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The mechanism of TGF- β signaling during palate development

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Abstract

Cleft palate, a malformation of the secondary palate development, is one of the most common human congenital birth defects. Palate formation is a complex process resulting in the separation of the oral and nasal cavities that involves multiple events, including palatal growth, elevation, and fusion. Recent findings show that transforming growth factor beta (TGF- β) signaling plays crucial roles in regulating palate development in both the palatal epithelium and mesenchyme. Here, we highlight recent advances in our understanding of TGF- β signaling during palate development.

Keywords

Cranial neural crest cells; TGF- β signaling mechanism; Cleft palate

Introduction

Cleft lip with or without cleft palate (CL/P) is among the most common craniofacial deformities in humans, with ethnic and geographic variations in prevalence, and affects feeding, digestion, speech, hearing, middle-ear ventilation, respiration, appearance, and more (Goudy *et al*, 2006; Berbert-Campos, 2007; Chen *et al*, 2008; Cooper-Brown *et al*, 2008). Patients with cleft palate require care from birth to adulthood from many disciplines, including nursing, plastic surgery, maxillofacial surgery, otolaryngology, speech therapy, audiology, psychological and genetic counseling, orthodontics, and dental treatment (De Bodt and Van Lierde, 2006; Prah-Andersen and Ju, 2006; Arosarena, 2007; Aminpour and Tollefson, 2008; Costello *et al*, 2008; Kasten *et al*, 2008; Panetta *et al*, 2008; Sitzman *et al*, 2008). The care for patients with cleft palate remains a cause for concern, particularly in developing countries where cleft palate is an economic burden, and the quality of care still varies substantially (Wehby and Cassell, 2010). Therefore, prevention of cleft palate is the ultimate objective. Here, we introduce some new ideas regarding the mechanisms of cleft palate, with the hope that we can prevent these congenital birth defects.

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Author contributions

J. Iwata made substantial contributions to the conception and design of the paper, drafted and revised the article critically for important intellectual content and was involved in the final approval of the version to be published. C. Parada revised it for intellectual content and was involved in the final approval of the version to be published. Y. Chai made substantial contributions to the conception and design of the paper, revised the article critically for important intellectual content, and was involved in the final approval of the version to be published. All authors read and approved the final manuscript.

Palate formation

Etiology of cleft lip with or without cleft palate

CL/P has an incidence of 1/500–1/1000 birth worldwide (Wong and Hagg, 2004; Mossey *et al*, 2009). Cleft palate is usually classified into the following four categories: (1) complete cleft palate with cleft lip; (2) cleft of primary (anterior) palate, in which the cleft is limited to the anterior incisive fossa and may or may not involve cleft lip; (3) cleft of the secondary (posterior) palate, in which the cleft defect is limited to the posterior incisive fossa; and (4) submucosal cleft including a bifid uvula (Chai and Maxson, 2006). The causes of these various cleft palate types are being dissected through studies of human populations and the use of animal models. The etiology of cleft palate correlates with ethnic, racial, and geographic variations, socio-economic status, single-gene disorders, chromosome aberrations, and exposure to teratogens (alcohol, tobacco, anti-convulsants etc.), suggesting that multiple genetic and environmental factors are involved in cleft palate (Cobourne, 2004; Little *et al*, 2004; Lidral *et al*, 2008; Scapoli *et al*, 2008; Vieira, 2008; Meng *et al*, 2009; Zhu *et al*, 2009). Almost 70% of human cleft palates are regarded as nonsyndromic, whereas the remaining approximately 30% of cleft palate cases are syndromic (Jugessur *et al*, 2009). Recent advances in our understanding of the molecular mechanisms of cleft palate have therefore resulted from the combination of genetic studies in humans and analysis of targeted mutations in mice.

Non-syndromic cleft lip with or without cleft palate (NSCL/P)

The genetics of NSCL/P is complex, involving variations in numerous genes together with epigenetic factors, such as maternal smoking, alcohol consumption, and exposure to certain drugs or toxins resulting in defects in normal palate development (Carinci *et al*, 2007; Yu *et al*, 2009). Mutations in *TGFB3* (see Table 1 for description of all genes abbreviated and cited herein), *IRF6*, *CYP*, *MSX1*, and *TBX10* have been associated with NSCL/P (Vieira *et al*, 2007; Yu *et al*, 2009). In addition, polymorphic variants associated with NSCL/P were identified by a genome-wide association scan (GWAS) in *IRF6*, chromosome 8q24.21, *ABCA4* on 1p22.1, and *MAFB* on 20q12 (Beaty *et al*, 2010). In parallel, several studies have shown that folic acid may have a protective effect on cleft palate and neural tube defects (Peer *et al*, 1958a,b; Czeizel and Dudas, 1992; Tolarova and Harris, 1995; Hartridge *et al*, 1999; Mostowska *et al*, 2010; Zhu *et al*, 2009; Wehby and Murray, 2010).

