Smad4-Irf6 genetic interaction and TGFβ-mediated IRF6 signaling cascade are crucial for palatal fusion in mice

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SUMMARY

Cleft palate is one of the most common human birth defects and is associated with multiple genetic and environmental risk factors. Although mutations in the genes encoding transforming growth factor beta (TGFβ) signaling molecules and interferon regulatory factor 6 (Irf6) have been identified as genetic risk factors for cleft palate, little is known about the relationship between TGFβ signaling and Irf6 activity during palate formation. Here, we show that TGFβ signaling regulates expression of Irf6 and the fate of the medial edge epithelium (MEE) during palatal fusion in mice. Haploinsufficiency of Irf6 in mice with basal epithelial-specific deletion of the TGFβ signaling mediator Smad4 (Smad4⁴/⁴;K14-Cre;Irf6⁺/R84C) results in compromised p21 expression and MEE persistence, similar to observations in Tgfbr2⁴/⁴;K14-Cre mice, although the secondary palate of Irf6⁺/R84C and Smad4⁺/⁴;K14-Cre mice form normally. Furthermore, Smad4⁺/⁺;K14-Cre;Irf6⁺/R84C mice show extra digits that are consistent with abnormal toe and nail phenotypes in individuals with Van der Woude and popliteal pterygium syndromes, suggesting that the TGFβ/SMAD4/IRF6 signaling cascade might be a well-conserved mechanism in regulating multiple organogenesis. Strikingly, overexpression of Irf6 rescued p21 expression and MEE degeneration in Tgfbr2⁺/⁺;K14-Cre mice. Thus, Irf6 and SMAD4 synergistically regulate the fate of the MEE, and TGFβ-mediated Irf6 activity is responsible for MEE degeneration during palatal fusion in mice.

KEY WORDS: TGFβ, Irf6, Palatal fusion, Mouse

INTRODUCTION

Cleft palate is a serious and common craniofacial birth defect affecting millions of people worldwide (Mossey et al., 2009; Wong and Hagg, 2004). Cleft palate has ethnic and geographic variations in prevalence, and it affects feeding, swallowing, speech, hearing, middle-ear ventilation, respiration and appearance (Iwata et al., 2011). Studies in mouse models and genetic screening in humans have implicated several factors in syndromic cleft palate, such as Irf6 mutation in Van der Woude syndrome (VWS) and popliteal pterygium syndrome (PPS), SMAD4 mutation in juvenile polypysis syndrome, and TGBFR1 or TGBFR2 mutation in Loeys-Dietz syndrome (previously called Marfan syndrome type II). Mutations in TGFβ3, Irf6, CYP (cytochrome P450), MSX1 and TBX10 have also been associated with non-syndromic cleft lip with or without cleft palate (NSCL/P) (Iwata et al., 2011). In addition, polymorphic variants associated with NSCL/P within human chromosomes 1q32 (Irf6), 1p22 (VAX1), 1q21, 1q25 (FAX1), 17q22 and 20q12 (MAFB) have been identified by genome-wide association studies (Dixon et al., 2011). Thus, mutations in Irf6, SMAD4 and TGBFR2 confer a significant attributable risk for cleft palate.

TGFβ signaling is one of the major signaling cascades crucial for craniofacial development (Iwata et al., 2011). Epithelial-specific deletion of Tgfr2 (Tgfr2⁺/⁻;K14-Cre) in mice results in persistence of the medial edge epithelium (MEE) and submucous cleft palate (Xu et al., 2006). TGFβ transmits signals through a membrane receptor serine/threonine kinase complex that phosphorylates SMAD2 and SMAD3, followed by the formation of transcriptional complexes with SMAD4 and translocation into the nucleus (Massagué, 2012; Ross and Hill, 2008; Schmierer and Hill, 2007; Shi and Massagué, 2003). TGFβ also activates SMAD-independent signaling cascades, including mitogen-activated protein kinase (MAPK) pathways, such as p38 MAPK (MAPK14 – Mouse Genome Informatics), under certain physiological and pathological conditions (Kang et al., 2009; Xu et al., 2008; Zhang, 2009). Studies using SMAD4-deficient cells, or dominant-negative SMADs, support the possibility that MAPK activation is independent of SMADs (Chen et al., 1998; Giehl et al., 2000; Hociev et al., 1999; Hu et al., 1999). p38 MAPK activation by TGFβ is accompanied by SMAD-independent, TRAF6 and TAK1 (MAP3K7 – Mouse Genome Informatics) phosphorylation (Iwata et al., 2012; Sorrentino et al., 2008; Yamashita et al., 2008). The balance between direct activation of SMADs and MAPK pathways often defines cellular responses to TGFβ.

