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

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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.*

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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 influences[1-3]. 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 palate[4]. 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 mechanisms[5]. 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,

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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 modification[6-8]. MicroRNAs (miRNAs) play a crucial role in silencing the expression of specific genes[8,9]. Environmental factors such as nutrition and smoking have a significant impact on DNA methylation patterns[10].

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 sequence[11], 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 signalling[12]. 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 abnormalities[12-14]. The mouse models of CL/P and CPO have been introduced in detail in previous reviews[15,16]. 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 regeneration[17]. 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 palate[18-20]. Downregulation of this signal in the palate and medial nasal processes (MNPs) 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 models[21] (Table 1). Transgenesis used in the SHH pathway mainly targets Ptch1 and Hedgehog acyltransferase (Hhat).

As an important factor in the initial transmembrane process of SHH signalling, Ptch1 is a negative regulator that inhibits the transmembrane protein Smo. Upon binding to Shh, Ptch1 is degraded. *Ptch1* 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 *Ptch1* knockout. This model results in CPO[22].

In addition to the important role played by Ptch1 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

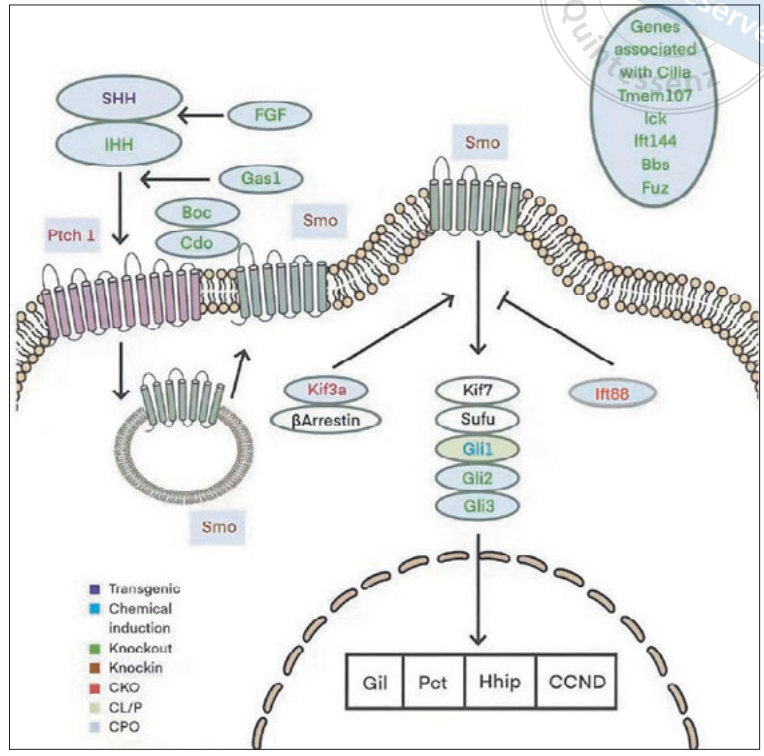

Fig 1 Genes associated with orofacial clefts in the SHH pathway.

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, cyclopamine 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 cyclopamine and vismodegib. Both are antagonists of the SHH pathway and the induced mouse phenotype is CL/P. Cyclopamine directly binds Smo, changing its conformation to inhibit Hh pathway activation. Vismodegib can be chemically modified by cyclopamine; its principle is the same as cyclopamine 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 cyclopamine 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 cyclopamine; 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

Table 1 Transgenic and chemical induction in SHH.