Syndromes with cleft lip with or without cleft palate

Approximately 400 known human syndromes have been described in which a cleft palate is part of the disorder (Jugessur *et al*, 2009). Several clefing factors have been implicated by studies in mouse models and genetic screening in humans: *IRF6* in Van der Woude syndrome, *TGFB1* or *TGFB2* in Loeys–Dietz syndrome, *PHF8* in Siderius X-linked mental retardation, *DHCR7* in Smith–Lemli–Opitz syndrome, *DHCR24* in desmosterolosis, *HSD3B7* in CDPX2 (X-linked dominant chondrodysplasia punctata type 2), *SC5D* in lathosterolosis (Engelking *et al*, 2006; Porter, 2006; Jugessur *et al*, 2009). Some of the biological mechanisms underlying cleft palate may be similar among these disorders. For example, inborn errors of lipid metabolism cause human malformation syndromes, such as Smith–Lemli–Opitz syndrome, lathosterolosis, desmosterolosis, X-linked dominant chondrodysplasia punctata type 2, congenital hemidysplasia with ichthyosiform erythroderma and limb defects (Engelking *et al*, 2006; Porter, 2006). All of these syndromes involve craniofacial anomalies including cleft palate. Moreover, animal models for a cholesterol synthesis defect, such as *Dhcr7*, *Sc5d*, and *Insig1/-2* conventional knockout mice, have cleft palate (Fitzky *et al*, 2001; Krakowiak *et al*, 2003; Engelking *et al*, 2006). Interestingly, these null mutant mice have severe malformations specifically in the craniofacial region. Another example is transforming growth factor beta (TGF- β) signaling

(see Table 2 for description of all proteins abbreviated and cited herein), which is crucial in regulating organogenesis during embryonic development (Ito *et al*, 2003; Sasaki *et al*, 2006; Oka *et al*, 2007a,b; Hosokawa *et al*, 2010; Iwata *et al*, 2010). For instance, heterozygous mutations in *TGFBR1* or *TGFBR2* are associated with Loeys–Dietz syndrome, which can manifest with craniofacial malformations such as cleft palate, craniosynostosis, and hypertelorism; skeletal defects such as scoliosis, arachnodactyly, and joint laxity; and vascular problems including arterial tortuosity with the potential for aneurysms and dissections (Mizuguchi *et al*, 2004; Loeys *et al*, 2005, 2006; Loeys and De Paepe, 2008). In addition, heterozygous mutations in *FBN-1*, which encodes an elastic extracellular matrix protein called fibrillin-1, lead to excessive TGF- β signaling and cause Marfan syndrome, which exhibits a clinical phenotype similar to Loeys–Dietz syndrome (Habashi *et al*, 2006; Brooke *et al*, 2008; Kalluri and Han, 2008). DiGeorge syndrome, which has a variably sized deletion on chromosome 22 (del22q11) including about 30 genes such as the transcription factor *TBX1* and the signal adaptor protein *CRKL*, exhibits a phenotype similar to that resulting from mutation in *TGFBR2* and shows altered TGF- β signaling (Lindsay *et al*, 1999, 2001; Lindsay, 2001; Vitelli and Baldini, 2003; Wurdak *et al*, 2005; Portnoi, 2009).

Developmental time course of palate formation

The formation of a continuous secondary palate separating the oral and nasal cavities is a complex process involving multiple events, including palatal shelf growth, elevation, and fusion. In humans, the palate develops from two primordia, which are the primary and secondary palate. The primary palate represents only a small part of the adult hard palate. The secondary palate contributes to the development of the hard and soft parts of the palate. Palatal shelves begin to develop early in the 6th week from the internal aspect of the maxillary primordia. The initial palatal shelves are positioned vertically along each side of the tongue. Subsequently, as the result of jaw growth and tongue descent, the palatal shelves re-orient into a horizontal position, expand, and fuse. In the hard palate, mesenchymal cells are replaced by intramembranous bone formation. In contrast, the posterior part of the secondary palate, which is the soft palate, does not undergo ossification. It contains five muscles, the tensor veli palatine, levator veli palatine, palatoglossus, palatopharyngeus, and musculus uvula, and extends posteriorly beyond the nasal septum, with the uvula at the end of the posterior border of the secondary palate. The fusion of palatal shelves is marked by the median palatine raphe. In mice, palate development is similar to that of humans (Chai and Maxson, 2006). Mouse palatal development also starts from two primordia. During mouse development, mouse palatogenesis initiates at embryonic day 11.5 (E11.5) as marked by the formation of palatal shelves extending from the internal aspects of the maxillae. Following initiation, the two developing palatal shelves first grow vertically along the lateral sides of the tongue by E13.5. During E13.5–E14.5, because of the growth of the jaws and descent of the tongue, space develops to accommodate the horizontal apposition of the palatal shelves above the tongue, thus allowing the two vertical palatal shelves to re-orient to a horizontal position above the dorsal side of the tongue, a process termed palatal elevation. The two elevated palatal shelves grow horizontally toward each other to meet along the facial midline and form the medial edge epithelial (MEE) seam. The MEE seam will soon undergo degradation, a process referred to as palatal fusion, which gives rise to mesenchymal confluence and the formation of a single continuous palate (Ferguson, 1988). Fusion of the palatal shelves starts at E14.5, with completion of a continuous palate by E16.5 (Xu *et al*, 2006). Both the elevation and fusion of the palatal shelves occur in an anterior to posterior sequence (Figure 1).