IRF6 belongs to a family of transcription factors that share a highly conserved, helix-turn-helix, DNA-binding domain and a less conserved, protein-binding domain. Among the genes that have been associated with NSCL/P, Irf6 has been implicated in the largest percentage of cases (Koillinen et al., 2005; Sririchomthong et al., 2005). Mutation of Irf6 can lead to the autosomal-dominant conditions VWS and PPS, which are characterized by oral clefting and lower lip pits (Kondo et al., 2002; Moretti et al., 2010). VWS and PPS are allelic variants of the same condition caused by different mutations of the same gene. PPS includes all the features of VWS, plus popliteal pterygia, synangithia, distinct toe and nail abnormality, syndactyly and genito-urinary malformations. An arginine 84 to cysteine (R84C) mutation in Irf6 is the most common mutation found in patients with PPS (Richardson et al., 2006). Although the function of the R84C mutation is still largely

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Accepted 31 December 2012
unknown, recent studies have demonstrated that it results in loss of DNA binding (Kondo et al., 2002; Little et al., 2009). The primary defect in Irf6-deficient mice is in keratinocyte differentiation and proliferation. Homozygous Irf6
null embryos exhibit abnormal skin, limb and craniofacial morphogenesis, including cleft palate (Ingraham et al., 2006). Mice homozygous for Irf6
WAP, which is an R84C knock-in resulting in expression of mutant Irf6 protein, exhibit a severe intraoral epithelial adhesion caused by a failure of terminal differentiation similar to that in homzygous Irf6
null embryos (Ingraham et al., 2006; Richardson et al., 2006).

Despite the established roles of TGFβ signaling and Irf6 activity during palate formation, the interaction between TGFβ signaling and Irf6 activity is poorly understood. In this study, we investigate the interaction between TGFβ signaling and Irf6 activity. We demonstrate that Irf6 and Smad4 interact genetically, and that TGFβ-mediated Irf6 expression is crucial for p21 (CDKN1A – Mouse Genome Informatics) expression and fate determination of the MEE cells during palatal fusion.

MATERIALS AND METHODS

Animals

To generate Smad4
; K14-Cre; Irf6
R84C mice, we mated Smad4
; K14-Cre; Irf6
R84C with Smad4
mice. To generate Tgbr2
; K14-Cre mice, we mated Tgbr2
; K14-Cre with Tgbr2
mice. Genotyping was performed using PCR primers as previously described (Ito et al., 2003; Richardson et al., 2009; Xu et al., 2008; Xu et al., 2006). Human keratin 14 (K14; KRT14 – Human Gene Nomenclature Database) promoter-driven Irf6
encoding transgene was prepared as follows: an EcoRI-HindIII blunt fragment (7.3 kb) encoding mouse Irf6 was subcloned into the BamHI blunted sites of the pGem 3Z-K14 vector to produce pG3ZK14-Irf6, resulting in a construct containing the K14 promoter (2.1 kb), the p-globin intron (736 bp), the coding sequence for Irf6 (4.1 kb) and the K14 polyadenylation signal (500 bp). The EcoRI-HindIII fragment was isolated free of vector sequence by preparative gel electrophoresis. DNA was further purified using an Elutip column (Schleicher and Schuell, Dessel, Germany) and microinjected in the pronuclei of fertilized oocytes (Ingraham et al., 2006; Richardson et al., 2006).

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Immunoblot analysis

Immunoblots were performed as described previously (Iwata et al., 2006; Iwata et al., 2010). Antibodies for immunoblotting were rabbit polyclonal antibodies against IRF6 (Aviva Systems Biology) and p21 (Abcam), and mouse monoclonal antibodies against p63 (Santa Cruz Biotechnology) and GAPDH (Chemicon).

Palatal shelf organ culture

Timed pregnant mice were sacrificed at E13.5. Genotyping was carried out as described above. The palatal shelves were microdissected and cultured in serum-free chemically defined medium as previously described (Ito et al., 2003; Xu et al., 2008). After 48 or 72 hours in culture, palates were harvested, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and processed. For p63 experiments, palatal shelves were transfected with small interfering RNA (siRNA) duplexes (500 nM) for p63 or control (Santa Cruz Biotechnology). The 500 nM siRNA solutions were prepared by diluting a siRNA stock (10 μM) in BGJb medium containing OligoFectamine (0.3%) (Invitrogen). The siRNA mixture in transfection medium was incubated with palatal shelves up to 72 hours after siRNA treatment at 37°C in a CO2 incubator. For experiments with p21, palatal shelves were transfected with a GFP-tagged p21 overexpression or control vector (Origene). The 2-μg p21 transfection solutions were prepared in BGJb medium containing Lipofectamine LTX and PLUS reagents (Invitrogen) according to the manufacturer’s protocol. The p21 transfection mixture was changed every 24 hours and incubated with palatal shelves up to 72 hours after transfection at 37°C in a CO2 incubator. All experiments were performed with at least five samples.

