Mouse Models of Orofacial Clefts: SHH and TGF-β Pathways

Yu Chen LI1#, Le Ran LI1#, Zi Han GAO1, Yi Ran YANG1, Qian Chen WANG1, Wei Yu ZHANG1, Li Qi ZHANG1, Tian Song XU1, Feng CHEN1

Birth defects have always been one of the most important diseases in medical research as they affect the quality of the birth population. Orofacial clefts (OFCs) are common birth defects that place a huge burden on families and society. Early screening and prevention of OFCs can promote better natal and prenatal care and help to solve the problem of birth defects. OFCs are the result of genetic and environmental interactions; many genes are involved, but the current research has not clarified the specific pathogenesis. The mouse animal model is commonly used for research into OFCs; common methods of constructing OFC mouse models include transgenic, chemical induction, gene knockout, gene knock-in and conditional gene knockout models. Several main signal pathways are involved in the pathogenesis of OFCs, including the Sonic hedgehog (SHH) and transforming growth factor (TGF-β) pathways. The genes and proteins in each molecular pathway form a complex network to jointly regulate the formation and development of the lip and palate. When one or more genes, proteins or interactions is abnormal, OFCs will form. This paper summarises the mouse models of OFCs formed by different modelling methods, as well as the key pathogenic genes from the SHH and TGF-β pathways, to help to clarify the pathogenesis of OFCs and develop targets for early screening and prevention.

Key words: mouse models, orofacial clefts, Sonic hedgehog pathway, transforming growth factor-β pathway.


As a result of socioeconomic progress and development, the mean age of the childbearing population is gradually increasing, leading to a rise in the incidence of birth defects and placing huge burdens on families and society for medical care as well as other influences1-3. Orofacial clefts (OFCs) account for a large proportion of birth defects, with an average of 1 in 600 to 800 newborns suffering from cleft lip and palate4. OFCs not only seriously affect the appearance of the face, but also directly impact development of the mouth and nose, often leading to upper respiratory tract infections and otitis media and causing serious psychological trauma to children and parents. It is therefore important to screen for and prevent birth defects.

OFCs are the most common congenital malformation in the oral and maxillofacial region. They can be regarded as a symptom of many syndromes or can occur independently and can be divided into two types: cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). An OFC is a gap formed by the improper fusion of facial prominences during early embryonic development. Studies have found that a combination of genetic and environmental factors lead to this occurrence, which is regulated by complex mechanisms5. Multiple signalling pathways and genes are involved. Lip and palatal development involve a series of highly coordinated, genetically programmed morphogenetic events. Gene mutations lead to palate shelf elevation,
epithelial–mesenchymal transformation and epithelial apoptosis abnormalities, which result in abnormal development of the lip and palate. In addition, an increasing number of studies have found that epigenetic mechanisms may be involved in the pathogenesis of OFCs, mainly including deoxyribonucleic acid (DNA) methylation, non-coding ribonucleic acid (ncRNA) and histone modification6-8. MicroRNAs (miRNAs) play a crucial role in silencing the expression of specific genes8,9. Environmental factors such as nutrition and smoking have a significant impact on DNA methylation patterns10.

Animal models are important for understanding the pathogenesis of OFCs. Because the early embryonic craniomaxillofacial development of Xenopus, zebrafish and mice is very similar to that of humans, they are suitable animal models for the study of OFCs. The mouse is the most commonly used animal model in OFC studies; advantages include its small size, rapid reproduction, high similarity with human genes, stable genetic background and clear gene sequence11, which make it suitable for a variety of experiments. The existing methods for constructing OFC mouse models include transgenesis, chemical induction, gene knockout (KO), gene knock-in and conditional gene knockout (CKO).

In the existing mouse model of OFCs, hundreds of genes have been studied. The pathogenesis of OFCs is complex, and multiple genes and signalling pathways are involved, such as fibroblast growth factor (FGF), bone morphogenetic protein (BMP), Wnt, Sonic hedgehog (SHH) and transforming growth factor (TGF)-β signalling12. Of the many different pathways, SHH and TGF-β play an important role in growth and development. These signalling pathways affect embryonic differentiation, tissue development and organ formation in early developmental stages, and perturbation of signalling pathways may underlie many human craniofacial abnormalities12-14. The mouse models of CL/P and CPO have been introduced in detail in previous reviews15,16. This paper focuses on the mouse models of the SHH and TGF-β pathways and provides supplements and updates to the previous models. Research on the genes and mechanisms involved in cleft lip and palate is conducive to understanding its pathogenesis and further developing methods for prevention and treatment, thus reducing its incidence rate.

**SHH signalling pathway**

Hedgehog proteins are a family of secreted signal proteins that jointly regulate many aspects of animal development, tissue homeostasis and regeneration17. The persistently activated SHH signalling pathway is involved in lung cancer cell proliferation, apoptosis, epithelial–mesenchymal transformation, angiogenesis and drug resistance recurrence. The pathway is composed of the Shh, Ptc, Smo, PKA and Gli proteins. Shh ligand can be produced by secretory cells of multiple organs and is an extracellular ligand (Fig 1).

The hedgehog pathway is relevant to many congenital diseases. Shh and Indian hedgehog (Ihh) are closely related to craniofacial development. Shh is expressed in the craniofacial ectoderm and regulates the development of the neural crest. It is significant for formation of the upper lip and secondary palate18-20. Downregulation of this signal in the palate and medial nasal processes (MNP) will lead to CL/P. During palatal fusion, however, if the signal is not downregulated in time, the medial marginal epithelium will fail to fuse, causing cleft palate (Fig 1).

**Transgenic**

In the early stage, transgenesis (the use of vectors to transfer specific foreign genes into a genome to increase, prevent or change the expression of a gene to study its functions) was widely used in the construction of OFC mouse models21 (Table 1). Transgenesis used in the SHH pathway mainly targets Ptc1 and Hedgehog acyltransferase (Hhat).

As an important factor in the initial transmembrane process of SHH signalling, Ptc1 is a negative regulator that inhibits the transmembrane protein Smo. Upon binding to Shh, Ptc1 is degraded. Ptc1 expression is essential for formation of the primitive nose and upper lip. Transfer of K14-Shh into embryos leads to overexpression of Shh in epithelial cells, similar to Ptc1 knockout. This model results in CPO22.

In addition to the important role played by Ptc1 in transmembrane processes, multimerisation, distribution and activity of Hedgehog protein are also significant. Autoproteolytic cleavage of Hedgehog (Hh) precursor molecules generates an N-terminal fragment (Hh-N) referred to as the mature form. Hh-N is then modified via the addition of a cholesterol moiety to its C-terminus, and then a palmitoyl moiety to its N-terminus. These lipid modifications are required for Hh protein multimerisation, distribution and activity. Mouse embryos engineered with AP2-Cre inserted into Hedgehog acyltransferase (Hhat) showed small size, craniofacial hypoplasia and limb defects. The mice displayed a defect in vertical extension and medial growth of the palatal shelves towards the midline, resulting in cleft palate. Hhat loss-of-function should disrupt the palmitoylation of Shh. The
signal gradient of Shh in the tissue is destroyed, resulting in abnormal SHH signal strength\(^{23}\).

**Chemical induction**

Chemical induction methods use chemicals such as retinoic acid to induce an OFC phenotype in mice\(^ {24}\). Common chemical inducers include retinoic acid, cyclopa mine and vismodegib (Table 1).

Retinoic acid–induced embryos show frontonasal process (FNP) and maxillary process (MXP) fusion dysfunction, which is caused by retinoic acid inhibition of the expression of SHH molecules. The lack of SHH causes the FNP and MXP to stop growing, resulting in bilateral cleft lip and palate\(^ {25}\).

Other SHH pathway–associated chemical inducers that cause OFCs in mice include cyclopa mine and vismodegib. Both are antagonists of the SHH pathway and the induced mouse phenotype is CL/P. Cyclopa mine directly binds Smo, changing its conformation to inhibit Hh pathway activation. Vismodegib can be chemically modified by cyclopa mine; its principle is the same as cyclopa mine but with a greater effect\(^ {26,27}\).