Cell lineage in the palate

The palatal structures are composed of cranial neural crest cell (CNCC)-derived ectomesenchymal cells, mesoderm-derived cells, and pharyngeal ectoderm-derived

epithelial cells (Ferguson, 1984; Shuler *et al*, 1992; Ito *et al*, 2003). The epithelia that cover the palatal shelves are regionally divided into oral, nasal, and medial edge MEE. The nasal and oral epithelia differentiate into pseudostratified and squamous epithelia, respectively, whereas the MEE is removed from the fusion line via programmed cell death, cell migration, or epithelial-mesenchymal transformation (EMT) (Fitchett and Hay, 1989; Shuler *et al*, 1992; Shuler, 1995; Martinez-Alvarez *et al*, 2000). Recent advances in cell fate analysis during palate formation have occurred using transgenic animal models that express a reporter gene. For example, using the *K14-Cre;R26R* model, it has been shown that MEE cells mainly disappear because of apoptosis and migration, but not EMT, during palatal fusion (Xu *et al*, 2006). Using the *Wnt1-Cre;R26R* model, it has been shown that more than 90% of palatal mesenchymal cells are derived from CNCC (Ito *et al*, 2003; Iwata *et al*, Submitted). Neural crest cells (NCC) are a transient population of cells created during higher vertebrate early embryonic development. During neurulation, NCC undergo successive epithelial-to-mesenchymal transformations along the cranial-caudal embryonic axis (Serbedzija *et al*, 1992), delaminate and migrate along defined pathways to contribute to the formation of a wide variety of target tissues, including peripheral neurons and glia, melanocytes, smooth muscle cells, connective tissues, secretory cells, most craniofacial cartilages and bones, and the developing aortic arch arteries and outflow track of the heart (Nichols, 1981; Kirby and Waldo, 1995; Sela-Donenfeld and Kalcheim, 1999; Chai *et al*, 2000; Jiang *et al*, 2000; Snider *et al*, 2007).

TGF- β signaling

SMAD-dependent TGF- β signaling pathway

The TGF- β superfamily includes activins, BMP, and TGF- β cytokines (Derynck and Zhang, 2003). The TGF- β pathway stimulates various signaling networks to control cell fate determination, growth, and differentiation (Massague and Wotton, 2000). TGF- β initiates signaling by assembling receptor complexes that activate Smad transcription factors (Massague and Wotton, 2000; Feng and Derynck, 2005). The extracellular region of the receptors is a small, tightly folded globular domain whereas the cytoplasmic region is formed by a short juxtamembrane segment, a protein kinase domain, and little else (Derynck and Feng, 1997; Derynck *et al*, 1998). Initially, the TGF- β 1, - β 2, or - β 3 isoform binds to the TGF- β type II receptor (T β RII). With the binding of the ligand, T β RII is phosphorylated and activated. Both TGF- β 1 and TGF- β 3 have a high affinity for T β RII. In contrast, TGF- β 2 has a low affinity for T β RII and requires an accessory receptor, T β RIII (also known as β -glycan), for high-affinity interaction with the heteromeric-signaling complex. Subsequently, the TGF- β type I receptor (T β RI; also known as ALK5, activin receptor-like kinase 5) is recruited into the complex and is activated by T β RII-mediated phosphorylation in the glycine-serine (GS) region (a GSGS sequence). Thus, TGF- β stimulation brings together heterotetrameric active receptor complexes. Receptor-associated SMADs (R-SMAD), SMAD2 and SMAD3, are recruited into the activated receptor complex in association with adaptor proteins and are specifically phosphorylated at the carboxyl terminus by T β RI. This phosphorylation event results in dissociation of R-SMAD from the receptor complex, association with the co-mediator SMAD (co-SMAD) SMAD4, nuclear translocation and subsequent transcriptional activation or repression (Figure 2). Each TGF- β ligand may have a choice of several receptors and adaptor proteins, and a given cell may express different receptor complexes and downstream signaling molecules or a given cell may express different receptor complexes resulting in divergent signaling outcomes.

SMAD-independent TGF- β signaling pathway

Transforming growth factor beta also activates SMAD-independent signaling cascades, including MAPK pathways such as the ERK, JNK, and p38 MAPK kinase pathways (Kang

et al, 2009; Zhang, 2009). Studies using SMAD4-deficient cells, or dominant-negative SMADs, support the model that MAPK pathway activation is independent of SMADs (Figure 2). JNK or p38 MAPK activation by TGF- β is accompanied by T β RI-kinase [Correction added after online publication 2 June 2011: TGF- β RI-kinase was changed to T β RIKinase] activity-independent TRAF6-TAK1 phosphorylation (Sorrentino *et al*, 2008; Yamashita *et al*, 2008). SMAD6 can bind to TAK1 and down-regulate its activity, whereas SMAD7 can enhance and sustain JNK activation (Kimura *et al*, 2000). However, it is still largely unknown how SMAD-independent pathways are specified and function. The balance between SMAD-dependent and SMAD-independent pathways may define cellular-specific responses to TGF- β .