Cell culture

Primary mouse keratinocytes were isolated from newborn mice and cultured in dermal cell basal medium (ATCC) supplemented with the Keratinocyte Growth Kit (ATCC). Primary mouse keratinocytes (2×10^6) cells were plated in a 60-mm cell culture dish until the cells reached 60-80% confluence. *Tgfbr2* and *irf6* siRNA duplexes were purchased from Santa Cruz Biotechnology. siRNA mixture in transfection medium was incubated with cells for 7 hours at 37°C in a CO2 incubator, as described previously (Iwata et al., 2010).

Quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated from dissected mouse MEE at E14.5 with the QiAshredder and RNeasy Micro Extraction Kit (QIAGEN), as described previously (Iwata et al., 2010). The following PCR primers were used: *irf6*, 5'-AGGGCTCTGTTATAAATTCAAG-3' and 5'-TGATTCCGGGCTGCAGTTTTC-3'; *p21* (Cdh1Lα), 5'-AGGCTGAAGACGTGATGG-3' and 5'-AAAGTTCACGCTTTCTGCG-3'; *ΔNp63*, 5'-AAAAAACCCC- TGGAGACGAAAA-3' and 5'-GGAGAGGCGTTGTCTGAATCTG-3'; and *Gapdh*, 5'-AATCTTGGCATTTGAAGG-3' and 5'-AAGCATGGGGTAGGAACA-3'.

Scanning electron microscopic (SEM) analysis

Samples were fixed with a modified Karnovsky fixative solution [2% paraformaldehyde and 2.5% glutaraldehyde in 0.067 M cacodylate buffer (pH 7.4)] for two days. After dehydration through a graded ethanol series, samples were critical-point dried in a Balzer Union apparatus (FL-9496), ion-sputtered with platinum-palladium (10-15 nm), and observed in JEOL JSM-6390 low vacuum scanning electron microscope (JEOL USA, MA, USA) at a low accelerating voltage of 10 kV.

Whole-mount skeletal staining and micro-CT analysis

The three-dimensional architecture of the skeleton was examined using a modified whole-mount Acantho Blue-Alizarin Red S staining protocol as previously described (Ito et al., 2003; Iwata et al., 2012). Micro-CT analysis was performed using SCANCO μCT 50 (nanoCT) at the University of Zurich.

Fig. 1. Decreased IRF6 expression in the MEE of *Tgfbr2^f/f*,K14-Cre mice. (A) Quantitative RT-PCR analyses of *irf6* expression in the palates of *Tgfbr2^f/f* (control, n=6) and *Tgfbr2^f/f*,K14-Cre (n=6) mice at E14.5. (B,C) Immunohistochemical analyses of IRF6 expression (red) in the palates of *Tgfbr2^f/f* control (B) and *Tgfbr2^f/f*,K14-Cre (C) mice at E14.5. Nuclei were counterstained with DAPI (blue). (D) Quantitative RT-PCR analyses of *irf6* expression in the palates of *Smad4^f/f* (control, n=6) and *Smad4^f/f*,K14-Cre (n=6) mice at E14.5. (E) Quantitative RT-PCR analyses of *irf6* expression in palate explants from *Smad4^f/f* (control) and *Smad4^f/f*,K14-Cre mice treated with p38 MAPK inhibitor (+) or vehicle (–) for 48 hours. n=3 per group. (F-H) H&E staining of palate explants from *Smad4^f/f*,K14-Cre and *Smad4^f/f* (control) mice treated with p38 MAPK inhibitor (F,H) or vehicle control (G,I) for 72 hours. n=5 per group. (J) Schematic of the upstream region of the mouse *irf6* gene (not to scale), showing locations of putative SMAD-binding (red) or MEF2-binding (green) sites tested in ChIP assays. Putative SMAD- and MEF2-binding sequences are shown below. Arrowheads indicate the position of primer used in ChIP analysis. (K) ChIP analysis of DNA fragments immunoprecipitated with a SMAD4-specific or MEF2-specific antibody or with an isotype-specific control antibody. Immunoprecipitates were PCR amplified with primers flanking the putative SMAD-binding or MEF2-binding region. Input lane shows PCR amplification of the sonicated chromatin before immunoprecipitation. Ab, antibody; BS, binding site. Error bars represent s.d. *p<0.05. Scale bars: 50 μm.
Southern California Molecular Imaging Center. The data were collected at a resolution of 10 μm. The reconstruction was performed using AVIZO 7.0 (Visualization Sciences Group).