Gene	Approach	Phenotype and penetrance	Embryonic development period	Gene/protein expression	Cause of death	Model generation method	Studies
Ptch1	Transgenic	CPO	E14.5–15.5	E13.5: oral epithelium of the palatal shelves, with a corresponding gradient of Ptch1 expression in the underlying mesenchyme	Perinatal lethality	EcoRI/HindIII double digest and pronuclear DNA injection of CBA/C56 BL6 embryos on E0.5. Isolating K14-SHH transgene	Cobourne et al[22]
Hedgehog acyltransferase (*Hhat*)	Transgenic	CPO	E9.5–14.5	The pharyngeal and midfacial hypoplasia evident in the form of a single nasal slit	Lymphatic and vascular anomalies	Hhat$^{+/Creface}$	Dennis et al[23]
	Chemical induction	Bilateral CLP	NR	Endoderm of the first, second and third pharyngeal pouches, and ectodermal-endodermal boundaries	NR	Retinoic acid induction	Helms et al[25]
	Chemical induction	CL/P	GD17	Decreased snout length and mandible length and increased interocular distance	NR	Cyclopamine induction	Chen et al[26], Lipinski et al[29]
	Chemical induction	CL/P	GD7.0-8.25	Highly arched palate	NR	Vismodegib induction	Heyne et al[27]
Gli1	Chemical induction	CL/P	GD11.0–14.0	Medial nasal processes (MNPs)	NR	Cycloparamide induction of female mice on GD8.25–9.375, 120 mg/kg/day	Everson et al[28]

NR, not reported.

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[32]. 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[33,34].

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[35]. 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[36].

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[37]. 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[38]. 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[39]. Bbs can reduce the level of Smo in cilia but can also regulate the transformation

Table 2 Gene knockout in SHH.

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

NR, not reported.

Table 3 Gene knock-in in SHH.

Gene	Phenotype and penetrance	Embryonic development period	Gene/protein expression	Cause of death	Model generation method	Studies
Fgf	CPO	E13.5	Posterior region and mesenchyme of the developing palate or nasal epithelium	NR	FGF-R1 recombinant virus with a hemagglutinin epitope tag	Crisera et al[45]
Smo	100% CPO	E14.5–15.5	Epithelial cells	The mice died shortly after birth for an undisclosed reason	K14-Cre; R26SmoM-2fl/+; Gli1-LacZ+/-	Li et al[44]

NR, not reported.

Table 4 CKO in SHH.

Gene	Phenotype and penetrance	Embryonic development period	Gene/protein expression	Cause of death	Model generation method	Studies
Ptch1	Complete cleft of the secondary palate	E13.5–16.5	Palate shelves and developing mandibles	NR	A reading frame of mouse Shh cloned downstream of a human K-14 promoter. EcoRI/HindIII double digest and pronuclear injection	Cobourne et al[22]
	CPO	E11.5–13.5	Palatal mesenchyme	NR	K14-Cre	Lan and Jiang[49]
Kif3a	CPO	E13.5	Palate shelves	NR	Wnt1-Cre; Kif3afl/fl	Li et al[51]
	CPO	E11.5	Neural crest cells	NR	Wnt1-Cre; Kif3afl/fl	Liu et al[52]
Ptch1	CL	E10.5–11.5	Subfacial mesenchyme	Embryonic lethality at E12.0 (ubiquitous inactivation of Ptch1 in mice leads to early embryonic lethality after 9.5 DPC)	Wnt1-Cre; Patch fl/fl	Metzis et al[50]
Ift88	CL/P	E10.5–newborn	Palatal epithelium and mesenchyme	Death after birth, cause not mentioned	Wnt1-Cre; Ift88 fl/fl	Tian et al[47]
	CPO	E12.5–newborn	Palatal epithelium and mesenchyme	NR	Osr2-Cre; Ift88 fl/fl	Tian et al[47]
	CPO	E18.5	Palate shelves	NR	Wnt1-Cre; Ift88 fl/fl	Watanabe et al[48]
	CPO	E14.5	Palatal rugae	NR	Shh-Cre; Ift88 fl/fl	Nakaniwa et al[53]

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 phenotype[40]. *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 malformation[41,42].

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 recombination[43,44] (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 vitro[45].

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 MEE[44].

CKO

CKO can modify specific genes in certain development stages, tissues, and cells of mice, thereby improving specificity[47,48]. 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.