Adapter proteins and modifiers

β -spectrin— β -spectrin (also known as ELF, embryonic liver fodrin) was first isolated from mouse E11 libraries and plays a crucial role in the propagation of TGF- β signaling (Mishra *et al*, 1999). *Elf*^{-/-} mice exhibit a phenotype similar to *Smad2*^{+/-}; *Smad3*^{+/-} mutant mice in that they undergo midgestational death because of gastrointestinal, liver, neural, and heart defects. Specifically, β -spectrin associates with SMAD3 and the TGF- β receptor complex and leads to their translocation to the nucleus. Disruption of β -spectrin expression in *Elf*^{-/-} mice results in mislocalization of SMAD3 and SMAD4 and disruption of TGF- β signaling following cell cycle disruption (Tang *et al*, 2003).

FKBP12—Adjacent to the kinase domain of T β RI is a conserved 30-amino acid segment known as the juxtamembrane GS domain. In the basal state, the GS domain forms a wedge that presses against the catalytic center. The immunophilins FKBP12 and FKBP12.6 bind to the GS domain and stabilize this inactive conformation (Wang and Donahoe, 2004).

Connective tissue growth factor (CTGF)—CTGF (also known as CCN2) is an extracellular protein that belongs to the CCN family. CTGF has been demonstrated to regulate a wide spectrum of cellular functions including proliferation, migration, adhesion, survival, differentiation, and synthesis of ECM proteins in various cell types, in both normal and pathological conditions (Chen and Lau, 2009). CTGF acts by modulating and mediating diverse signaling pathways, such as TGF- β , BMP, and WNT (Abreu *et al*, 2002). *Ctgf*^{-/-} mice exhibit skeletal dysmorphology that is caused by impaired chondrocyte proliferation and altered ECM composition within the hypertrophic zone of long bones during development. *Ctgf*^{-/-} mice also exhibit cleft palate because of failure of palatal shelf elevation (Ivkovic *et al*, 2003). Studies in diverse cell types and organisms have shown that TGF- β 1 is a strong inducer for *Ctgf* expression and that CTGF acts as a downstream mediator of the TGF- β signaling pathway (Grotendorst, 1997; Shiwen *et al*, 2006; Arnott *et al*, 2007; Woods *et al*, 2009). In addition, CTGF modulates TGF- β activation and signal transduction by the binding of TGF- β through its von Willebrand type C/cysteine-rich (vWC/CR) domain, which enhances the affinity of TGF- β ligands for their receptors (Abreu *et al*, 2002; Chen and Lau, 2009).

TGF- β and cleft palate

Human syndromes related to TGF- β signaling

Human linkage studies have shown that point mutations in the fibrillin-1 gene (*FBN-1*) cause Marfan syndrome, in which patients are typically abnormally tall with long limbs and long thin fingers and have defects of the heart valves and aorta, lungs, eyes, the dural sac surrounding the spinal cord, skeleton, and the hard palate (Neptune *et al*, 2003; Dietz *et al*, 2005; Judge and Dietz, 2005; Singh *et al*, 2006). Fibrillin-1 binds to latent TGF- β ligands to inhibit TGF- β activation, and patients with Marfan syndrome usually have up-regulated

TGF- β signaling (Dietz *et al*, 2005; Judge and Dietz, 2005; Habashi *et al*, 2006; Brooke *et al*, 2008; Kalluri and Han, 2008). Patients with Loeys–Dietz syndrome, who have heterozygous mutations in *TGFBR1* or *TGFBR2*, exhibit a similar, but more severe, clinical phenotype than Marfan syndrome, with craniofacial malformations such as cleft palate, craniosynostosis, and hypertelorism, skeletal defects such as scoliosis, arachnodactyly, and joint laxity, and vascular problems including arterial tortuosity, with the potential for aneurysms and dissections (Mizuguchi *et al*, 2004; Loeys *et al*, 2005, 2006).

Mouse craniofacial phenotypes after deletion of genes related to TGF- β signaling

Tgfb1 null (*Tgfb1*^{-/-}) mice—Transforming growth factor beta 1 (TGF- β 1) is strongly expressed in MEE cells prior to adherence of the opposing palatal shelves (Fitzpatrick *et al*, 1990). Following adherence of the palatal shelves, TGF- β 1 gradually ceases to be expressed in mesenchymal cells (Li *et al*, 2007; Mu *et al*, 2008). *Tgfb1* null newborn mice grow normally for the first 2 weeks and then develop a rapid wasting syndrome with diffuse inflammatory infiltrates and die by 3–4 weeks of age (Letterio *et al*, 1996; McCartney-Francis *et al*, 1996; Sudarshan *et al*, 1998). Moreover, about half of *Tgfb1* homozygous embryos die at E10 because of hematopoietic and vasculogenetic defects in the yolk sac (Goumans *et al*, 1999). Heterozygous *Tgfb1* knockout mice are developmentally normal.