**Statistical analysis**
A two-tailed Student’s *t*-test was applied for statistical analysis. For all graphs, data are represented as mean±s.d. A *P*-value of less than 0.05 was considered statistically significant.

**RESULTS**

**TGFβ signaling in the MEE regulates Irf6 expression during palatal fusion**
Loss of TGFβ signaling in the basal epithelium in mice (Tgfb2−/−;K14-Cre) results in diminished Irf6 expression and failure of apoptosis in MEE cells, followed by MEE persistence, suggesting that TGFβ-mediated Irf6 expression might play a role in degeneration of the MEE (Xu et al., 2006). The MEE is composed of a basal columnar cell layer covered by flat cells that constitute the periderm (Cuervo and Covarrubias, 2004). Irf6 is expressed in the periderm at E13.5, and Irf6 expression shows an obvious and consistent transition from the periderm to regions of expressed in the periderm at E13.5, and constitute the periderm (Cuervo and Covarrubias, 2004). In degeneration of the MEE (Xu et al., 2006). The MEE is suggesting that TGFβ disappearance.

Because SMAD4 and p38 MAPK are functionally redundant in regulating the MEE disappearance (Xu et al., 2008), we performed ex vivo organ culture of E13.5 palatal shelf explants from Smad4−/−;K14-Cre and Smad4fl/fl control mice with p38 MAPK inhibitor or with vehicle as control. We found that p38 MAPK inhibitors blocked both Irf6 expression (Fig. 1E) and MEE disappearance (Fig. 1F) in Smad4fl/fl;K14-Cre palates but did not affect Irf6 expression (Fig. 1E) or MEE disappearance (Fig. 1H) in control samples, indicating that SMAD and p38 MAPK activation are functionally redundant in regulating Irf6 expression and MEE disappearance.

Next, we analyzed the sequence of the mouse Irf6 gene (including 2.5 kb upstream and 2.5 kb downstream of the transcription start site). The mouse Irf6 genomic region has 43 potential SMAD recognition sites (three of which are conserved in at least six mammals) (Fig. 1J; supplementary material Table S1). The MEF2 transcription factor is regulated by the p38 MAPK pathway (Han and Molkentin, 2000; Toro et al., 2004). We found a potential MEF2 recognition site in the Irf6 genomic region (Fig. 1J). We performed chromatin immunoprecipitation (ChIP) assays to test whether SMAD and MEF2 could bind to the promoter region of Irf6. Binding sites for SMAD (SMAD binding site 1: −274 bp to −271 bp; site 2: −1695 bp to −1692 bp; site 3: −2375 bp to −2372 bp) and MEF2 (MEF2 binding site: −1387 bp to −1375 bp) immunoprecipitated with SMAD4 or MEF2 antibodies, but not with control antibody (Fig. 1K). Taken together, our results suggest that Irf6 gene expression is regulated by both SMAD and p38 MAPK pathways.

Next, we investigated the functional significance of Irf6 in regulating the disappearance of MEE cells during palatal fusion. Because homozygous mutation of Irf6 results in severe intraoral epithelial adhesion, it is not known whether Irf6 is crucial for palatal fusion (Ingraham et al., 2006; Richardson et al., 2006). To investigate the fate of the MEE in Irf6fl/fl;K14-Cre mice, we used the ex vivo organ culture system. E13.5 wild-type control (n=17) and Irf6fl/fl;R84C (n=39) palate explants fused and the MEE disappeared following two days of culture (Fig. 2A,B). By contrast, Irf6fl/fl;R84C mutant (n=19) palatal shelves failed to fuse (Fig. 2C) and the MEE persisted with continuous cell proliferation (Fig. 2D-F) and compromised cell death (Fig. 2G-I) following two days of culture with complete phenotype penetrance. We quantified the increased cell proliferation as well as decreased apoptosis in Irf6fl/fl;R84C MEE cells compared with wild-type littermate control and Irf6−/− MEE cells during palatal fusion (Fig. 2J,K). The MEE in control and Irf6−/− explants disappeared completely following three days in culture; however, the MEE persisted in Irf6fl/fl;R84C explants (supplementary material Fig. S2). These data indicate that IRF6 is crucial for MEE disappearance during palatal fusion.
Genetic interaction between Smad4 and Irf6 during palatal fusion

We hypothesized that haploinsufficiency of Irf6 in a Smad4 mutant background would cause an additional reduction of IRF6 activity and result in cleft palate. To investigate a possible genetic interaction between Irf6 and Smad4, we generated Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> mice. Smad4<sup>fl/fl</sup>;K14-Cre<sup>−/−</sup> mice have normal palates although they have open eyes, short fingers, and nail abnormalities (<i>n</i>=17/20) (Fig. 3; supplementary material Fig. S3). Interestingly, Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> mice exhibited extra digits with complete phenotype.