In addition to chemical modifications, transcription of target genes can also be blocked. Gli1, as the most common transcriptional activator activated by Smo, participates in the transcription of target genes and functions in craniofacial and finger development, as well as central nervous system and gastrointestinal development, and is also involved in cell proliferation and differentiation through its role in SHH signalling, while cyclopa mine is its common blocker. Stimulation of cranial neural crest cells (CNCCs) with SHH ligands causes significant upregulation of Gli1 and Foxf2 expression, which can be blocked completely by the addition of cyclopa mine; outgrowth of the MNP is attenuated after blockade, leading to deficient frontonasal prominence–derived MNP, preventing contact with the MXP and subsequent fusion, which causes the CL/P phenotype\(^ {28}\).

**Gene KO**

KO techniques are used to construct OFC mouse models via deletion of target genes in mice by homologous recombination (Table 2).

The SHH-based mouse model of OFCs includes genes such as Gli2, Gli3, Ihh, Ick and Tmem107; the mouse phenotype is dominated by incomplete penetrant CPO.

Ihh is a ligand in the HH pathway that plays an important role in craniofacial development. It can form a negative feedback loop with parathyroid hormone– associated protein to indirectly regulate chondrocyte differentiation and affect chondrocyte proliferation and osteoblast specification. Ihh KO mice have the CPO phenotype\(^ {30,31}\). The proteins encoded by Cdo and Boc are...
located in the cell membrane and both proteins act as SHH signal coreceptors to promote signal transduction. Cdo/Boc double heterozygous mice showed decreased SHH signalling, CPO and forebrain malformation phenotypes. The protein encoded by Gas1, located in the cell membrane, can bind Shh together with Ptch and play an antagonistic role in the SHH pathway; however, Gas1 can enhance the effect of SHH in early facial development. When the gene was knocked out, the mice developed CPO and miniature forebrain malformation.

Gli is another important transcriptional factor after Ihh. In the presence of Shh, Smo activation generates intracellular signals that induce dissociation of the Gli-Sufu complex and facilitate translocation of transcriptional activator Gli into the nucleus. Gli2 is expressed in the palatal epithelium and mesenchyme; when knocked out, it blocks the SHH pathway, resulting in impaired palatal elevation (no elevation or partial elevation) or delayed fusion. Gli3 functions as both an activator and a repressor; the phosphorylated full-length form acts as an activator, while Gli3R, a C-terminal truncated form, acts as a repressor. There is a proper balance between Gli3 activator and inhibitor Gli3R. An imbalance will cause over- or underexpression of the SHH pathway, resulting in extensive separation of the palatal shelves from the underlying sphenoid bone, defects and incomplete mineralisation of the maxillary ramus, elevated palatal shelves and fusion disorders.

In addition to the above, cilia also have an impact on downstream genes. Tmem107, Ick, Ift144, Bbs and Fuz are related to the formation and function of cilia. Knockout of these genes causes changes in downstream genes, leading to the CPO model. Tmem107 combined with Gli2 and Gli3 also plays a role in ciliogenesis and embryogenesis. Ick regulates intra-flagellar transport velocity and negatively regulates ciliary length. Knockout of Ick results in abnormal primary cilia and distribution of Smo, which affects conduction of the SHH pathway, resulting in CPO and other malformations. Ift144, which is related to ciliary transport, may mediate bone migration during development, such as fusion of the MNP and lateral nasal process as well as fusion with the MXP. Bbs can reduce the level of Smo in cilia but can also regulate the transformation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Approach</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
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<tbody>
<tr>
<td>Ptch1</td>
<td>Transgenic</td>
<td>CPO</td>
<td>E14.5–15.5</td>
<td>E13.5: oral epithelium of the palatal shelves, with a correspond-</td>
<td>Perinatal lethality</td>
<td>EcoRI/HindIII double digest and pronuclear DNA injection</td>
<td>Cobourne et al&lt;sup&gt;22&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td></td>
<td>ing gradient of Ptch1 expression in the underlying mesenchyme</td>
<td></td>
<td>of CBA/C56 BL6 embryos on E0.5. Isolating K14-SHH transgene</td>
<td></td>
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<tr>
<td>Hedgehog acyltransferase (Hhat)</td>
<td>Chemical induction</td>
<td>Bilateral CLP</td>
<td>NR</td>
<td>The pharyngeal and midfacial hypoplasia evident in the form of a single nasal slit</td>
<td>Lymphatic and vascular anomalies</td>
<td>Hhat&lt;sup&gt;+&lt;/sup&gt;/Creface</td>
<td>Dennis et al&lt;sup&gt;23&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Chemical induction</td>
<td>CL/P</td>
<td>GD17</td>
<td>Decreased snout length and mandible length and increased interocular distance</td>
<td>NR</td>
<td>Cyclopamine induction</td>
<td>Helms et al&lt;sup&gt;25&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Chemical induction</td>
<td>CL/P</td>
<td>GD7.0–8.25</td>
<td>Highly arched palate</td>
<td>NR</td>
<td>Vismodegib induction</td>
<td>Heyne et al&lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gli1</td>
<td>Chemical induction</td>
<td>CL/P</td>
<td>GD11.0–14.0</td>
<td>Medial nasal processes (MNPs)</td>
<td>NR</td>
<td>Cycloparamide induction of female mice on GD8.25–9.375, 120 mg/kg/day</td>
<td>Everson et al&lt;sup&gt;28&lt;/sup&gt;</td>
</tr>
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NR, not reported.
### Table 2  Gene knockout in SHH.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
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</thead>
<tbody>
<tr>
<td>Ihh</td>
<td>CPO</td>
<td>E13.5–17.5</td>
<td>Developing palatine bone</td>
<td>Murine lethality not mentioned</td>
<td>Ihh&lt;sup&gt;+/−&lt;/sup&gt;; Ptc-lacZ</td>
<td>Levi et al&lt;sup&gt;30&lt;/sup&gt;, Ohba&lt;sup&gt;31&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cdo, Boc</td>
<td>CPO</td>
<td>E11.5–15.5</td>
<td>Dorsal regions of the developing CNS</td>
<td>Perinatal death, cause not mentioned</td>
<td>Cdo&lt;sup&gt;+/−&lt;/sup&gt;; gene targeting in embryonic stem (ES) cells, deletion of exon 1 and addition of marker fragment BOC&lt;sup&gt;+/−&lt;/sup&gt;; gene targeting of ES cells, deletion of exon 1 fragment and addition of marker gene fragment to exon 2</td>
<td>Zhang et al&lt;sup&gt;32&lt;/sup&gt;, Cole and Krauss&lt;sup&gt;46&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gas1</td>
<td>CPO, 60%</td>
<td>E13.5–15.5</td>
<td>Early craniofacial region</td>
<td>Almost died within the first 3 days of life</td>
<td>Gas1 gene was knocked out in ES cells, and recombinant positive cells were screened out to establish chimeric blastocysts</td>
<td>Lee et al&lt;sup&gt;33&lt;/sup&gt;, Seppala et al&lt;sup&gt;34&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gli2</td>
<td>CPO, 64%</td>
<td>13.5–14.5dpc</td>
<td>Epithelium and mesenchyme of jawbone</td>
<td>NR</td>
<td>Gli2 zinc finger domain was isolated from a 129/Sv genomic library. Genomic DNA was digested with EcoRV and hybridised with a 0.7 kb XbaI-BamHI 5’ probe, or with BamHI and hybridised with a 1.0 kb XbaI-EcoRI 3’ probe</td>
<td>Mo et al&lt;sup&gt;35&lt;/sup&gt;</td>
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<tr>
<td>Gli3</td>
<td>CPO</td>
<td>E13.5–14.0</td>
<td>Mesenchyma and epithelium of palatal shelves</td>
<td>NR</td>
<td>Gli3&lt;sup&gt;+/−&lt;/sup&gt; mice with C57/BL6 genetic background</td>
<td>Huang et al&lt;sup&gt;36&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tmem107</td>
<td>CPO</td>
<td>E13.5–15.5</td>
<td>The front and middle of the palatal shelves</td>
<td>NR</td>
<td>Tmem107&lt;sup&gt;−/−&lt;/sup&gt; mice (all 5 exons of Tmem107 were replaced with a targeting cassette via homologous recombination)</td>
<td>Cela et al&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ick</td>
<td>CPO</td>
<td>E15.5</td>
<td>Epithelial cells</td>
<td>Third trimester embryonic death</td>
<td>Ick&lt;sup&gt;tm1a&lt;/sup&gt;(KOMP)Mbp allele (Ick&lt;sup&gt;tm1a&lt;/sup&gt;) contains an embedded splice receptor sequence and a β-galactosidase reporter gene located between exons 5 and 6</td>
<td>Moon et al&lt;sup&gt;38&lt;/sup&gt;</td>
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<tr>
<td>Ift144</td>
<td>CL/R, 84%</td>
<td>13.5–15.5dpc</td>
<td>Palatal shelves</td>
<td>NR</td>
<td>FVB/C57BL6 F1 twt&lt;sup&gt;+/−&lt;/sup&gt; intercross or FVB/C57BL6 F1 twt&lt;sup&gt;+/−&lt;/sup&gt; × FVB twt&lt;sup&gt;+/−&lt;/sup&gt; backcross</td>
<td>Ashe et al&lt;sup&gt;39&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bbs</td>
<td>CPO</td>
<td>E12.5</td>
<td>Bbs7: endothelial cells, etc; Ift88: bronchial epithelial cells, etc</td>
<td>Prenatal death, double mutant embryos, pericardial oedema</td>
<td>Knockout of Bbs7 combined with a hypomorphic Ift88 allele (orpk as a model for SHH dysfuction)</td>
<td>Zhang et al&lt;sup&gt;40&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fuz</td>
<td>CPO</td>
<td>E18.5</td>
<td>Brain, spinal cord, eye, craniofacial, etc</td>
<td>Death after birth, cause not mentioned</td>
<td>Gene trap cassette inserted in the second exon of the Fuz gene</td>
<td>Gray et al&lt;sup&gt;41&lt;/sup&gt;</td>
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NR, not reported.