Tgfb2 null (*Tgfb2*^{-/-}) mice—Transforming growth factor beta 2 is expressed in palatal mesenchymal cells during adherence of the opposing palatal shelves. *Tgfb2* null mice exhibit perinatal mortality and a wide range of developmental defects including cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital defects (Sanford *et al*, 1997). Heterozygous *Tgfb2* knockout mice are developmentally normal.

Tgfb3 null (*Tgfb3*^{-/-}) mice—Transforming growth factor beta 3 begins to be expressed strongly in the MEE cells prior to adherence of the opposing palatal shelves and continues to be expressed during palatal fusion (Tudela *et al*, 2002; Dudas *et al*, 2004; Yang and Kaartinen, 2007; Martinez-Sanz *et al*, 2008; Yang *et al*, 2008). TGF- β 3 plays an essential role in MEE cell degradation and palatal fusion (Kaartinen *et al*, 1995; Proetzel *et al*, 1995). *Tgfb3* homozygous mice have cleft palate caused by defects in MEE seam degeneration and palate fusion (Kaartinen *et al*, 1995; Proetzel *et al*, 1995; Taya *et al*, 1999), and this defect can be rescued by *Smad2* overexpression (Cui *et al*, 2005). The loss of TGF- β 3 function leads to changes in metalloproteinase 13 (MMP-13) expression and loss of cell surface filopodia (Taya *et al*, 1999; Blavier *et al*, 2001). Heterozygous *Tgfb3* knockout mice are developmentally normal.

Tgfr1 null (*Tgfr1*^{-/-}/*Alk5*^{-/-}) mice—*Tgfr1* conventional knockout mice die around embryonic day 10.5 (E10.5) because of defects in vascular development of the yolk sac and placenta and an absence of circulating red blood cells (Larsson *et al*, 2001). Heterozygous *Tgfr1* knockout mice are developmentally normal.

Tgfr2 null (*Tgfr2*^{-/-}) mice—*Tgfr2* conventional knockout mice have defects of yolk sac hematopoiesis and vasculogenesis, resulting in embryonic lethality around E10.5 (Oshima *et al*, 1996). Heterozygous *Tgfr2* knockout mice are developmentally normal.

Tgfr3 null (*Tgfr3*^{-/-}) mice—*Tgfr3* homozygous mice die around E13.5 with proliferative defects in the heart and increased apoptosis in the liver (Stenvers *et al*, 2003). Heterozygous *Tgfr3* knockout mice are developmentally normal.

Smad2 null (*Smad2*^{-/-}) mice—Homozygous *Smad2* mice fail to form an organized egg cylinder and lack mesoderm (Nomura and Li, 1998; Weinstein *et al*, 1998). About 20

percent of heterozygous *Smad2* mice have severe gastrulation defects and lack mandibles or eyes (Nomura and Li, 1998).

Smad3 null (*Smad3*^{-/-}) mice—Homozygous *Smad3* null mice die between 1 and 8 months because of a primary defect in immune function (Yang *et al*, 1999). Heterozygous *Smad3* knockout mice are developmentally normal.

Smad4 null (*Smad4*^{-/-}) mice—Homozygous *Smad4* mice die between E6.5 and E8.5. All *Smad4*^{-/-} mice are developmentally delayed at E6 and show little or no elongation in the extraembryonic portion of late egg-cylinder-stage embryos (Yang *et al*, 1998). Heterozygous *Smad4* knockout mice are developmentally normal.

Alk5^{fl/fl};K14-Cre mice—Epithelial-specific deletion of the TGF- β type I receptor gene (*Alk5*^{fl/fl};K14-Cre) causes clefts in the posterior part of the palate (soft palate) and the anterior portions of the hard palate (Dudas *et al*, 2006). *Alk5*^{fl/fl};K14-Cre mice also have submucous cleft palate because of failure to undergo apoptosis during MEE disappearance between E14.5 and E16.5 (Dudas *et al*, 2006).

Tgfb2^{fl/fl};K14-Cre mice—Epithelial-specific deletion of the TGF- β type II receptor gene (*Tgfb2*^{fl/fl};K14-Cre) causes clefts in the posterior part of the palate (soft palate) and the anterior portions of the hard palate (Xu *et al*, 2006). *Tgfb2*^{fl/fl};K14-Cre mice also exhibit submucous cleft palate because of failure to undergo apoptosis during MEE disappearance between E14.5 and E16.5 (Xu *et al*, 2006).

