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 (Smad4⁺/-,K14-Cre;Irf6⁺/R84C) results in compromised p21 expression and MEE persistence, similar to observations in Tgbr2⁺/-,K14-Cre mice, although the secondary palate of Irf6⁺/-R84C and Smad4⁺/-,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 Tgbr2⁺/-,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 polyposis syndrome, and TGFBR1 or TGFBR2 mutation in Loeys-Dietz syndrome (previously called Marfan syndrome type II). Mutations in TGFBR3, 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 (ABC48), 8q24.21, 10q25 (IRX1), 17q22 and 20q12 (MAFB) have been identified by genome-wide association studies (Dixon et al., 2011). Thus, mutations in Irf6, SMAD4 and TGFBR2 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; Hocevar 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; Sirichomthong 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, syndactyly, distinct toe and nail abnormality, syndactyly and genito-urinary malformations. An arginine 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
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 \(Irfg6^{+/+}\) (null) embryos exhibit abnormal skin, limb and craniofacial morphogenesis, including cleft palate (Ingraham et al., 2006). Mice homozygous for \(Irfg6^{R44C}\), 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 homozygous \(Irfg6^{+/+}\) (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 \(Smad^{+/+}\);K14-Cre;\(irf6^{R44C}\) mice, we mated \(Smad^{+/+}\);K14-Cre;\(irf6^{R44C}\) with \(Smad^{+/+}\);K14-Cre mice. To generate \(Tg\beta rb^{+/+}\);K14-Cre mice, we mated \(Tg\beta rb^{+/+}\);K14-Cre with \(Tg\beta rb^{+/+}\) mice. Genotyping was performed using PCR primers as previously described (Ito et al., 2003; 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 \(EcoRI\)-\(HindIII\) blunted 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 \(\beta\)-globin intron (736 bp), the coding sequence for \(irf6\) (4.1 kb) and the K14 polycadenylation 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, Dessell, Germany) and microinjected in the pronuclei of fertilized oocytes (Jackson/B6D2F1) following standard procedures. Transgenic founder mice were identified by PCR analysis. PCR amplification of tail genomic DNAs (0.5-1 μg) was performed on a thermal cycler, with 35 cycles consisting of 94°C, 62°C and 72°C for 1 minute each. An aliquot (15 μl) of each reaction was resolved in 1% agarose gel, and amplified fragments were visualized by ethidium bromide staining. The PCR primers used were 5’-TCAGGAGCAGGTGCACACAAGATT-3’ and 5’-ACTCGCACTCCTTTCAATTTAAT-3’.

To generate \(Tg\beta rb^{+/+}\);K14-Cre;\(irf6^{R44C}\) mice, we mated \(Tg\beta rb^{+/+}\);K14-Cre;\(irf6^{R44C}\) with \(Tg\beta rb^{+/+}\) mice. Genotyping was performed using PCR primers as previously described (Ito et al., 2003; Xu et al., 2008; Xu et al., 2006). All mouse experiments were conducted in accordance with protocols approved by the Department of Animal Resources and the Institutional Animal Care and Use Committee of the University of Southern California.