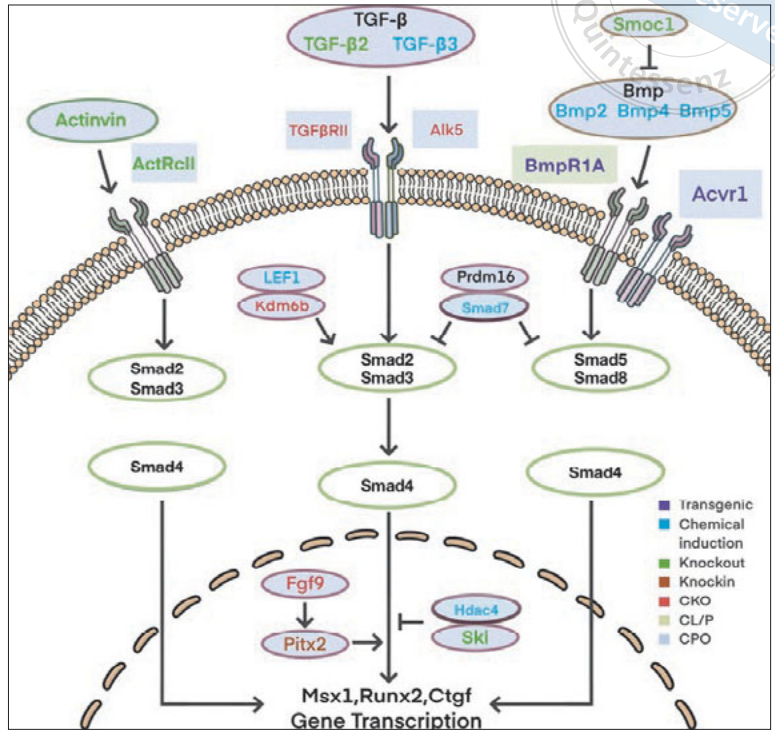

Fig 2 Genes associated with orofacial clefts in the TGF-β pathway.

K14 can also be used to induce Shh gene knockout in epithelial cells[22,49].

Besides K14-induced overexpression of Shh in epithelial cells, overactivation of the SHH pathway can also be achieved by direct CKO of *Ptch1*. Conditional *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 Ptch1 is essential for formation of the nose and upper lip[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[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[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, apoptosis of cells in the palatal shelves was significantly increased, and proliferation was decreased. The mice showed the CL/P phenotype[47,48,53,54]. Osr2 is specifically expressed in the palatal shelves and mesenchyme from E12.5 to birth; *Osr2KI-Cre; Ift88*$^{fl/fl}$ mice exhibited CPO[47].

TGF-β signalling pathway

The TGF-β signalling pathway is believed to have emerged early in multicellular evolution. TGF-β mainly mediates a variety of embryonic and adult signal functions, providing differentiation, proliferation and control of cell- or tissue-specific movement[55]. In most cells, TGF-β combines with TGF-β receptor II and initiates downstream Smad protein-mediated signal transduction. Smad2 and Smad3 transcription factors in the cytoplasm form heteropolymer complexes and enter

Table 5 Transgenic and chemical induction in TGF-β.

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

NR, not reported.

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[56,57]. 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[58]. K14-Cre-mediated *Acvr1* overexpression in palatal epithelium also specifically enhances Smad-dependent BMP signalling, resulting in submucosal cleft palate[59].

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-

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 palatine 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[60]. 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[61].

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[62]. 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[63]. Hdac4 acts as a co-repressor of TGF-β/Smad3-mediated Runx2 functional transcriptional repression in osteoblasts64. Retinoic acid-induced reduction of *Smad7* and *Lef1* expression[65] and cis-element methylation of Smad3 and Hdac4[63] 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 palatal 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*[67].

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)[68].

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[69]. 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[70].

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[71].

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[72].

Msx1 is a target of the WNT/β-catenin pathway and also regulates Bmp4[73]. In *Msx1* knockdown mutants, angiogenesis of the MXP is disrupted and its growth is inhibited, which may lead to the cleft palate phenotype[74,75]. 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[76]. 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[77].

Table 6 Gene knockout in TGF-β.