Fig. 3. A haploinsufficiency of Irf6 in Smad4<sup>fl/fl</sup>;K14-Cre mice causes submucous cleft palate. (A-F) Morphologies of newborn control (A,B), Smad4<sup>fl/fl</sup>;K14-Cre (C,D) and Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> (E,F) mice. Lower panels (B,D,F) show macroscopic appearance of palates at newborn stage. Arrows indicate open eyes. (G,H) SEM analysis of newborn control (G) and Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> (H) mice. Arrows indicate lack of rugae formation. (I-N) H&E staining of sections of newborn Smad4<sup>fl/fl</sup> control (I,J;<i>n</i>=42), Smad4<sup>fl/fl</sup>;K14-Cre (K,L;<i>n</i>=20) and Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> (M,N;<i>n</i>=19) mice. Arrows indicate MEE persistence. (O) Schematics showing the position from which the sections shown in I-N were taken. Blue lines indicate the anterior region of the palate (hard palate; I,K,M), and red lines indicate the posterior region of the palate (soft palate; J,L,N). The MEE was scored for persistence in I-N (shown below). Scale bars: 500 μm in G,H; 100 μm in I-N.

Fig. 4. Generation of K14-Irf6 transgenic mice. (A) Construct used to generate K14-Irf6 transgenic mice. (B) Quantitative RT-PCR analyses of Irf6 in the palates of control and K14-Irf6<sup>Tg</sup> mice at E14.5. <i>n</i>=6 per genotype. *<i>P</i>&lt;0.05. (C) Immunoblotting analysis of IRF6 in the MEE of control and K14-Irf6<sup>Tg</sup> mice. GAPDH was used as a loading control. (D) Bar graph shows the ratio of IRF6 per GAPDH following quantitative densitometry analysis of immunoblotting data. Three samples were analyzed. (E) Immunohistochemical analysis of IRF6 in the palate of E14.5 K14-Irf6<sup>Tg</sup> mice. Arrows indicate IRF6 expression in the MEE. (F,G) H&E staining of littermate control (F) and K14-Irf6 transgenic (Irf6<sup>Tg</sup>;G) mice at E15.5. Scale bars: 50 μm. (H-J) MicroCT analysis of the soft tissues of E18.5 control (H) and K14-Irf6<sup>Tg</sup> (I,J) mice. Seventy-eight percent of K14-Irf6<sup>Tg</sup> mice exhibit apparently normal development (I), and 22% exhibit severe craniofacial deformities (J). Arrowhead indicates palatal fusion in the K14-Irf6<sup>Tg</sup> mice with severe craniofacial deformities. Arrow indicates calvarial defect in the K14-Irf6<sup>Tg</sup> mice.

(K-M) MicroCT analysis of the hard tissue of E18.5 control mice (K) and K14-Irf6<sup>Tg</sup> mice with normal development (L) or with severe craniofacial deformities (M). Arrow indicates hypoplasia of the palatine bones in K14-Irf6<sup>Tg</sup> mice with severe craniofacial deformities. Error bars represent s.d.
penetration (n=19/19) (supplementary material Fig. S3). Thus, Smad4<sup>fl/fl</sup>;K14-Cre;Ir6<sup>f6<sub>R84C</sub></sup> mice have a more severe phenotype than do Smad4<sup>fl/fl</sup>;K14-Cre mice, suggesting that IRF6 and SMAD4 function synergistically in regulating embryogenesis. In addition, the extra digits in Smad4<sup>fl/fl</sup>;K14-Cre;Ir6<sup>f6<sub>R84C</sub></sup> mice are consistent with the abnormal toe and nail phenotype in individuals with VWS and PPS. Because some individuals with VWS and PPS have cleft palate, we investigated the palate in Smad4<sup>fl/fl</sup>;K14-Cre;Ir6<sup>f6<sub>R84C</sub></sup> mice in detail by scanning electron microscopy (SEM), and detected compromised rugae formation (Fig. 3G,H). By histological analysis, we found that all Smad4<sup>fl/fl</sup>;K14-Cre;Ir6<sup>f6<sub>R84C</sub></sup> mice exhibited submucous cleft palate at birth with persistence of the MEE (n=19/19) and in some cases a stretched epithelial bridge (n=6/19) (Fig. 3I-O). Taken together, our findings suggest that a genetic interaction between Smad4 and Irf6 is responsible for MEE disappearance during palatal fusion.