### Table 3  Gene knock-in in SHH.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fgf</td>
<td>CPO</td>
<td>E13.5</td>
<td>Posterior region and mesenchyme of the developing palate or nasal epithelium</td>
<td>NR</td>
<td>FGF-R1 recombinant virus with a hemagglutinin epitope tag</td>
<td>Crisera et al&lt;sup&gt;45&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smo</td>
<td>100% CPO</td>
<td>E14.5–15.5</td>
<td>Epithelial cells</td>
<td>The mice died shortly after birth for an undislosed reason</td>
<td>K14-Cre; R26SmolM-2/+; Gli1-LacZ+/-</td>
<td>Li et al&lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NR, not reported.
of Smo from the inactive state to the activated state. SHH pathway activation in mouse embryonic fibroblast cells of Bbs7 knockout and Ift88/orpk homozygous mice decreased by 20% to 30% in a study, showing the CPO phenotype40.

Fuz encodes a planar cell polarity protein involved in ciliogenesis and cargo transport between the base and the tip of the cilium. Knockout of this gene can lead to disorder of cilia development, downregulation of HH signalling and cilia malformation41,42.

Gene knock-in
Gene knock-in, similar to knockout, involves the introduction of foreign functional genes into homologous sequences in cells and genomes via homologous recombination43,44 (Table 3).

FGF can induce epithelial cell proliferation and Shh expression at the very beginning of SHH signalling; the latter can lead to mesenchymal cell proliferation, thus FGF and SHH signalling have a synergistic effect. Recombinant FGF-R1 virus was transfected into mouse palatal shelf cells cut from E13.5 to induce CPO in this tissue in vitro45.

HH signalling is gained in epithelial cells in the K14-Cre; R26SmoM2 mouse model, which exhibits CPO. Normally, during palatal fusion, SHH signalling in MEE cells must be downregulated to ensure palatal shelf fusion. This mouse model enhances HH signalling, leading to maintenance of p63, upregulation of p63 target genes, cell adhesion-associated genes and epithelial progenitor cell-associated genes, and persistence of MEE44.

CKO
CKO can modify specific genes in certain development stages, tissues, and cells of mice, thereby improving specificity47,48. Many OFC models are created in this way (Table 4).

The CKO mouse models of OFCs are relatively few, involving Kif3a, Ift88 and Ptch1. Ptch1 is a membrane receptor for Shh. Activation of the SHH pathway can be regulated by changing the expression levels of Shh or Ptch1. Kif3a and Ift88 are both cilia-associated proteins that also regulate the SHH pathway.

Excessive activation of the SHH pathway leads to failure of secondary palate fusion. A mouse model of K14-induced overexpression of Shh in epithelial cells can be used to mimic the CKO model of Ptch1. Upregulation of Shh signals in epithelial cells of these mice results in severe bone and skin defects, as well as severe craniofacial deformities: a complete cleft of the secondary palate but an intact primary palate in K14-Shh mice.

### Table 4 CKO in SHH.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptch1</td>
<td>Complete cleft of the secondary palate E13.5–16.5</td>
<td>Palate shelves and developing mandibles</td>
<td>NR</td>
<td>A reading frame of mouse Shh cloned downstream of a human K-14 promoter. EcoRl/Hindlll double digest and pronuclear injection</td>
<td>Cobourne et al²²</td>
<td></td>
</tr>
<tr>
<td>CPO</td>
<td>E11.5–13.5</td>
<td>Palatal mesenchyme</td>
<td>NR</td>
<td>K14-Cre</td>
<td>Lan and Jiang⁴⁹</td>
<td></td>
</tr>
<tr>
<td>Kif3a</td>
<td>CPO</td>
<td>E13.5</td>
<td>Palate shelves</td>
<td>NR</td>
<td>Wnt1-Cre; Kif3afl/fl</td>
<td>Li et al⁵¹</td>
</tr>
<tr>
<td>CPO</td>
<td>E11.5</td>
<td>Neural crest cells</td>
<td>NR</td>
<td>Wnt1-Cre; Kif3afl/fl</td>
<td>Liu et al⁵²</td>
<td></td>
</tr>
<tr>
<td>Ptch1</td>
<td>CL</td>
<td>E10.5–11.5</td>
<td>Subfacial mesenchyme</td>
<td>Embryonic lethality at E12.0 (ubiquitous inactivation of Ptch1 in mice leads to early embryonic lethality after 9.5 DPC)</td>
<td>Wnt1-Cre; Patch fl/fl</td>
<td>Metzis et al⁵⁰</td>
</tr>
<tr>
<td>Ift88</td>
<td>CL/P</td>
<td>E10.5–newborn</td>
<td>Palatal epithelium and mesenchyme</td>
<td>Death after birth, cause not mentioned</td>
<td>Wnt1-Cre; Ift88 fl/fl</td>
<td>Tian et al⁴⁷</td>
</tr>
<tr>
<td>CPO</td>
<td>E12.5–newborn</td>
<td>Palatal epithelium and mesenchyme</td>
<td>NR</td>
<td>Osr2-Cre; Ift88 fl/fl</td>
<td>Tian et al⁴⁷</td>
<td></td>
</tr>
<tr>
<td>CPO</td>
<td>E18.5</td>
<td>Palate shelves</td>
<td>NR</td>
<td>Wnt1-Cre; Ift88 fl/fl</td>
<td>Watanabe et al⁴⁶</td>
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<tr>
<td>CPO</td>
<td>E14.5</td>
<td>Palatal rugae</td>
<td>NR</td>
<td>Shh-Cre; Ift88 fl/fl</td>
<td>Nakaniwa et al⁵³</td>
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NR, not reported.
K14 can also be used to induce Shh gene knockout in epithelial cells.\textsuperscript{22,49}