**Comparative analysis of transcription factor-binding sites**

Genomic sequences of the entire human and murine \(IRF6\) (RefSeq accession NM_001114980/hg19 and NM_011641.2/mm10) and CDKN1A/p21 (RefSeq accession NM_000389.4/hg19 and NM_006147.3/mm10) genes were obtained from the University of California, Santa Cruz (UCSC) genome browser, including 2.5 kb upstream and 2.5 kb downstream of the respective transcription start sites (TSSs), based on mouse genome Build 38. These sequences were mapped to seven additional mammalian genomes \(\text{chimpzanpe\(z\)}\text{e} \text{Build} 2.1.4, \text{orugut\(an\)}\text{an} \text{Build} 2.0.2, \text{thesc\(u\)macque \text{Build} 1.0, \text{human \text{Build} 19, \text{rat \text{Build} 5, \text{dog \text{Build} 3.1 and horse \text{Build} eqsc\(a\)b2\} using the BLAT tool. Multiple alignments for these sequences were obtained using the ClustalW2 tool with default parameters and settings (Larkin et al., 2007). To account for uncertainty in the quality of the horse and dog draft genome sequences, sequence alignments with and without information from these species were performed. Transcription factor-binding motifs relevant to SMAD, p63 (TRP63 – Mouse Genome Informatics) and p38 MAPK pathway elements were searched with and proximal to these genes (i.e. 2.5 kb upstream and 2.5 kb downstream of the mouse TSS for each gene). The recognition sequences searched for SMAD binding sites were 5’-GTCT-3’, 5’-AGAC-3’ and 5’-GCTTAGAC-3’ (Denissova et al., 2000; Zawel et al., 1998) and for MEF2 family member binding was 5’-TAYAWWWTAR-3’ (Black and Olson, 1998). In addition, we evaluated the following potential p63 recognition sites: the canonical p53 family 20-base recognition sequence formed from duplicates of the half-site recognition sequence 5’-RRRCWGWYY-3’ separated by any combination of 0 to 13 bases (Cai et al., 2012; de-Deiry et al., 1992) and the p63-preferential recognition sequence 5’-RRRCWWGYYRRCWGYYY-3’ (Perez et al., 2007). To account for the possibility of degenerate p53 family member-binding sites, we used the p53scan and p63scan algorithms with default options (Kouwenhoven et al., 2010), which allow the consensus half-site to vary slightly in composition. Finally, we also searched for the eight-base recognition sequence 5’-AANNGAAA-3’ for the DNA-binding domains of IRF family members (Fujii et al., 1999) and the more specific ten-base recognition sequence 5’-AACCAGAACY-3’ for the IRF6 DNA-binding domain (Little et al., 2009).

**Promoter-proximal search of p53-family responsive elements**

The aforementioned consensus recognition sequences of p53 family members (see previous section) were used to search the murine and human p21 genomic sequences for putative p53 and p63 recognition sites proximal to the promoter. Genomic sequences were obtained from the UCSC genome browser, spanning a 5-kb window centered around the transcription start site of each p21 RefSeq transcript variant in the murine genome (build mm10, 2 transcript variants) and human genome (build hg19, 4 transcript variants).

**Chromatin immunoprecipitation (ChIP) assay**

Back skin was dissected from embryonic day (E) 13.5 C57B6/J mouse embryos in PBS. For the separation of skin epithelium from mesenchyme, the explants were incubated in 0.25% dispase (Invitrogen) for 30 minutes at 37°C. The epithelium was further dissociated in a 0.25% trypsin-EDTA solution for 10 minutes at 37°C, fixed with 1% formaldehyde for 15 minutes at room temperature and lysed in cell lysis buffer (Cell Signaling Technology) with a cocktail of proteinase inhibitors (Ultra Complete Mini, Roche). After pre-clearing treatment, cell extracts were incubated with anti-SMAD4, anti-MEF2 (Cell Signaling Technology), anti-p63 or IgG control (Santa Cruz Biotechnology) antibodies for two hours according to the manufacturer’s protocol (Cell Signaling Technology). We assayed for the presence of putative target sites in the immune complexes by PCR using primers amplifying the following genomic regions: mouse \(irf6\) promoter SMAD-binding site (BS) 1, 5’-GACGGCTCTGCTGATTC-3’ and 5’-TGCTGCTTGGTCACCTTTAC-3’; mouse \(irf6\) promoter SMAD2 BS 2, 5’-GAAACGTATTCTGGGGCTC-3’ and 5’-GACGACCTTTATTGGGCT-3’; mouse \(irf6\) promoter SMAD3 BS 3, 5’-GCTGCTTATTATTTTGGTGGT-3’; mouse \(irf6\) promoter SMAD3 BS 4, 5’-TCTGGTGCTATTACCAAGAATTCC-3’ and 5’-TGAGGTGGGGGTTGATGTGG-3’; mouse \(irf6\) promoter MEF2 BS2, 5’-CAGATCAACACACAGCATCTCG-3’ and 5’-CAGAGCTCTGTCACCTTTGA-3’; mouse \(p21\) promoter p63 BS 1, 5’-CTCGACAGACAGGCTCTTT-3’ and 5’-AcCCTCTCGGCTGCTTCTTA-3’; mouse \(p21\) promoter p63 BS 2, 5’-TGGTCTTCCATCGGGAATTTGTT-3’ and 5’-TGTATGCGGCTGCTTCTTA-3’; mouse \(p21\) promoter MEF2 BS, 5’-CGATCAACACACAGCATCTCG-3’. Positions of PCR fragments correspond to National Center for Biotechnology Information (NCBI) mouse genome Build 33.1.