Smad2^{fl/fl};K5-Cre mice—Mice with an epithelial-specific conditional knockout of *Smad2* (*Smad2*^{fl/fl};K5-Cre) survive, but exhibit accelerated formation and malignant progression of chemically induced skin tumors. They exhibit no detectable phenotype during palatal formation (Hoot *et al*, 2008).

Smad4^{fl/fl};K14-Cre mice—Mice with epithelial-specific deletion of *Smad4* (*Smad4*^{fl/fl};K14-Cre) die at birth, but exhibit no detectable phenotype during palatal formation (Xu *et al*, 2008).

Alk5^{fl/fl};Wnt1-Cre mice—Mice with neural crest cell-specific deletion of *Tgfb1/Alk5* (*Alk5*^{fl/fl};Wnt1-Cre) are born alive, but severely disfigured, and die soon after birth with a hypoplastic cranium, split snout, small mandible, cleft lip and palate, and small tongue (Dudas *et al*, 2006; Li *et al*, 2008; Zhao *et al*, 2008).

Tgfb2^{fl/fl};Wnt1-Cre mice—Mice with neural crest cell-specific deletion of *Tgfb2* (*Tgfb2*^{fl/fl};Wnt1-Cre) are born alive, but die soon after birth with cardiovascular defects, and thymic, parathyroid, and craniofacial anomalies, such as complete cleft palate, small mandible, small tongue, small maxilla, and calvaria defects (Chai *et al*, 2003; Ito *et al*, 2003; Hosokawa *et al*, 2010; Iwata *et al*, 2010). The defects in *Tgfb2*^{fl/fl};Wnt1-Cre embryos mimic defects in patients with Loeys–Dietz and DiGeorge syndromes (Ito *et al*, 2003; Wurdak *et al*, 2005).

Smad4^{fl/fl};Wnt1-Cre mice—*Smad4*^{fl/fl};Wnt1-Cre embryos are arrested at E11.5–E12.5 because of decreased blood circulation to the yolk sacs by E12.0. *Smad4*^{fl/fl};Wnt1-Cre embryos show defective phenotypes by E10.5, including underdevelopment of the first branchial arch and failure of fusion in the middle of the frontonasal process and the mandibular process of the first branchial arch (Ko *et al*, 2007).

Alk5^{fl/fl};Nestin-Cre mice—*Alk5^{fl/fl};Nestin-Cre* mice die at E15 with unilateral or bilateral cleft lip because of increased cell death and retarded development of palatal shelves (Li *et al*, 2008).

Tgfb^{fl/fl};Myf5-Cre mice—Mesoderm-derived cells in the palate consist of less than 10% of all palatal mesenchymal cells. Mesoderm-derived cell-specific deletion of *Tgfb^{fl/fl}* (*Tgfb^{fl/fl};Myf5-Cre*) causes defects of the supraoccipital bone with meningoencephalocele and discontinuity of the neural arch of the C1 vertebra, but no apparent phenotype in palatal formation (Hosokawa *et al*, 2007; and our unpublished data).

Gene expression patterning and tissue-tissue interaction

Recent discoveries have highlighted the existence of genetic heterogeneity along the anterior–posterior (A–P) and medial–lateral axes of the developing palate (Hilliard *et al*, 2005; Han *et al*, 2009). Constant and reciprocal interactions between the palatal epithelium and the mesenchyme are responsible for setting up this genetic heterogeneity along the A–P axis and may provide differential regulatory mechanisms for the fusion of the anterior versus posterior region of the palate (Zhang *et al*, 2002; Murray and Schutte, 2004; Rice *et al*, 2004). Multiple genes have been found to be critical for the development of the anterior region of the palate. For example, *Msx1*, *Bmp4*, *Bmp2*, *Fgf10*, and *Shox2* show restricted expression patterns in the anterior region of the palate (Rice *et al*, 2004; Hilliard *et al*, 2005). Loss of *Msx1* results in a cell proliferation defect in the CNCC-derived palatal mesenchyme in the anterior region of the secondary palate, followed by a reduction in *Bmp4* and *Bmp2* gene expression in the mesenchyme (Zhang *et al*, 2002). *Fgf10* is expressed in the anterior palatal mesenchyme and functions in the palatal epithelium to mediate *Shh* expression, which in turn regulates *Bmp2* expression in the mesenchyme to promote cell proliferation. *Shox2* is expressed in the anterior palatal mesenchyme, and loss of *Shox2* results in an incomplete cleft of the anterior palate, whereas fusion of the posterior palate is normal (Yu *et al*, 2005). In contrast, we know less about the specific gene expression patterns in the posterior region of the palate. *Fgfr2* is expressed in the epithelium and the mesenchyme in the middle and posterior palate. FGF8 induces the expression of *Pax9* in the posterior region of palatal mesenchyme, and loss of *Pax9* results in cleft palate (Peters *et al*, 1998; Hilliard *et al*, 2005). In addition to the differential gene expression patterns along the A–P axis of the developing palate, there is also mesenchymal heterogeneity between the medial and lateral regions of the palatal shelf. For example, the *odd skipped-related genes* *Osr1* and *Osr2* are expressed in a medial–lateral gradient in the palate. Mutation in *Osr2* results in the compromised development of the medial aspect of the palatal shelf and retards palatal shelf elevation (Lan *et al*, 2004). The expression of *Fgfr2* is limited in the medial aspect of the developing palatal shelf, suggesting a possible functional significance in regulating the development and elevation of palatal shelves. There are also oral–nasal gene expression patterns that control the heterogeneity of palatal mesenchyme and determine the fate of CNC-derived cells during palatogenesis (Han *et al*, 2009). These studies demonstrate that there are different regulatory mechanisms that control the development and fusion of various regions of the palate.