Gene	Phenotype and penetrance	Embryonic development period	Gene/protein expression	Cause of death	Model generation method	Studies
Prdm16	CPO	GD14.5	Widely expressed	NR	Prdm16 expression was inactivated by a gene-trap that inserted the gene for β-galactosidase between exons 1 and 2	Warner et al[68]
	CPO, 23%; skeletal abnormalities	E14	Widely expressed	Heart defect, pulmonary insufficiency	Blastocysts were prepared from C57BL/6J mice and the E14.1 ES cells were derived from 129/Ola blastocysts. Male germline chimeras were bred to outbred Black Swiss females (Taconic) to produce F1 offspring heterozygous for the TGF-β2 locus	Sanford et al[85]
Bmp7	CPO, 100%	E15.5	Palate, tongue, lower lip and other orofacial structures	NR	Delete a conditional Bmp7wt/flx allele by Cre-mediated recombination in the germline	Kouskoura et al[67]
Ctgf	CPO, 100%	E15.5	Lung, adipocyte, kidney, spleen and thyroid	NR	Replace a 500-bp SmaI fragment containing exon 1, the TATA box and the transcription start site with the neomycin resistance gene under the control of a PGK promoter	Ivkovic et al[86]
Ski	Facial fissures with abnormal formation of fingers and eyes; skeletal muscle defects	E14	Thyroid and pancreas	Death after birth, cause not mentioned	Specific mutation of the exon by targeted vector	Luo et al[69], Berk et al[70]
Myf5, MyoD	CPO	E18.5	Muscle	NR	Myf5cre allele is used to ablate Myf5-expressing cells in Myf5-NN/R-DTA embryos.	Rot and Kablar[71]
Gdf11	CPO	E15.5	Maxillary, mandible, palate	NR	Gdf11-/- mice (null mutation)	Rot and Kablar[71]
Smoc1	CPO	E14.5	Developing pharynx arch and frontal nasal region	Died at or shortly after birth, possibly related to CP	Mice with a targeted pre-conditional mutation in Smoc1 containing a LacZ reporter allele	Rainger et al[72]
Msx1	NSCP, 100%(cleft secondary palate)	E14.5	Anterior palatal mesenchyme	NR	1. *Msx1*$^{-/-}$ mice (null mutation) 2. *Msx1-Bmp4* transgenic mice 3. *Msx1*$^{-/-}$*/Tg*	Zhang et al[76]
Msx homology domain 6 (MH6)	Hypopplasia of lower incisors with or without cleft palate; hypoplasia of molars	E18.5, 4-week-old	Developing limb buds and craniofacial structures, dental papilla and follicle	NR	CRISPR/Cas-mediated genome editing	Mitsui et al[77]
ActRcII	CPO, 22%, Other deformities as mandibular dystrophy	E18.5	Mandibular component of the first branchial arch	NR	ActRcII-deficient mice. Mutating the ActRcII gene using ES cell technology to delete exon I, hybridisation of mutant heterozygotes. (activin-βA/βB double-mutant mice)	Matzuk et al[78]
Activin	Missing whiskers and lower incisors, defective second palate, including cleft palate	NR	Mesenchymal cells of the developing face, whiskers, hair follicles, heart and digestive tract	Developed to term but died within 24 hours of birth	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)	Matzuk et al[79]
Runx2	CPO with skeletal abnormalities, dental defects and failed eyelid fusion	P0	Dental mesenchyme	Died after birth due to respiratory failure	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	Lee et al[80], Afzal et al[81], Aberg et al[82], Otto et al[87]

NR, not reported.

Table 7 Gene knock-in in TGF-β.

Gene	Phenotype and penetrance	Embryonic development period	Gene/protein expression	Cause of death	Model generation method	Studies
Pitx2	CPO, with abnormal mandibular prominence and developmental arrest of the teeth	E14.5	Palatal mesenchyme	NR	Gene targeting by phage-mediated targeting vector	Iwata et al[83], Lu et al[84]

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[78]. By contrast, knockout of *Activin* leads to primary defects in the beard, lower incisors, eyelids and palate[79]. 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[80]. 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[81]. 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[82].

