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 oro-facial 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).
| 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                |
| Bmp11a/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               |
| Chrhd             | Und                                                                                     | CL                 |
| Chuk/Ikk1/Tcf16   | Cococon 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                |
| Ctnmb1            | 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/Ozca1            | 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/Fkh15     | 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                 |
| Sec         | 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                 |
| Jmjdc/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/Objf2b/lhSSB1 | 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                                                      |
| Pds2               | 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; iridogoniodygenesis, type 2; Peters anomaly | Und                                                      |
| Pkdcc/Vlk          | Und                     | Und                                                      |
| Pnn                | Und                     | Und                                                      |
| Prdm16             | Cardiomyopathy, dilated, ILL; left ventricular noncompaction 8 | Und                                                      |
| Prickle1           | Epilepsy, progressive myoclonic | Und                                                      |
| Prrx1/Prx1/Mhox    | Agnathia-otocephaly complex | CL/P                                                     |
| Ptc1/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                                     |
| Rsop2        | Und                     | Und                                      |
| Rtnx2        | 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/Viat | 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                                     |
| Snat2        | 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                                     |
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.

| 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               |
| Tgfβ2  | Loeys-Dietz syndrome, type 4 | CL/P               |
| Tgfβ3  | Arrhythmogenic right ventricular dysplasia 1 | CL/P               |
| Tgfβr1/Alk5 | Loeys-Dietz syndrome, type 1 | CL/P               |
| Tgfβr2 | Loeys-Dietz syndrome, type 2 | CL/P               |
| Trp63/Tp63 | Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome 5; 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                 |
| Wdp5c  | Und                     | Und                 |
| Wnt5c1 | Und                     | Und                 |
| Wnt5c1 | Und                     | Und                 |
| Wnt5a  | Robinow syndrome, autosomal dominant | CL/P               |
| Wnt9h  | 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.
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/Mbh1     | 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               |
| Tgfr1/Alk5     | Loeys-Dietz syndrome, type 1 | CL/P               |
| Tgfr2          | 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].
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 |
|-------------|-----------------------------------------------------------|------------|
| 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.

| 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 \textit{Wnt9b}. Expressed between the facial processes, alterations in signaling of this molecule have shown to express clefting in mice. Additionally, \textit{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 \textit{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 \textit{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 \textit{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 \textit{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. Homebox proteins

Homebox proteins and their respective KO/mutant mouse models are used to represent easily observable phenotypes. Some of the most well-studied homebox 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 homebox genes act in a sitespecific 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 homoeogenes which control bone patterning in the limb buds; similarly, there are separate homeoegenes 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 homoeogenes, 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.
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.

| 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; iridogoniodygenesis, 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.
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 patient 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.

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