Besides K14-induced overexpression of Shh in epithelial cells, overactivation of the SHH pathway can also be achieved by direct CKO of \textit{Ptch1}. Conditional \textit{Ptch1} knockout mice in facial mesenchyme derived from neural crest cells were obtained using Wnt1-Cre, and exhibited cleft lip. Inhibitory signalling of Hedgehog by \textit{Ptch1} is essential for formation of the nose and upper lip.\textsuperscript{50}

The bind of Shh and Ptch1 is crucial for the formation and function of cilia. When Shh binds Ptch1, the latter loses its inhibitory effect on Smo. Smo localises to cilia to mediate downstream molecules. Thus, the SHH pathway can be regulated by altering cilia-associated proteins or inhibiting cilia-material transport. Kif3a is a microtubule-based anterograde transporter that functions in primary ciliogenesis; it is also required for ciliary basal formation and microtubule anchoring to centrioles. CKO mice show broad midface processes and nasal pits separated by fissures because of ciliary dyskinesia. At the same time, CNCCs do not extend primary cilia, and the bones of the palatal and ventral cranial midline (maxilla, trabecular lamina, palatal and sphenoid) are either laterally displaced or absent, exhibiting the CPO phenotype.\textsuperscript{51,52} Kif3a establishes crosstalk between the SHH and WNT pathways. After primary cilia are established, midline CNCCs require Kif3a function to integrate and respond to WNT signals from the surrounding epithelium.\textsuperscript{52}

Ift88 is involved in primary ciliogenesis and apoptosis and limits bone formation in the MXP. In SHH signalling, Ift88 is located downstream of Smo and upstream of Gli1. In Wnt1-Cre-mediated Ift88 knockout mice, the FNP was widened, the distance between nasal pits was increased, the medial nasal depression was rotated, proliferation of cells in the palatal shelves was decreased, proliferation of neural crest cells in the FNP was significantly decreased, proliferation of neural crest cells in the palatal shelves was significantly increased, and proliferation was decreased. The mice showed the CL/P phenotype.\textsuperscript{47,48,53,54} Osr2 is specifically expressed in the palatal shelves and mesenchyme from E12.5 to birth; Osr2KI-Cre; Ift88\textsuperscript{fl/fl} mice exhibited CPO.\textsuperscript{47}

\textbf{TGF-\beta signalling pathway}

The TGF-\beta signalling pathway is believed to have emerged early in multicellular evolution. TGF-\beta mainly mediates a variety of embryonic and adult signal functions, providing differentiation, proliferation and control of cell- or tissue-specific movement. In most cells, TGF-\beta combines with TGF-\beta receptor II and initiates downstream Smad protein-mediated signal transduction. Smad2 and Smad3 transcription factors in the cytoplasm form heteropolymer complexes and enter

\textbf{Fig 2} Genes associated with orofacial clefts in the TGF-\beta pathway.
the nucleus, thus participating in various physiological and pathological processes (Fig 2).

TGF-β signalling regulates the proliferation, differentiation, migration and apoptosis of epithelial and mesenchymal cells in the lip and palate, thus affecting the fusion of facial prominences, the elevation and fusion of palatal shelves and the development of cartilage and bone. Pathway conduction disorder will lead to CPO and other abnormalities (Fig 2).

**Transgenic**

Transgenesis used in the TGF-β pathway mainly targets BmpRIA and Acvr1, which are receptors in this pathway (Table 5). OFC mouse models have been created using transgenic technology to overexpress BmpRIA and Acvr1 in the TGF-β signalling pathway. Overexpression of BmpRIA in the cranial neural crest mediated by WNT1-Cre leads to CL/P in mice. BMP signalling can regulate cell proliferation in the anterior palatal mesenchyme and maintain the integrity of the posterior palatal epithelium. BmpRIA-mediated BMP signalling activity is enhanced, the cell proliferation rate of the anterior palatal mesenchyme is changed, and ectopic expression of Msx1 and Shox2 in the posterior palatal mesenchyme leads to ectopic chondrogenesis and delayed palatal elevation, resulting in cleft palate formation. K14-Cre-mediated Acvr1 overexpression in palatal epithelium also specifically enhances Smad-dependent BMP signalling, resulting in submucosal cleft palate.

**Chemical induction**

Chemical induction mainly targets TGF-β3 and Bmp, which are ligands in the TGF-β pathway (Table 5). TGF-β3, an important ligand of the TGF-β pathway, plays a domi-

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**Table 5** Transgenic and chemical induction in TGF-β.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Approach</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BmpRIA/Alk3</td>
<td>Transgenic</td>
<td>CL/P</td>
<td>P0</td>
<td>Epithelium and mesenchyme of anterior palate, epithelium of posterior palate</td>
<td>All binary transgenic mice died shortly after birth</td>
<td>Wnt1-Cre mice were mated to pMes-caBmpRia mice</td>
<td>Li et al&lt;sup&gt;58&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acvr1</td>
<td>Transgenic</td>
<td>Submucosal cleft palate, 100%</td>
<td>NR</td>
<td>Palatal epithelium</td>
<td>NR</td>
<td>Mice carrying the caACVR1 allele were mated with mice carrying the K14-Cre allele</td>
<td>Noda et al&lt;sup&gt;59&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Chemical induction</td>
<td>CPO</td>
<td>GD12.5, GD13.5, GD14.5, GD15.5, GD16.5</td>
<td>Pre-fusion palatal midline epithelium</td>
<td>NR</td>
<td>Pregnant mice were given tetrachlorodibenzo-p-dioxin on GD10 with or without folic acid. The control mice received sesame oil on GD10</td>
<td>Li et al&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP receptor</td>
<td>Chemical induction</td>
<td>Partial anterior cleft palate or complete cleft palate</td>
<td>E16.5</td>
<td>NR</td>
<td>NR</td>
<td>Pregnant C57Bl/6J mice were intraperitoneally injected with LDN-193189 from E10.5 to E15.5 at a dose of 3, 6 or 9 mg/kg twice a day</td>
<td>Lai et al&lt;sup&gt;61&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bmp2,4,5 mRNA</td>
<td>Chemical induction</td>
<td>CPO, 100%</td>
<td>E14-16</td>
<td>Many regions of the developing embryo</td>
<td>NR</td>
<td>BALB/c mice exposed to retinoic acid in E12</td>
<td>Lu et al&lt;sup&gt;62&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smad7</td>
<td>Chemical induction</td>
<td>CPO</td>
<td>E14.5</td>
<td>NR</td>
<td>NR</td>
<td>Retinoic acid induced C57BL/6 mice</td>
<td>Yu et al&lt;sup&gt;63&lt;/sup&gt;, Shu et al&lt;sup&gt;65&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lef1</td>
<td>Chemical induction</td>
<td>CPO</td>
<td>E14</td>
<td>NR</td>
<td>NR</td>
<td>Retinoic acid induced C57BL/6 mice</td>
<td>Yu et al&lt;sup&gt;63&lt;/sup&gt;; Shu et al&lt;sup&gt;65&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smad3</td>
<td>Chemical induction</td>
<td>CPO</td>
<td>E10.5-14.5</td>
<td>NR</td>
<td>NR</td>
<td>Retinoic acid induction</td>
<td>Kang et al&lt;sup&gt;64&lt;/sup&gt;, Shu et al&lt;sup&gt;66&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hdac4</td>
<td>Chemical induction</td>
<td>CPO</td>
<td>E10.5-14.5</td>
<td>NR</td>
<td>NR</td>
<td>Retinoic acid induction</td>
<td>Kang et al&lt;sup&gt;64&lt;/sup&gt;, Shu et al&lt;sup&gt;66&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NR, not reported.
nant role in palatogenesis and its fine-tuned expression is temporally and spatially correlated with the critical events surrounding palatal shelf adhesion. Tetrachlorodibenzo-p-dioxin can inhibit the expression of TGF-β3 during palate development and induce abnormal apoptosis in medial edge epithelial (MEE) cells, leading to CPO. Folic acid has no protective effect on 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced cleft palate. Ldn-193189 can manipulate BMP signalling by selectively targeting the BMP/Smad signalling pathway, resulting in a significant reduction of BMP/Smad signalling (p-Smad1/5/8) and unchanged BMP noncanonical signalling (p-p38, p-Erk1/2); the palatal shelves thus fail to adhere properly, resulting in partial anterior or complete cleft palate.