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; corresponding mouse models can be established, resulting in cleft palate with abnormal cardiac morphogenesis, abnormal maxillary and mandibular facial prominences and arrested tooth development. As a downstream transcription factor of the TGF-β pathway, Pitx2 is widely expressed in epithelium and mesenchyme. The TGF-β-Fgf9-Pitx2 signalling cascade involving Pitx2 promotes the proliferation of mesenchymal cells in the process of palatal formation. As a downstream target of Fgf9, Pitx2 can regulate cell proliferation by directly activating expression of the genes cyclin *D1* and *D3*[83]. Loss of function of Pitx2 may inhibit cell proliferation during palatal formation by blocking the TGF-β-Fgf9-Pitx2 signalling cascade, leading to delayed palatal shelf elevation, extension failure and ultimately, CPO[84].

CKO

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[88]. 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[88].

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 *Msx1*, *Fgf8* and *Tgif*, abnormal apoptosis and cell proliferation in the palatal shelves, and abnormalities in other skeletal craniofacial structures that may also contribute to CPO[89].

Prdm16 is a transcriptional corepressor of TGF-β signalling that partly inhibits the differentiation of osteoblasts into osteocytes[90]. Prdm16 is also a Smads-binding protein that can form a complex with Smads2/3 and recruit Hdac1, thereby inhibiting TGF-β pathway

Table 8 CKO in TGF-β.

Gene	Phenotype and penetrance	Embryonic development period	Gene/protein expression	Cause of death	Model generation method	Studies
BmpRIA/Alk3	100% bilateral CLP; dental arrest	10.5 dpc, 11.5 dpc, 14.5 dpc, 18.5 dpc	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	NR	*Nestin-Cre; BmpRIA* $^{null/flox}$	Liu et al[88]
Bmp4	CL	12.0 dpc, 14.5 dpc	Bmp4 was deleted by 10.5 dpc in the edge epithelium of the MNP and MXP	NR	*Nestin-Cre; Bmp-4*$^{null/flox(n/f)}$	Liu et al[88]
Prdm16	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%	E18.5	The anterior part of the secondary palate, pharyngeal arch and head fold	NR	Transducing mice with a pInducer20 lentivirus23 expressing Prdm16	Zeng et al[90], Warner et al[91], Shull et al[92]
Bmp I receptor/Alk2	CPO; Mandibular dystrophy	E14, mutant palatal shelves fail to elevate, unilaterally or bilaterally	The first two pharyngeal arches	Death at birth or shortly after birth with multiple craniofacial defects	Alk2/Wnt1-Cre-mediated	Dudas et al[93]
Fgf9	Obvious secondary CP, 100%	E18.5	E9.5–E12.5: ectoderm of the craniofacial region, with spatiotemporal variation E13.5: palatal epithelium E14.5: epithelium and mesenchyme	Death after birth, cause not mentioned	*Ddx4-Cre*	Iwata et al[83], Li et al[94]
TGFbr2	Cleft secondary palate and cranial hypoplasia, 100%	E14.5-16.5 (palatal fusion failure)	Palatal mesenchyme	Defects of yolk sac hematopoiesis and vasculogenesis	*Wnt1-Cre; TGFbr2*$^{fl/fl}$	Ito et al[95]
	Cleft soft palate, submucosal cleft, and primary and secondary palate fusion failure, 100%	E14.5 (cell proliferation rate and Cyclin D1 were significantly reduced)	Palatal epithelium	Died shortly after birth, lack of milk in the stomach	*K14-Cre; TGFbr2*$^{fl/fl}$	Xu et al[97]
Alk5	CPO, 100% (especially anterior and posterior to the second palate)	E14, E14.5, E15, E17	Palatal epithelium	Died shortly after birth, lack of milk in the stomach	K14-Cre-mediated	Dudas et al[89]
	Cranial hypoplasia; oronasal cleft; micromandible; uvula; CPO	E10, E11, E14	Palatal mesenchyme	Severely disfigured, died shortly after birth	Wnt1-Cre-mediated	Dudas et al[89]
Hdac3	CPO	E17.5	E9.5-10: widely expressed in the head, including neural crest, ectoderm and endoderm	Cleft palate pups are unable to generate suction and suckling, and subsequently die at P0 from dehydration and air accumulation in the digestive tract	Wnt1-Cre-mediated	Singh et al[96]
Kdm6b (Jmjd3)	Complete secondary CP, 66% No CL/P	NR	Cranial neural crest-derived cells	NR	*Wnt1-Cre; Kdm6b*$^{fl/fl}$ *Krt14-Cre; Kdm6b*$^{fl/fl}$	Guo et al[98], Fueyo et al[99], Lee et al[100]

NR, not reported.

signalling[91]. Prdm16 deficiency results in the failure of palatal shelf elevation to meet and fuse at the midline, eventually leading to CPO[92]. 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[93].