**Rescue of palatal fusion in Tgfbr2<sup>fl/fl</sup>;K14-Cre mice by overexpression of Irf6**

To test whether Irf6 expression level controls MEE cell fate, we generated K14-driven Irf6 transgenic (K14-Irf6<sup>Tg</sup>) mice to overexpress Irf6 in MEE cells (Fig. 4A). Most K14-Irf6<sup>Tg</sup> mice were born healthy and fertile without any noticeable pathological phenotype up to the age of one year (n=114/146) (supplementary material Table S2). We analyzed Irf6 gene expression level by quantitative RT-PCR and found that there is a significant increase in Irf6 expression (2.2-fold) in the MEE of K14-Irf6<sup>Tg</sup> mice compared with control (Fig. 4B). Moreover, we confirmed the overexpression of IRF6 protein in the MEE by immunoblotting (Fig. 4C,D) and immunostaining (Fig. 4E) at E14.5. Approximately 22% of Irf6 transgenic mice (n=32/146) exhibited absence of the calvaria and died within one day of birth (supplementary material Table S2 and Fig. S4). Although these Irf6 transgenic mice exhibited severe calvarial defects and hypoplasia of the palatine bones (Fig. 4I,M), their palatal shelves fused normally without obvious cleft palate or persistence of the MEE (Fig. 4I). Palatal fusion appeared to be unaffected in all K14-Irf6<sup>Tg</sup> mice (n=146) (Fig. 4F-M; supplementary material Table S2).

Next, we generated Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice to restore Irf6 gene expression in Tgfb2<sup>fl/fl</sup>;K14-Cre mice. By SEM analysis, rugae formation was detectable in control mice, but not in their Tgfb2<sup>fl/fl</sup>;K14-Cre and Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> littermates (Fig. 5A-C). We also found that Tgfb2<sup>fl/fl</sup>;K14-Cre mice exhibit cleft soft palate, which was partially rescued (in the anterior of the soft palate) in Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice (Fig. 5B,C). In addition, Tgfb2<sup>fl/fl</sup>;K14-Cre mice have submucous cleft palate throughout the anterior to posterior regions of the hard palate, based on histology (Fig. 5K). At E15.5 and E16.5, the MEE disappeared in control mice, whereas MEE persistence was detectable in Tgfb2<sup>fl/fl</sup>;K14-Cre mice (Fig. 5D,E,G,H). Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice show a one-day delay in the disappearance of the MEE (Fig. 5F,I). In histological sections, we detected MEE persistence (n=14/14) corresponding with the groove in the midline of the palate in Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice (Fig. 5K). Proper apoptotic degradation of the MEE was restored in Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice (n=7/7), based on histological analysis (Fig. 5L), indicating that TGFβ-mediated Irf6 signaling is functionally important and sufficient for MEE disappearance.

**IRF6 regulates MEE disappearance via p21 expression**

Apoptosis is one of the ultimate fates of the MEE during palatal fusion (Cuervo and Covarrubias, 2004). Loss of Tgfb2 in the epithelium results in absence of apoptosis and maintenance of proliferation in MEE cells (Xu et al., 2006). We analyzed the cellular defect in persistent MEE cells by assaying both cell proliferation and apoptosis activities. We found that altered cell proliferation in the MEE was restored in Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice to a level comparable to the control (Fig. 6A-D). Similarly, we found that altered apoptotic activity in the MEE was also restored in Tgfb2<sup>fl/fl</sup>;K14-Cre;Ir6<sup>Tg</sup> mice compared with the control (Fig. 6E-H). Thus, our data demonstrate that loss of Tgfb2 results in compromised Irf6 expression, continuous cell proliferation, and failure of apoptosis in MEE cells, indicating that TGFβ-mediated Irf6 expression plays a role in the disappearance of the MEE. We previously reported that p21 (also known as Cdkn1a) was decreased in the MEE of Tgfb2<sup>fl/fl</sup>;K14-Cre mice (Xu et al., 2008). We hypothesized that p21 expression might be regulated by the TGFβ-mediated IRF6 pathway. To investigate p21 expression, we performed immunohistochemical (Fig. 6I-K), immunoblotting
expression was detectable in basal epithelial cells in formation (Fakhouri et al., 2012). Indeed, increased ∆hypothesized that IRF6 might regulate mammals, 5 kb upstream and 5 kb downstream of the TSS), we Westfall et al., 2003). Because there is no conserved IRF6-binding mice, correlating with that p21 expression was restored in E14.5 (Fig. 6L) and quantitative RT-PCR (Fig. 6M,N) analyses. We found that p21 expression is regulated by p63, gene expression was significantly (∆Np63 expression levels in control, Np63 and that this is linked with ∆Np63. [Fig. 7C,F]. We also evaluated signaling cascade at E14.5 and E15.5 (Fig. 7A-F). Moreover, CRE mice, consistent with a TGFβ/IRF6/p63 signaling cascade (Fig. 8J). Finally, we investigated the functional significance of the Irf6/p21 signaling cascade using an ex vivo organ culture system. We found that either reduction of p63 or overexpression of p21 could restore the degeneration of the MEE (Fig. 8A-F). In addition, primary mouse keratinocytes were treated with Tgfbr2fl/fl;K14-Cre;Irf6Tg and Tgfbr2fl/fl;K14-Cre;Irf6R84C/R84C mice by immunoblotting data. Tgfbr2fl/fl;K14-Cre;Irf6Tg (red, n=3) and Tgfbr2fl/fl;K14-Cre;Irf6R84C/R84C (green, n=3) mice at E14.5. Error bars represent s.d. *P<0.05. Scale bars: 50 μm.