Conclusion

The utility of animal models with cleft palate is impressive as we have begun to discover the cellular and molecular mechanisms of palate formation. To date, over 200 genetically mutated mice are reported to exhibit cleft palate, and approximately 400 known human syndromes exhibit cleft palate as a part of their clinical symptoms. However, the molecular and cellular mechanisms associated with cleft palate are still not completely defined. We propose that rescue experiments using genetic and pharmacological approaches may lead to

a greater understanding of the gene–gene and gene–environment interactions in craniofacial development.

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Figure 1.

Time course of palate formation during embryonic development in mice. (a–e) Hematoxylin and eosin staining at E12.5 (a), E13.5 (b), E14.5 (c), E15.5 (d), and E16.5 (e). (f–k) Scanning electron microscopic (SEM) images at E12.5 (f), E13.0 (g), E13.5 (h), E14.0 (i), E14.5 (j), and E15.0 (k)

Figure 2.

Schematic diagram of TGF- β signaling. Schematic diagram indicates the mechanism of TGF- β signaling. TGF- β initiates signaling by assembling receptor complexes that activate SMAD transcription factors. Initially, the TGF- β 1, - β 2, or - β 3 isoform binds to the TGF- β type II receptor (T β RII). With the binding of the ligand, T β RII is phosphorylated and activated. Both TGF- β 1 and TGF- β 3 have a high affinity for T β RII. In contrast, TGF- β 2 has a low affinity for T β RII and requires T β RIII. Subsequently, the T β RI is recruited into the complex and is activated by T β RII-mediated phosphorylation. Receptor-associated SMADs (R-SMAD), SMAD2 and SMAD3, are recruited into the activated receptor complex and are specifically phosphorylated by T β RI. This phosphorylation event results in dissociation of R-SMAD from the receptor complex, association with the SMAD4, nuclear translocation, and subsequent transcriptional activation or repression. TGF- β also activates SMAD-independent signaling cascades, including MAPK pathways, such as the ERK, JNK, and p38 MAPK kinase pathways, in some physiological and pathological conditions

Table 1

Summary of genes cited

Gene	Official name	Function	References
<i>ABCA4</i>	ATP-binding cassette, sub-family A, member 4	ATP-binding cassette transporters	Beaty <i>et al</i> (2010)
<i>BMP2</i>	Bone morphogenetic protein 2	TGF- β superfamily ligand	Zhang <i>et al</i> (2002) Hilliard <i>et al</i> (2005)
<i>BMP4</i>	Bone morphogenetic protein 4	TGF- β superfamily ligand	Zhang <i>et al</i> (2002) Hilliard <i>et al</i> (2005)
<i>CRKL</i>	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	Protein kinase containing SH2 and SH3 domains	Vitelli and Baldini (2003)
<i>CYP</i>	Cytochrome P450	Monooxygenase, and involved in drug metabolism and cholesterol or steroid synthesis	Yu <i>et al</i> (2009)
<i>DHCR7</i>	7-dehydrocholesterol reductase	Enzyme which removes the C7–C8 double bond of 7-dehydrocholesterol and produces cholesterol	Fitzky <i>et al</i> (2001) Porter (2006)
<i>DHCR24</i>	24-dehydrocholesterol reductase	A flavin adenine dinucleotide (FAD)-dependent oxidoreductase	Porter (2006)
<i>FBN1</i>	Fibrillin 1	Extracellular calcium-binding microfibrils	Brooke <i>et al</i> (2008) Habashi <i>et al</i> (2006) Kalluri and Han (2008)
<i>FGF10</i>	Fibroblast growth factor 10	FGF family ligand	Rice <i>et al</i> (2004) Hosokawa <i>et al</i> (2010)
<i>FGFR2</i>	Fibroblast growth factor receptor 2	FGF receptor	Rice <i>et al</i> (2004)
<i>HSD3B7</i>	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	Enzyme of the short-chain dehydrogenase/reductase superfamily	Porter (2006)
<i>Insig1/2</i>	Insulin induced gene 1 and 2	A cholesterol synthesis regulator	Engelking <i>et al</i> (2006) Porter (2006)
<i>IRF6</i>	Interferon regulatory factor 6	Transcriptional factor	Vieira <i>et al</i> (2007) Jugessur <i>et al</i> (2009)
<i>MAFB</i>	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B	Basic leucine zipper transcriptional factor	Beaty <i>et al</i> (2010)
<i>MSX1</i>	msh homeobox 1	Transcriptional factor	Hilliard <i>et al</i> (2005)
	Muscle segment homeobox 1		Yu <i>et al</i> (2009)
<i>MYF5</i>	Myogenic factor 5	Mitogenic factor	Hosokawa <i>et al</i> (2010)
<i>OSR1</i>	Odd-skipped related 1	Transcriptional factor	Lan <i>et al</i> (2004)
<i>OSR2</i>	Odd-skipped related 2	Transcriptional factor	Lan <i>et al</i> (2004) Hilliard <i>et al</i> (2005)
<i>PAX9</i>	Paired box 9	Transcriptional factor	Peters <i>et al</i> (1998) Hilliard <i>et al</i> (2005)
<i>PHF8</i>	PHD finger protein	Histone lysine demethylase	Jugessur <i>et al</i> (2009)
<i>SC5D</i>	Sterol-C5-desaturase homolog	Enzyme for cholesterol biosynthesis	Krakowiak <i>et al</i> (2003) Porter (2006)
<i>SHH</i>	Sonic hedgehog	Signal molecule	Rice <i>et al</i> (2004) Hilliard <i>et al</i> (2005)
<i>SHOX2</i>	Short stature homeobox protein 2	Homeobox gene	Hilliard <i>et al</i> (2005) Yu <i>et al</i> (2005)
<i>TBX1</i>	T-box 1	Transcriptional regulator	Lindsay <i>et al</i> (2001) Portnoi (2009)
<i>TBX10</i>	T-box 10	Transcriptional factor	Yu <i>et al</i> (2009)
<i>TGFB3</i>	Transforming growth factor, beta	TGF- β ligand	Yu <i>et al</i> (2009)
<i>TGFBR1</i>	Transforming growth factor, beta receptor 1	TGF- β receptor	Jugessur <i>et al</i> (2009)