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[94]. 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[94]. TGF-β regulates cell proliferation through the Fgf9-Pitx2 signalling cascade during palate formation[83]. 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[94].

Loss of Tgfbr2 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[95].

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 Msx1 and Msx2 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[96].

Progressive disintegration of the midline epithelial seam as well as removal of the transient epithelial seams begin following contact of the palatal shelves[16]. 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[89]. 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 MEE[97].

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[98]. Kdm6b (Jmjd3) is a cofactor of the TGF-β pathway and is required for enhancer activation when TGF-β is stimulated[99]. Ras-activated Kdm6b contributes to TGF-βI-induced Smad2 and Smad3 activation by promoting syntenin-mediated TGF-βRI/Smad2/3 complex formation, thereby promoting TGF-β-induced epithelial–mesenchymal transition[100]. A lack of Kdm6b leads to inhibition of the epithelial–mesenchymal transition, limited proliferation and differentiation of CNCCs and development failure, resulting in CPO[98].

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*, *Ptch1*, *Cdo*, *Boc* and *Glis* are among the Hedgehog signalling genes. Except for loss of *Gli1* and *Ptch1*, all mice defective in these genes exhibit CPO. *Gli1* knockout mice show CL/P, while *Ptch1* 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. *Ptch1* 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 *Bbs*, 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, Msx1, 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-BmpRIA* pathway plays an important role in lip fusion. Nestin-Cre-mediated CKO of *BmpRIA* leads to CL and CP, while *Bmp4* leads

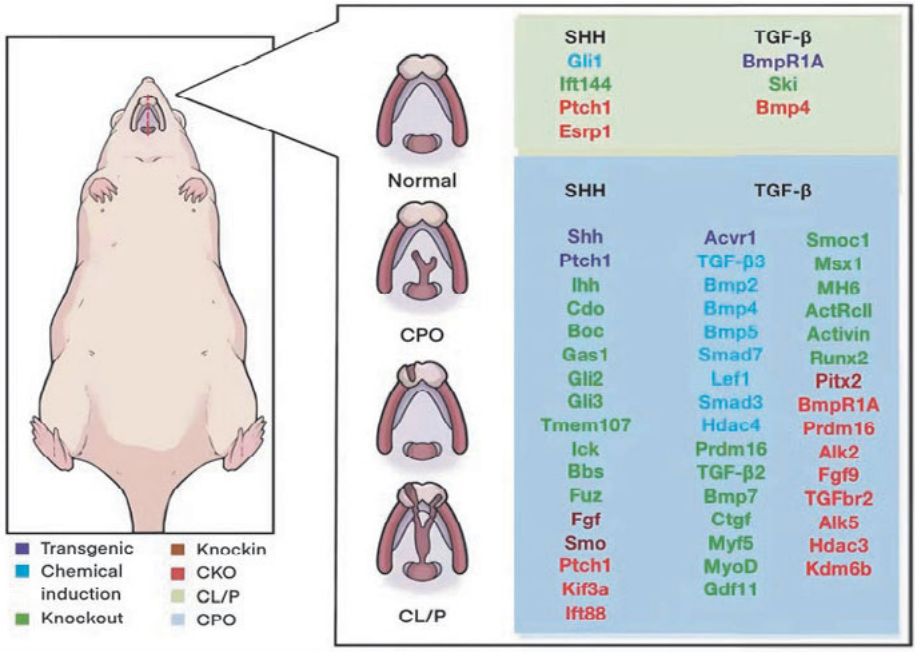

Fig 3 Phenotypic classification of OFC mouse models in the SHH and TGF-β pathways.

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, Fgfs, Msx1* 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[101]. 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[101]. 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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