Bmp is also a ligand of the TGF-β pathway. Smads are signal transduction molecules downstream of the Bpm receptor. Following induction with retinoic acid, mice showed a CPO phenotype that was related to Bmp-2/4/5, Smad7, Smad3 and Hdac4. The content of Bmp-2/4/5 mRNA in both condensed and dispersed mesenchyme is reduced, and the ability of undifferentiated mesenchyme to differentiate into osteoblasts is also reduced, resulting in abnormal bone shape development. Smad7 is an inhibitory signal transduction molecule downstream of TGF-β family receptors. Lef1 is a cofactor of the TGF-β pathway that forms a complex with Smad2/Smad4, corresponding to the TGF-β signal. Hdac4 acts as a co-repressor of TGF-β/Smad3-mediated Runx2 functional transcriptional repression in osteoblasts. Retinoic acid-induced reduction of Smad7 and Lef1 expression and cis-element methylation of Smad3 and Hdac4 may be involved in CPO formation.

**Gene knockout**

In the TGF-β signalling pathway, knockout mouse models generally affect the elevation of mouse palatal shelves, leading to the occurrence of OFCs. The genes involved include TGF-β2, Prdm16, Ctgf and Bmp7 (Table 6).

TGF-β2 is a ligand of the TGF-β family and plays a role in the epithelial–mesenchymal transition. Bmp7 is a ligand of the BMP pathway. Ctgf mediates Smad-dependent TGF-β signalling to regulate mesenchymal cell proliferation during palate development. At the same time, Ctgf is a downstream target of TGF-β signalling. The mechanism of CPO after Tgf-β2, Bmp7 or Ctgf knockout is similar to that of Prdm16.

Prdm16 can bind Smads linked to TGF-β and BMP to regulate the transcription of downstream genes such as Gdf6 and Gsc. Following knockout of Prdm16, the arch development of mice is defective, the tongue cannot be properly repositioned and the palatal shelves cannot be properly elevated, leading to CPO (this gene model was also found in CKO, with a complete secondary cleft palate phenotype).

Transcriptional repressors also work in the TGF-β pathway. Ski is a transcriptional repressor of the TGF-β pathway that can bind the Smad2/3/4 signal complex activated by TGF-β signalling and recruit nuclear receptor co-repressor (N-CoR) and the transcription corepressor Hdac. Hdac interacts directly with N-CoR/mSin3A to promote histone deacetylation; this leads to transcription shutdown. Loss of Ski function may lead to dysregulation of TGF-β pathway transcription, resulting in defects in craniofacial morphogenesis, abnormal neural tube and skeletal muscle formation and the CL/P phenotype.

In Myf5−/−;MyoD−/− mice, Tgfbr2 and Bmp7 are downregulated. Myf5 and MyoD are expressed in muscle tissue and participate in muscle paracrine signalling during palate development, affecting palatal shelf fusion. Knockout of Myf5 and MyoD results in CPO. Furthermore, downregulation of Gdf11 expression after Myf5 and MyoD knockout may affect downstream genes, leading to CPO.

Normal palatal development requires multiple mechanisms to balance the effects of agonists and antagonists on BMP signalling. Smoc1 encodes a BMP antagonist, and knockout will affect stability of the BMP gradient. There may be an interaction between Smoc1 and BMP4 leading to the cleft palate phenotype, but this will require experimental verification.

Msx1 is a target of the WNT/β-catenin pathway and also regulates Bmp4. In Msx1 knockout mutants, angiogenesis of the MXP is disrupted and its growth is inhibited, which may lead to the cleft palate phenotype. Knockdown of Msx1 also results in defective proliferation of anterior palatal mesenchymal cells, causing CPO. Several growth factors, including Bmp2, Bmp4 and Shh, can be downregulated. Ectopic expression of Bmp4 in palatal mesenchyme can restore normal cell proliferation and rescue the cleft palate phenotype. The authors hypothesised that Msx1 regulates epithelial–mesenchymal interactions through a network of growth factors: in the anterior palatal shelves, Msx1, which is induced by Bmp4, is required for mesenchymal Bmp4 expression, which in turn functions upstream of Shh and Bmp2, thereby regulating mammalian palate development. Frameshift mutations in Msx homeodomain 6 (MH6, the highly conserved C-terminal domain of Msx1) cause hypoplasia of mandibular incisor teeth with or without cleft palate in mice at embryonic day 16.5 (E16.5), highlighting the role of MH6 in tooth and palate development.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prdm16</td>
<td>CPO, 23%; skeletal abnormalities</td>
<td>E14</td>
<td>Widely expressed</td>
<td>Heart defect, pulmonary insufficiency</td>
<td>Blastocysts were prepared from C57BL/6J mice and the E14.1 ES cells were derived from 129/Ola blastocysts. Male germine chimeras were bred to outbred Black Swiss females (Taconic) to produce F1 offspring heterozygous for the TGF-β2 locus</td>
<td>Sanz et al [85]</td>
</tr>
<tr>
<td>Bmp7</td>
<td>CPO, 100%</td>
<td>E15.5</td>
<td>Palate, tongue, lower lip and other orofacial structures</td>
<td>NR</td>
<td>Delete a conditional Bmp7wt/flx allele by Cre-mediated recombination in the germine</td>
<td>Kouskoura et al [67]</td>
</tr>
<tr>
<td>Ctgf</td>
<td>CPO, 100%</td>
<td>E15.5</td>
<td>Lung, adipocyte, kidney, spleen and thyroid</td>
<td>NR</td>
<td>Replace a 500-bp Smal fragment containing exon 1, the TATA box and the transcription start site with the neomycin resistance gene under the control of a PGK promoter</td>
<td>Ivkovic et al [86]</td>
</tr>
<tr>
<td>Ski</td>
<td>Facial fissures with abnormal formation of fingers and eyes; skeletal muscle defects</td>
<td>E14</td>
<td>Thyroid and pancreas</td>
<td>Death after birth, cause not mentioned</td>
<td>Specific mutation of the exon by targeted vector</td>
<td>Luo et al [69], Berk et al [70]</td>
</tr>
<tr>
<td>Myf5, MyoD</td>
<td>CPO</td>
<td>E18.5</td>
<td>Muscle</td>
<td>NR</td>
<td>Myf5cre allele is used to ablate Myf5-expressing cells in Myf5-NN/R-DTA embryos.</td>
<td>Rot and Kablar [71]</td>
</tr>
<tr>
<td>Gdf11</td>
<td>CPO</td>
<td>E15.5</td>
<td>Maxillary, mandible, palate</td>
<td>NR</td>
<td>Gdf11−/− mice (null mutation)</td>
<td>Rot and Kablar [71]</td>
</tr>
<tr>
<td>Smoc1</td>
<td>CPO</td>
<td>E14.5</td>
<td>Developing pharynx arch and frontal nasal region</td>
<td>Died at or shortly after birth, possibly related to CP</td>
<td>Mice with a targeted pre-conditional mutation in Smoc1 containing a LacZ reporter allele</td>
<td>Rainiger et al [72]</td>
</tr>
<tr>
<td>Msx1</td>
<td>NSCP, 100%(cleft secondary palate)</td>
<td>E14.5</td>
<td>Anterior palatal mesenchyme</td>
<td>NR</td>
<td>1. Msx1−/− mice (null mutation) 2. Msx1-Bmp4 transgenic mice 3. Msx1−/−/Tg</td>
<td>Zhang et al [76]</td>
</tr>
<tr>
<td>Msx homology domain 6 (MH6)</td>
<td>Hypoplasia of lower incisors with or without cleft palate; hypoplasia of molars</td>
<td>E18.5, 4-week-old</td>
<td>Developing limb buds and craniofacial structures, dental papilla and follicle</td>
<td>NR</td>
<td>CRISPR/Cas-mediated genome editing</td>
<td>Mitsui et al [77]</td>
</tr>
<tr>
<td>ActRcll</td>
<td>CPO, 22%, Other deformities as mandibular dystrophy</td>
<td>E18.5</td>
<td>Mandibular component of the first branchial arch</td>
<td>NR</td>
<td>ActRcll-deficient mice. Mutating the ActRcll gene using ES cell technology to delete exon I, hybridisation of mutant heterozygotes. (activin-βA/βB double-mutant mice)</td>
<td>Matzuk et al [78]</td>
</tr>
<tr>
<td>Activin</td>
<td>Missing whiskers and lower incisors, defective second palate, including cleft palate</td>
<td>NR</td>
<td>Mesenchymal cells of the developing face, whiskers, hair follicles, heart and digestive tract</td>
<td>Developed to term but died within 24 hours of birth</td>
<td>1. Disrupted activin-βA allele by embryonic stem cell technology, hybridisation of mutant heterozygotes. (Activin-βA-deficient mice) 2. Activin-βA/βB double-mutant mice (hybridisation of mutant heterozygotes)</td>
<td>Matzuk et al [79]</td>
</tr>
<tr>
<td>Runx2</td>
<td>CPO with skeletal abnormalities, dental defects and failed eyelid fusion</td>
<td>P0</td>
<td>Dental mesenchyme</td>
<td>Died after birth due to respiratory failure</td>
<td>Mutations in ES cells were produced using a substitutional targeting vector. The correct targeted G418-resistant colonies were identified by southern blot analysis of the genomic DNA of SACC-digested ES cells</td>
<td>Lee et al [80], Afzal et al [81], Aberg et al [82], Otto et al [87]</td>
</tr>
</tbody>
</table>