**DISCUSSION**

Submucous cleft palate, which can result from MEE defects during palatal fusion, is one of the most common forms of cleft palate in...
humans, but the molecular and developmental mechanism of submucous cleft palate are not well studied, in part because of the paucity of animal models that exhibit this phenotype (Funato et al., 2006; Pauws et al., 2009). MEE persistence affects palatal bone formation in the hard palate and muscle development in the soft palate; consequently, patients with submucous cleft palate need surgical and other procedures to develop precise physiological functions such as speech and swallowing.

We have generated an animal model in which loss of TGFβ signaling in MEE cells results in submucous cleft palate (Xu et al., 2006). In this study, we show that TGFβ-mediated Irf6 expression is crucial for the fate of MEE cells. MEE degeneration in Tgfbir2fl/fl;K14-Cre mice was restored by overexpression of Irf6, indicating that Irf6 is functionally important for the MEE disappearance mediated by TGFβ signaling. Previous studies indicate that mice with loss of Irf6 function exhibit cleft palate owing to failure of palatal shelf elevation resulting from adhesion between the palatal shelves and the tongue, following a defect in epithelial differentiation (Ingraham et al., 2006; Richardson et al., 2009). Because of the failure of palatal elevation in Irf6R84C/R84C mice, we were previously not able to investigate the role and function of Irf6 during MEE disappearance fully. In this study, using an ex vivo organ culture system, we show for the first time that Irf6R84C/R84C mutation results in the absence of apoptosis and the maintenance of proliferation in the MEE, indicating that Irf6 is crucial for MEE cell fate during palatal fusion. Although heterozygous Irf6 mutant mice have normal palate formation, a combination of loss of Smad4 and inhibition of p38 MAPK only led to ~50% reduction of Irf6 expression and persistence of the MEE in ex vivo organ culture. This result suggests that there are other factors or signaling pathways that might regulate Irf6 expression in addition to SMAD4/p38 MAPK signaling pathways (Ferretti et al., 2011; Letra et al., 2012). A recent study has shown that integration of IRF6 and the Notch ligand jagged 2 signaling is essential for controlling palatal adhesion and fusion during palatogenesis (Richardson et al., 2009). Interestingly, overexpression of Irf6 in Tgfbir2fl/fl;K14-Cre mice did not rescue the developmental defects in the palatal mesenchyme (supplementary material Fig. S5), suggesting that the TGFβ-mediated Irf6 signaling cascade plays a cell-autonomous role in regulating the fate of MEE cells during palatal fusion and that TGFβ regulates other downstream target genes that control the development of muscles in the soft palate through tissue-tissue interactions. Furthermore, using mutant mouse models, we found that TGFβ signaling regulates Irf6 and p21 expression and MEE disappearance via both SMAD-dependent and -independent pathways during palatal fusion.