**Histological examination**

Hematoxylin and Eosin (H&E), immunohistochemical and 5-bromo-2’-deoxyuridine (BrDU) staining were performed as described previously (Iwata et al., 2012; Iwata et al., 2010). Antibodies used for immunohistochemistry were rabbit polyclonal antibodies against p21 (Santa Cruz Biotechnology), IRF6 (Aviva Systems Biology), phosphorylated histone H3 (Millipore) and Lex/SSEA1 (FUT4 – Mouse Genome Informatics). Cell Signaling Technology), and mouse monocular antibody against p63 (Santa Cruz Biotechnology). Fluorescence images were obtained using a fluorescence microscope (Model IX71, Olympus).
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 in BGJb medium containing Lipofectamine LTX and PLUS reagents (Invitrogen) according to the manufacturer’s protocol. For p21, palatal shelves were transfected with a GFP-tagged p21 overexpression or control vector (Origene). The 2-μg 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. 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 (2×106 cells) were plated in a 60-mm cell culture dish until the cells reached 60-80% confluence. Tgfr2 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′-AGGGCTCTGTCAATTACCCAG-3′ and 5′-TGATCCGGGCT-GCACTTTTC-3′; p21 (Cdh1α), 5′-AGCTGGAAGACTGTGATGGG-3′ and 5′-AAAAGTTCACCGTTCCTCAG-3′; ΔNp63, 5′-AAGACCAACT-TGGAGGACGAAAA-3′ and 5′-AGGAGGCGGTCTGAATCTG-3′; and Gapdh, 5′-AACTTTGCGATTGTGGAAAG-3′ and 5′-AACATGT-GGGTATGCAA-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 Alcian Blue-Alizarin Red S staining protocol as previously described (Iwata et al., 2010). Antibodies used for immunoblotting were rabbit polyclonal antibodies against IRF6 (Aviva Systems Biology) and p21 (Abcam), and mouse monoclonal antibodies against IRF6 (Aviva Systems Biology) and p21 (Abcam). Samples were processed as described previously (Iwata et al., 2010). The following PCR primers were used: irf6, 5′-AGGGCTCTGTCAATTACCCAG-3′ and 5′-TGATCCGGGCT-GCACTTTTC-3′; p21 (Cdh1α), 5′-AGCTGGAAGACTGTGATGGG-3′ and 5′-AAAAGTTCACCGTTCCTCAG-3′; ΔNp63, 5′-AAGACCAACT-TGGAGGACGAAAA-3′ and 5′-AGGAGGCGGTCTGAATCTG-3′; and Gapdh, 5′-AACTTTGCGATTGTGGAAAG-3′ and 5′-AACATGT-GGGTATGCAA-3′.
RESULTS

TGFβ signaling in the MEE regulates Irf6 expression during palatal fusion

Loss of TGFβ signaling in the basal epithelium in mice (Tgfbr2<sup>fl/fl</sup>;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 E14.5 (Fakhouri et al., 2012).

Because SMAD4 and p38 MAPK are functionally redundant in regulating the MEE disappear (Xu et al., 2008), we performed ex vivo organ culture of E13.5 palatal shelf explants from Smad4<sup>fl/fl</sup>;K14-Cre and Smad4<sup>fl/fl</sup> 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 Smad4<sup>fl/fl</sup>;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; Torο 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: −1695 bp to −1692 bp; site 2: −2373 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 Irf6<sup>R84C/R84C</sup> mice, we used the ex vivo organ culture system. E13.5 wild-type control (n=17) and Irf6<sup>R84C/R84C</sup> (n=39) palate explants fused and the MEE disappeared following two days of culture (Fig. 2A,B). By contrast, Irf6<sup>R84C/R84C</sup> 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 Irf6<sup>R84C/R84C</sup> MEE cells compared with wild-type littermate control and Irf6<sup>R84C/R84C</sup> MEE cells during palatal fusion (Fig. 2J,K). The MEE in control and Irf6<sup>R84C/R84C</sup> explants disappeared completely following three days in culture; however, the MEE persisted in Irf6<sup>R84C/R84C</sup> 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 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.