NR, not reported.
Activin is a TGF-β family ligand and a TGF-β pathway receptor. Knockdown of ActRcll results in mandibular dystrophy, Meckel cartilage abnormalities, craniofacial skeletal abnormalities, secondary cleft palate and loss of incisors in 22% of mice, but the main defect in most mice is in reproduction. By contrast, knockout of Activin leads to primary defects in the beard, lower incisors, eyelids and palate. The different phenotypes suggest that ActRcll is likely not a receptor in the Activin-mediated pathway.

Homozygous mutation of Runx2 causes loss of function of Runx2, which is manifested as cleft palate accompanied by skeletal abnormalities, dental defects, eyelid fusion failure and death after birth. Runx2 is a downstream transcription factor of the TGF-β pathway. It can mediate the transcription of corresponding effector genes, and thus promotes the differentiation of mesenchymal precursor cells and induces the differentiation of osteoblasts and bone formation. Loss of function of Runx2 leads to the inhibition of transcription of downstream effector genes of TGF-β/BMP2- and MAPK-dependent signals, as well as the blockage of signal transmission, which leads to inhibition of osteoblast differentiation and bone formation, failure of palatal fusion and CPO.

**Gene knock-in**

Gene knock-in in the TGF-β pathway mainly targets Pitx2 (Table 7). Gene targeting of Pitx2 with phage-mediated targeting vectors results in gene loss, but the nulling cascade, leading to delayed palatal shelf elevation, extension failure and ultimately, CPO.

**Table 7 Gene knock-in in TGF-β.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitx2</td>
<td>CPO, with abnormal mandibular prominence and developmental arrest of the teeth</td>
<td>E14.5</td>
<td>Palatal mesenchyme</td>
<td>NR</td>
<td>Gene targeting by phage-mediated targeting vector</td>
<td>Iwata et al, Lu et al</td>
</tr>
</tbody>
</table>

NR, not reported.

In CKO mouse models, dysfunctions of related genes affect various palatal development stages and finally lead to cleft palate and other OFCs (Table 8).

BmpRIA knockout in the MXP epithelium and stroma mediated by Nestin-Cre results in cleft lip, cleft palate and arrest of tooth development. BmpRIA is a receptor of the TGF-β pathway. Its deletion leads to downregulation of Fgf8, P63 and Pitx1 expression, and premature apoptosis of epithelial cells at the MNP margin results in cleft lip. Abnormal spatiotemporal expression of Barx1 and Pax9, increased apoptosis of mesonasal ectoderm and mesenchymal cells, and defects in proliferation and anterior posterior patterning of maxillary mesenchymal cells lead to cleft palate. The growth and merger of the MNPs with each other and the MXP create the maxillomandibular segment consisting of the upper lip, maxilla and primary palate. Failure of adequate growth or fusion between the processes generates a spectrum of OFCs. Nestin-Cre-mediated knockdown of Bmp4 in the marginal epithelium of the MNP and MXP results in delayed fusion of bilateral MNPs and the MXP, which leads to cleft lip. Eventually, however, most mutants spontaneously repair the cleft lip. Bmp4 functions in the ectoderm of the nasal processes, and the authors hypothesised that Bmp4-BmpRIA signalling plays an important role in lip fusion.

Alk5 is a receptor of the TGF-β pathway. Wnt1-Cre-mediated knockout of Alk5 results in craniomaxillofacial deformities including cleft palate. The authors observed significant changes in the expression of downstream genes Mnx1, Fgf8 and Tgfβ, abnormal apoptosis and cell proliferation in the palatal shelves, and abnormalities in other skeletal craniofacial structures that may also contribute to CPO.