Gene	Official name	Function	References
<i>TGFBR2</i>	Transforming growth factor, beta receptor 2	TGF- β receptor	Jugessur <i>et al</i> (2009)

Table 2

TGF- β signaling molecules

Protein	Official name	Function	References
β -spectrin/ELF	Embryonic liver fodrin	Membrane cytoskeletal protein	Mishra <i>et al</i> (1999) Tang <i>et al</i> (2003)
CTGF/CCN2	Connective tissue growth factor/CCN family member 2	Mitogen	Grotendorst (1997) Oka <i>et al</i> (2007a)
FKBP12	FK506 binding protein 1A, 12 kDa	Immunophilin protein family	Wang and Donahoe (2004)
SMAD2	Smad family member 2	Transcriptional modulator	Nomura and Li (1998) Weinstein <i>et al</i> (1998) Hoot <i>et al</i> (2008)
SMAD3	Smad family member 3	Transcriptional modulator	Yang <i>et al</i> (1999)
SMAD4	Smad family member 4	Transcriptional modulator	Yang <i>et al</i> (1998) Ko <i>et al</i> (2007) Xu <i>et al</i> (2008)
TAK1	Transforming growth factor (TGF)-beta-activated kinase 1	Serine/threonine protein kinase family	Kimura <i>et al</i> (2000) Sorrentino <i>et al</i> (2008) Yamashita <i>et al</i> (2008)
TGF- β 1	Transforming growth factor beta 1	TGF- β ligand	Fitzpatrick <i>et al</i> (1990) Letterio <i>et al</i> (1996) McCartney-Francis <i>et al</i> (1996) Sudarshan <i>et al</i> (1998) Goumans <i>et al</i> (1999) Li <i>et al</i> (2007) Mu <i>et al</i> (2008)
TGF- β 2	Transforming growth factor beta 2	TGF- β ligand	Sanford <i>et al</i> (1997)
TGF- β 3	Transforming growth factor beta 3	TGF- β ligand	Sanford <i>et al</i> (1997)
T β RI/ALK5	Transforming growth factor beta receptor I/ Activin receptor-like kinase 5	TGF- β receptor	Larsson <i>et al</i> (2001) Dudas <i>et al</i> (2006) Li <i>et al</i> (2008) Zhao <i>et al</i> (2008)
T β RII	Transforming growth factor beta receptor II	TGF- β receptor	Oshima <i>et al</i> (1996) Chai <i>et al</i> (2003) Ito <i>et al</i> (2003) Wurdak <i>et al</i> (2005) Sasaki <i>et al</i> (2006) Xu <i>et al</i> (2006) Oka <i>et al</i> (2007a) Oka <i>et al</i> (2007b) Hosokawa <i>et al</i> (2010) Iwata <i>et al</i> (2010)
T β RIII	Transforming growth factor beta receptor III	TGF- β receptor	Stenvers <i>et al</i> (2003)
TRAF6	Tumor necrosis factor (TNF) receptor-associated factor 6	A ubiquitin ligase	Sorrentino <i>et al</i> (2008) Yamashita <i>et al</i> (2008)