**SMAD4 mutations** have been found in patients with unselected hereditary hemorrhagic telangiectasia (HHT), which is an autosomal dominant disease of vascular dysplasia (Gallione et al., 2006). The symptoms of HHT include epistaxis, telangiectases, and arteriovenous malformations, which are most often found in the lungs, brain, liver and gastrointestinal tract. In addition, **SMAD4 mutations** have been identified in families with juvenile polyposis, aortopathy, and mitral valve dysfunction (Andradi et al., 2011). Approximately 15% of people with juvenile polyposis syndrome have other abnormalities, such as cleft palate, polydactyly, intestinal malrotation, heart or brain abnormalities, and abnormalities of the genitalia or urinary tract. Although most Smad4fl/fl;K14-Cre mice have normal palates, one copy of the R84C IRF6 mutation in a Smad4 mutant background (Smad4fl/fl;K14-Cre:Irf6R84C/R84C mice) resulted in fully penetrant submucous cleft palate and polydactyly. It is important to note that Smad4fl/fl;K14-Cre:Irf6R84C/R84C mice closely phenocopy individuals affected by PPS as described in
humans with IRF6 mutation, whereas IRF6+/R84C mice do not show digit developmental defects. These results suggest that a compromised SMAD-dependent, TGFβ-mediated IRF6 signaling cascade might be responsible for developmental defects associated with PPS. Furthermore, an impaired TGFβ/IRF6 signaling cascade may cause submucosal cleft palate.

The secondary palate is divided into two parts: the anterior bony hard palate, which is about two-thirds of the secondary palate and is composed of bone, and the posterior fleshy soft palate, which is about one-third of the secondary palate and is composed of muscles. Our previous study suggests that loss of Tgbr2 in the epithelium causes cleft soft palate owing to failure of muscle development and misorientation of muscle fibers (Xu et al., 2006). Overexpression of Irf6 in the MEE of Tgbr2f/f;K14-Cre mice failed to restore epithelial-mesenchymal interaction and to support soft palate muscle development. We are currently investigating the molecular and cellular mechanisms of soft palate muscle defects in Tgbr2f/f;K14-Cre mice.

Cleft soft palate in Tgbr2f/f;K14-Cre mice was not rescued in Tgbr2f/f;K14-Cre;Irf6f/f mice. One possible explanation is that there are differential gene expression patterns in the anterior versus the posterior in the MEE. Expression of Irf6, p63 and Tgfb3 is detectable throughout the oral epithelia in wild-type mice with no variation along the antero-posterior axis of the secondary palate (Fakhouri et al., 2012; Richardson et al., 2009; Yang et al., 2008). However, gene expression of Tgfb3 was compromised specifically in the posterior palate in Irf6R84C/R84C mice, indicating that Tgfb3 expression is differentially regulated between the anterior and posterior MEE (Richardson et al., 2009). It also suggests that IRF6 provides a feedback in regulating TGFβ signaling in the posterior region of developing palate.

WNT/β-catenin signaling acts upstream of IRF6 in the lip epithelium during lip fusion (Ferretti et al., 2011). A previous study has shown that loss of WNT/β-catenin signaling results in downregulation of TGFβ, whereas expression of stabilized β-catenin in the palatal epithelium can cause ectopic Tgfb3 expression and fusion of the palatal shelf and mandible (He et al., 2011). This study clearly demonstrates that a precisely controlled TGFβ signaling level is crucial for regulating the fate of MEE cells during palatal fusion. Ablation of p63 results in diminished Irf6 expression in the palate (Moretti et al., 2010; Thomason et al., 2010), and IRF6 can regulate ΔNp63 degradation in human keratinocytes (Moretti et al., 2010). However, we found that upregulated p63 expression in wild-type mice did not affect the expression of Irf6 in MEE cells (data not shown), indicating that there is no apparent feedback loop from upregulated p63 to Irf6 expression.

In summary, our data indicate that a combination of genetic mutations in Irf6 and Smad4 cause submucous cleft palate and that TGFβ-mediated IRF6 activity is crucial for MEE disappearance. This is a significant advancement in our understanding of the mechanism of TGFβ signaling-associated cleft palate. Our findings that TGFβ-mediated Irf6 expression is responsible for MEE disappearance and that overexpression of Irf6 normalizes MEE cell fate determination in Tgbr2f/f;K14-Cre mice might provide potential therapeutic approaches for individuals with altered TGFβ signaling and submucous cleft palate. In addition, we propose that combined mutations in IRF6 and SMAD4 in humans might be useful diagnostic biomarkers for patients with cleft palate.
Acknowledgements
We are grateful to Dr Julie Mayo for critical reading of the manuscript, and to Dr Harold Slavkin for discussion. We thank Dr Harold Moses for Tgfb2rtf mice. We thank Xuemei Deng, Toshiaki Yokota, Pablo Bringas Jr. and the Molecular Imaging Center and Center for Electron Microscopy and Microanalysis of USC for technical assistance.

Funding
This study was supported by grants from the National Institutes of Health National Institute of Dental and Craniofacial Research [DE020065 and DE012711 to Y.C.], and from the UK Medical Research Council [G0901539 to M.J.D.]. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi:10.1242/dev.089615/-/DC1

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