Prdm16 is a transcriptional corepressor of TGF-β signalling that partly inhibits the differentiation of osteoblasts into osteocytes. Prdm16 is also a Smads-binding protein that can form a complex with Smads2/3 and recruit Hdac1, thereby inhibiting TGF-β pathway-mediated signals that partly inhibit cell proliferation during palatal formation by blocking the TGF-β-Fgf9-Pitx2 signalling.
<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Gene Phenotype and penetrance</th>
<th>Embryonic development period</th>
<th>Gene/protein expression</th>
<th>Cause of death</th>
<th>Model generation method</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BmpRIA/Alk3</td>
<td>100% bilateral CLP; dental arrest</td>
<td>10.5 dpc, 11.5 dpc, 14.5 dpc, 18.5 dpc</td>
<td>Complete removal of BmpRIA from the epithelium and mesenchyme of the MXP by 10.5 dpc, and mosaic deletion in the epithelium of the mandibular and nasal processes</td>
<td>NR</td>
<td>Nestin-Cre; BmpRIA null/fox</td>
<td>Liu et al88</td>
</tr>
<tr>
<td>Bmp4</td>
<td>CL</td>
<td>12.0 dpc, 14.5 dpc</td>
<td>Bmp4 was deleted by 10.5 dpc in the edge epithelium of the MNP and MXP</td>
<td>NR</td>
<td>Nestin-Cre; Bmp4 null/fox (n/f)</td>
<td>Liu et al88</td>
</tr>
<tr>
<td>Prdm16</td>
<td>Complete secondary CP; 66%; middle ear defect with severe dysplasia of the tympanic ring, abnormal sex blastoid formation, and dysplasia of the incus and malleus, 100%</td>
<td>E18.5</td>
<td>The anterior part of the secondary palate, pharyngeal arch and head fold</td>
<td>NR</td>
<td>Transducing mice with a producer20 lentivirus23 expressing Prdm16</td>
<td>Zeng et al90, Warner et al91, Shull et al92</td>
</tr>
<tr>
<td>Bmp receptor/Alk2</td>
<td>CPO; Mandibular dysplasty</td>
<td>E14, mutant palatal shelves fail to elevate, unilaterally or bilaterally</td>
<td>The first two pharyngeal arches</td>
<td>Death at birth or shortly after birth with multiple craniofacial defects</td>
<td>Alk2/Wnt1-Cre-mediated</td>
<td>Dudas et al93</td>
</tr>
<tr>
<td>Fgf9</td>
<td>Obvious secondary CP, 100%</td>
<td>E18.5</td>
<td>E9.5–E12.5: ectoderm of the craniofacial region, with spatiotemporal variation E13.5: palatal epithelium E14.5: epithelium and mesenchyme</td>
<td>Death after birth, cause not mentioned</td>
<td>Ddx4-Cre</td>
<td>Iwata et al83, Li et al84</td>
</tr>
<tr>
<td>TGFbr2</td>
<td>Cleft secondary palate and cranial hypoplasia, 100%</td>
<td>E14.5-16.5 (palatal fusion failure)</td>
<td>Palatal mesenchyme</td>
<td>Defects of yolk sac hematopoesis and vasculogenesis</td>
<td>Wnt1-Cre; TGFbr2fl/fl</td>
<td>Ito et al85</td>
</tr>
<tr>
<td></td>
<td>Cleft soft palate, submucosal cleft, and primary and secondary palate fusion failure, 100%</td>
<td>E14.5 (cell proliferation rate and Cyclin D1 were significantly reduced)</td>
<td>Palatal epithelium</td>
<td>Died shortly after birth, lack of milk in the stomach</td>
<td>K14-Cre; TGFbr2fl/fl</td>
<td>Xu et al87</td>
</tr>
<tr>
<td>Alk5</td>
<td>CPO, 100% (especially anterior and posterior to the second palate)</td>
<td>E14, E14.5, E15, E17</td>
<td>Palatal epithelium</td>
<td>Died shortly after birth, lack of milk in the stomach</td>
<td>K14-Cre-mediated</td>
<td>Dudas et al89</td>
</tr>
<tr>
<td></td>
<td>Cranial hypoplasia; oronasal cleft; micromandible; uvula; CPO</td>
<td>E10, E11, E14</td>
<td>Palatal mesenchyme</td>
<td>Severely disfigured, died shortly after birth</td>
<td>Wnt1-Cre-mediated</td>
<td>Dudas et al89</td>
</tr>
<tr>
<td>Hdac3</td>
<td>CPO</td>
<td>E17.5</td>
<td>E9.5-10: widely expressed in the head, including neural crest, ectoderm and endoderm</td>
<td>Cleft palate pups are unable to generate suction and suckling, and subsequently die at P0 from dehydration and air accumulation in the digestive tract</td>
<td>Wnt1-Cre-mediated</td>
<td>Singh et al86</td>
</tr>
<tr>
<td>Kdm6b (Jmjd3)</td>
<td>Complete secondary CP 66%; No CL/P</td>
<td>NR</td>
<td>Cranial neural crest-derived cells</td>
<td>NR</td>
<td>Wnt1-Cre; Kdm6bfl/fl; Krt14-Cre; Kdm6bfl/fl</td>
<td>Guo et al88, Fueyo et al90, Lee et al100</td>
</tr>
</tbody>
</table>

NR, not reported.
signalling. Prdm16 deficiency results in the failure of palatal shelf elevation to meet and fuse at the midline, eventually leading to CPO. Wnt1-Cre-mediated knockout of Alk2 leads to hypoplasia of the jaw; a smaller mouth obstructs normal movement of the tongue, which in turn results in delayed and unsynchronised palatal shelf elevation and secondary cleft palate.

Ddx4-Cre-mediated specific knockout of Fgf9 in germ cells results in significant secondary cleft palate and death shortly after birth. Fgf9 is widely expressed in epithelial and mesenchymal cells. It promotes palatal growth and timely elevation by regulating cell proliferation and accumulation of hyaluronic acid. By influencing tongue descent and morphology and mandibular growth, it ensures there is sufficient space for the process of palate elevation. TGF-β regulates cell proliferation through the Fgf9-Pitx2 signalling cascade during palate formation. The germ-specific knockdown of Fgf9 may lead to obstruction of the Fgf9-Pitx2 signalling cascade and inhibition of palatal formation. At E18.5, the vertical growth of palatal shelves is small, elevation is delayed and contact fails, ultimately leading to CPO.

Loss of Tgfbrr2 in palatal mesenchyme inhibited cyclin D1 expression and affected the proliferation of CNCCs in palatal mesenchyme and palatogenesis, resulting in impaired palatal shelf extension and failure of palatal shelf fusion. The mutant mice presented with cleft secondary palate and skull hypoplasia.

Neural crest cells show a demand for class I histone deacetylase Hdac3 during craniofacial development. Following Hdac3 knockout, G1/S arrest is caused by abnormal cell cycle regulation in mouse neural crest cells. Upregulation of Msh1 and Msh2 in the precranial mesenchyme leads to a marked increase in apoptosis and a decrease in proliferation without proper migration or proliferation. In addition, Bmp4 upregulation results in failure of palatal shelf expansion and ultimately, cleft palate.

Progressive disintegration of the midline epithelial seam as well as removal of the transient epithelial seams begins following contact of the palatal shelves. A mouse model of K14-Cre-mediated ectodermal epithelial-specific knockout of Alk5 showed cleft palate. Loss of Alk5 leads to the failure of palatal epithelial seam disappearance. TGF-βR2 is a receptor of the TGF-β pathway. K14-Cre-mediated knockout leads to the downregulation of Irf6 and Mmp13, interfering with apoptosis in MEFs.

Wnt1-Cre-mediated specific knockout of Kdm6b results in complete cleft palate with defects in the soft palate and death shortly after birth. Kdm6b is widely expressed in the palate, but K14-Cre-mediated epithelial-specific knockout of Kdm6b did not cause CL/P, indicating that palate development depends on Kdm6b in CNCCs rather than epithelial cells. Kdm6b (Jmjd3) is a cofactor of the TGF-β pathway and is required for enhancer activation when TGF-β is stimulated. Ras-activated Kdm6b contributes to TGF-β-induced Smad2 and Smad3 activation by promoting syntenin-mediated TGF-βRI/Smad2/3 complex formation, thereby promoting TGF-β-induced epithelial–mesenchymal transition. A lack of Kdm6b leads to inhibition of the epithelial–mesenchymal transition, limited proliferation and differentiation of CNCCs and development failure, resulting in CPO.

Discussion

The present review offers a systematic summary of various mouse models of OFCs and focuses on elucidating the roles of defective genes involved in the SHH and TGF-β signalling pathways and the genetic aetiology of corresponding phenotypes.

Shh, Ihh, Smo, Pitx1, Cdo, Boc and Glis are among the Hedgehog signalling genes. Except for loss of Glil1 and Pitx1, all mice defective in these genes exhibit CPO. Glil1 knockout mice show CL/P, while Pitx1 loss causes cleft lip. Deletion of Ihh affects osteogenesis of the secondary palate. Deletion of Smo leads to upregulation of p63 and its target gene. Deletion of Gli is mainly related to failure of elevation and fusion of the palatal shelves. Pitx1 is crucial to the formation of the original nose and upper lip. Hedgehog signalling during embryogenesis depends on primary cilia function and intra-flagellar transport. Ick, Tmem107, Ift144, Ift88, Fuz and Kif3a are closely related to the development of cilia. Deletion of these genes leads to a disorder of cilia development and blocks normal Hedgehog signalling. Except for Ift144 deletion and Wnt-1-Cre-mediated Ift88 CKO, other deletions lead to CPO. Ablation of Gas1, FGF and Bhs, which regulate Hedgehog signalling, results in CPO (Fig 1).

Among the TGF-β signalling molecules, members of the Smads family are important molecules that transmit extracellular signals to the nucleus. Ctgf, Prdm16, Myf5, MyoD, Gdf11, Smoc1, Msh1, Kdm6b and Fgf9 regulate the signalling pathway. Pitx2, Runx2, Ski and Hdac3 are targets. The loss of most molecules leads to CPO (Fig 2).

