Sep [cited 2016 Jul 18];16(9):e15393. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25593724

[23] Kang SW, Kim JS, Park KS, Cha BH, Shim JH, Kim JY, et al. Surface modification with fibrin/hyaluronic acid hydrogel on solid-free form-based scaffolds followed by BMP-2 loading to enhance bone regeneration. Bone [Internet]. Elsevier; 2011 Feb [cited 2016 Jul 19];48(2):298–306. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20870047

[24] Krishnamoorthy G, Sehgal PK, Mandal AB, Sadulla S. Novel collagen scaffolds prepared by using unnatural D-amino acids assisted EDC/NHS crosslinking. J Biomater Sci Polym

Ed [Internet]. 2013 [cited 2016 Jul 19];24(3):344–364. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23565652

[25] Guda T, Walker JA, Pollot BE, Appleford MR, Oh S, Ong JL, et al. In vivo performance of bilayer hydroxyapatite scaffolds for bone tissue regeneration in the rabbit radius. J Mater Sci Mater Med [Internet]. 2011 Mar [cited 2016 Jul 19];22(3):647–656. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21287244

[26] Diao Y, Ma Q, Cui F, Zhong Y. Human umbilical cord mesenchymal stem cells: osteogenesis in vivo as seed cells for bone tissue engineering. J Biomed Mater Res A [Internet]. 2009 Oct [cited 2016 Jul 19];91(1):123–131. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18767055

[27] Pourebrahim N, Hashemibeni B, Shahnaseri S, Torabinia N, Mousavi B, Adibi S, et al. A comparison of tissue-engineered bone from adipose-derived stem cell with autogenous bone repair in maxillary alveolar cleft model in dogs. Int J Oral Maxillofac Surg [Internet]. Elsevier; 2013 May [cited 2016 Jul 19];42(5):562–568. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23219713

[28] Behnia H, Khojasteh A, Soleimani M, Tehranchi A, Atashi A, Behnia H, et al. Repair of alveolar cleft defect with mesenchymal stem cells and platelet derived growth factors: a preliminary report. J Craniomaxillofac Surg [Internet]. Elsevier; 2012 Jan [cited 2016 Jul 19];40(1):2–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21420310

[29] Chin M, Ng T, Tom WK, Carstens M. Repair of alveolar clefts with recombinant human bone morphogenetic protein (rhBMP-2) in patients with clefts. J Craniofac Surg [Internet]. 2005 Sep [cited 2016 Jul 19];16(5):778–789. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16192856

[30] Shirvan MK, Alamdari DH, Ghoreifi A, Arrowsmith SD, Ruminjo J, Landry EG, et al. A novel method for iatrogenic vesicovaginal fistula treatment: autologous platelet rich plasma injection and platelet rich fibrin glue interposition. J Urol [Internet]. Elsevier; 2013 Jun [cited 2016 Jul 19];189(6):2125–2129. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022534712060053

[31] Oyama T, Nishimoto S, Tsugawa T, Shimizu F, Boyne P, Sand N, et al. Efficacy of platelet-rich plasma in alveolar bone grafting. J Oral Maxillofac Surg [Internet]. Elsevier; 2004 May [cited 2016 Jul 19];62(5):555–558. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0278239104000345