Conditional activation of BmpR1A mediated by Wnt1-Cre leads to CL/P. The Bmp4-BmpR1A pathway plays an important role in lip fusion. Nestin-Cre-mediated CKO of BmpR1A leads to CL and CP, while Bmp4 leads
to CL, both causing MNP and MXP proliferation defect and fusion delay. Knockout of Tgf-β2, Bmp7, Pitx2, Fgf9, Prdm16 and Alk2 result in CPO due to delay or obstruction of palatal shelf elevation. Among these, loss of Prdm16 and Alk2 lead to mandibular development defects, resulting in an inability to reposition the tongue properly. In Acvr1, Tgf-β3 and Alk5 KO mice and K14-Cre; TGF-βR2 CKO mice, MEE cells fail to disappear. Wnt1-Cre; TGFβ-R2 CKO and Hdac3 defects affect the proliferation of CNCCs in the palatal mesenchyme, leading to failure of palatal shelf extension and fusion. Although the different TGF-βR2 CKO models both show cleft palate, the affected stages differ because the knockout happens in the epithelium and mesenchyme, respectively. The loss of Bmp-2/4/5, Ctgf, Runx2, Prdm16 and Kdm6b is related to the abnormal development of cartilage and bone, thus leading to CPO.

Knockout of different genes in the two pathways may lead to OFCs through similar mechanisms (such as palatal shelf elevation disorder or abnormal fusion of facial prominences) (Fig 3). The same gene can also function in multiple pathways. These signalling pathways do not act in isolation during lip and palate development; instead, they interact with each other through several important molecules including p63, Fgf5, Mx1 and Kif3a.

Different modelling methods have their own characteristics. As the earliest method used in this field, chemical induction is relatively simple and intuitive, but the impact of the environment cannot be excluded, and the specific mechanism involved cannot be proven. As in the TGF-β pathway, retinoic acid induction can lead to changes in several molecules, thereby weakening the correlation between any single molecule and OFCs. Knockout mice have improved these problems, but the important genes related to morphogenesis often play multiple roles in embryonic development. Even in the lip and palate, they may also have different functions in different parts and types of cells. Moreover, complete knockout in the embryo may lead to serious embryonic lethality and severe syndromes, which hampers in-depth research. As an improvement, knock-in enhances pertinence through site-directed mutagenesis; however, the abnormal phenotype may be hidden due to compensation. In recent years, CKO has solved the obstacle of early embryo lethality and greatly improved accuracy through tissue-specific gene knockout, thus gradually becoming the most powerful and most commonly used method.

The present study is not without limitations. Certain genes might possess multiple functions across both the lip/palate and other regions of the body, leading to severe syndromes and fatal malformations. This complexity hampers our ability to distinctly elucidate the specific mechanisms driving OFCs. Additionally, distinguishing primary OFCs from those secondary to other craniomaxillofacial malformations can pose challenges. The redundancy in gene function can also obscure certain abnormalities. While the present authors have comprehensively summarised gene mutation sites within the TGF-βs, SHH and WNT pathways in recent years, numerous mutation sites outside these
pathways remain unexplored. Other pathways relevant to OFCs, not discussed in this article, are also waiting to be uncovered and consolidated. Furthermore, our understanding of the intricate interconnections among genes in specific pathways is still incomplete, and we have yet to fully elucidate how, when and where signalling pathways intersect and converge. To advance our research, a more profound grasp of the intricate interactions governing lip/palate development through these signalling pathways is crucial.

The integration of single-cell multiomics into the study of mouse models of OFCs presents an innovative avenue for unravelling the complex pathogenic mechanisms underlying these congenital anomalies. By enabling high-resolution characterisation of gene expression patterns at the level of individual cells, single-cell multiomics techniques have the potential to offer unparalleled insights into the molecular and cellular events that contribute to the development of OFCs. The recent use of single-cell RNA sequencing (scRNA-seq) datasets in craniofacial research underscores the potency of this approach in deciphering the heterogeneity of cell populations during critical palate formation stages. This encompasses the identification of specific cell types, exploration of shared expression patterns across datasets and unveiling of potential regulatory networks involving pivotal candidate genes. As we navigate the era of single-cell multiomics, future research should concentrate on refining data analysis methodologies to ensure precise cluster resolution and cross-dataset comparisons. Tackling technical challenges related to data integration, cell type annotation and noise reduction will be pivotal in unlocking the full potential of this technology. Additionally, coupling the application of single-cell multiomics with spatial transcriptomic analyses will help unveil the spatial organisation of gene expression within tissue structures, offering a holistic perspective on how molecular events impact tissue architecture during craniofacial development. Collaborative efforts between bioinformaticians, developmental biologists and clinicians will play a crucial role in translating these findings into clinically relevant insights, ultimately advancing our comprehension of the aetiology of CL/P and facilitating the development of targeted therapeutic interventions.

Currently, most mouse models primarily target functional gene regions. While the majority of attention has historically been directed towards protein-coding genes, emerging evidence underscores the pivotal role of ncRNA, particularly miRNA, in orchestrating gene expression networks that govern tissue development and differentiation, and homeostasis of the lip and palate. Moving forward, the creation of mouse models for OFCs based on miRNA and other ncRNA mutations should be explored. Furthermore, given the growing recognition that non-coding RNAs often exhibit tissue- and developmental stage-specific expression patterns, mouse models offer a unique opportunity to investigate the context-dependent roles of miRNA in palate formation. By manipulating miRNA expression during distinct developmental stages, researchers can pinpoint critical windows of vulnerability and better understand how miRNA dysregulation contributes to the aetiology of OFCs. These insights hold promise for the development of targeted interventions that aim to normalise miRNA expression and restore proper craniofacial development. Additionally, innovative strategies for modulating miRNA activity, such as miRNA mimics or inhibitors, can be explored in mouse models to assess their potential as therapeutic interventions.

At present, clinical application of OFC pathogenic genes is mainly used to provide a basis for early screening of familial genetic disorders, and the treatment of OFCs is still mainly focused on traditional repair surgery. In the future, the application of small molecule inhibitors or specialised nutritional elements during early embryonic development could hold the potential to reverse the occurrence of OFCs. By targeting key signalling pathways and molecular processes implicated in craniofacial development, these interventions could potentially mitigate the disruptions that give rise to OFCs. This approach could address the underlying molecular and cellular disruptions that lead to cleft formation, offering the advantage of avoiding surgical procedures and their associated risks. Additionally, intervening at the embryonic stage might allow for more natural and holistic corrections in tissue development, potentially yielding better functional and aesthetic outcomes. However, while this concept holds great promise, its implementation requires careful consideration and extensive research. The precise identification of critical developmental time windows and the specific signalling pathways amenable to modulation are essential to maximise the effectiveness of such interventions. Rigorous preclinical studies using mouse models should be conducted to validate the safety, efficacy and potential long-term consequences of employing small molecule inhibitors or nutritional elements in altering embryonic development.

Conclusion

In summary, the horizon of research into OFCs is undergoing a transformative shift, propelled by the innovative
potential of mouse models. Alongside the traditional paradigms of functional gene targeting, emerging avenues such as single-cell multiomics and the exploration of ncRNA mutations are set to reshape the landscape of our understanding. The integration of single-cell multiomics techniques promises an unprecedented resolution in characterising gene expression patterns across individual cells, unravelling the intricate molecular events that shape CL/P development. Furthermore, the recognition of the role of miRNA and other ncRNA in the aetiology of CL/P has opened up a compelling avenue. Researchers will increasingly zero in on core target molecules within pathways, striving to elucidate comprehensive chains of events and crosstalk across diverse pathways. Having a deeper understanding of the intricate mechanisms and complex interactions that underpin OFCs will enhance prospects for treatment and prevention, ultimately fostering improved natal and prenatal care as well as nurturing practices. As a pivotal genetic disorder within the maxillofacial domain, OFC-related research has the potential to substantially mitigate the profound impact of birth defects on both individuals and society as a whole.

Conflicts of interest
The authors declare no conflicts of interest related to this study.

Author contribution
Drs Yu Chen LI, Le Ran LI, Zi Han GAO, Yi Ran YANG, Qian Chen WANG and Wei Yu ZHANG contributed to drafting the manuscript; Drs Tian Song XU, Li Qi ZHANG and Feng CHEN contributed to the review and revision of the manuscript; Dr Feng CHEN provided the idea and made the conceptual framework.

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