DEVELOPMENT
penetration \((n=19/19)\) (supplementary material Fig. S3). Thus, Smad4/IRF6;K14-Cre;Irf6/; mice have a more severe phenotype than do Smad4/IRF6;K14-Cre mice, suggesting that Irf6 and SMAD4 function synergistically in regulating embryogenesis. In addition, the extra digits in Smad4/IRF6;K14-Cre;Irf6/; 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/IRF6;K14-Cre;Irf6/; mice in detail by scanning electron microscopy (SEM), and detected compromised rugae formation (Fig. 3G,H). By histological analysis, we found that all Smad4/IRF6;K14-Cre;Irf6/; 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 Tgfbr2fl/fl;K14-Cre mice by overexpression of Irf6**

To test whether Irf6 expression level controls MEE cell fate, we generated K14-driven Irf6 transgenic \((K14-Irf6fl)\) mice to overexpress Irf6 in MEE cells (Fig. 4A). Most K14-Irf6fl mice were born healthy and fertile without any noticeable pathological phenotype up to the age of one year \((n=14/146)\) (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 \((2.2\text{-}fold)\) in the MEE of K14-Irf6fl 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 an open eye phenotype 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-Irf6fl mice \((n=146)\) (Fig. 4F-M; supplementary material Table S2).

Next, we generated Tgfbr2fl/fl;K14-Cre;Irf6fl mice to restore Irf6 gene expression in Tgfbr2fl/fl;K14-Cre mice. By SEM analysis, rugae formation was detectable in control mice, but not in their Tgfbr2fl/fl;K14-Cre and Tgfbr2fl/fl;K14-Cre;Irf6fl littermates (Fig. 5A-C). We also found that Tgfbr2fl/fl;K14-Cre mice exhibit cleft soft palate, which was partially rescued (in the anterior of the soft palate) in Tgfbr2fl/fl;K14-Cre;Irf6fl mice (Fig. 5B,C). In addition, Tgfbr2fl/fl;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, but not in Tgfbr2fl/fl;K14-Cre;Irf6fl mice (Fig. 5D,E,G,H). Tgfbr2fl/fl;K14-Cre;Irf6fl 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 Tgfbr2fl/fl;K14-Cre mice (Fig. 5K). Proper apoptotic degradation of the MEE was restored in Tgfbr2fl/fl;K14-Cre;Irf6fl mice \((n=7/7)\), based on histological analysis (Fig. 5L), indicating that TGFB-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 Tgfbr2 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 Tgfbr2fl/fl;K14-Cre;Irf6fl 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 Tgfbr2fl/fl;K14-Cre;Irf6fl mice compared with the control (Fig. 6E-H). Thus, our data demonstrate that loss of Tgfbr2 results in compromised Irf6 expression, continuous cell proliferation, and failure of apoptosis in MEE cells, indicating that TGFB-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 Tgfbr2fl/fl;K14-Cre mice (Xu et al., 2008). We hypothesized that p21 expression might be regulated by the TGFB-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 site in the promoter-proximal genomic region of Westfall et al., 2003). Because there is no conserved IRF6-binding that p21 expression was restored in E14.5 (Fig. 6L) and quantitative RT-PCR (Fig. 6M,N) analyses. We found -mediated Irf6-mediated IRF6 activity regulates MEE disappearance by regulating p21 expression.

Previous studies have demonstrated that IRF6 induces degradation of the p63 isoform ΔNp63 and that this is linked with the pathogenesis of VWS and PPS (Moretti et al., 2010; Thomason et al., 2010). In addition, ΔNp63 represses transcription of p21 in vitro and in vivo (Laurikkala et al., 2006; Welsh and O’Brien, 2009; Westfall et al., 2003). Because there is no conserved IRF6-binding site in the promoter-proximal genomic region of p21 (in at least six mammals, 5 kb upstream and 5 kb downstream of the TSS), we hypothesized that IRF6 might regulate p21 expression via ΔNp63. ΔNp63 is only expressed in the basal epithelial layer during palate formation (Fakhouri et al., 2012). Indeed, increased ΔNp63 expression was detectable in basal epithelial cells in Tgfrbr2fl/fl;K14-Cre mice, consistent with a TGFβ-mediated Irf6/ΔNp63/p21 signaling cascade at E14.5 and E15.5 (Fig. 7A-F). Moreover, ΔNp63 expression was reversed by overexpression of Irf6 (Fig. 7C,F). We also evaluated ΔNp63 expression levels in control, Tgfrbr2fl/fl;K14-Cre and Tgfrbr2fl/fl;K14-Cre;Irf6f8g mice by immunoblotting (Fig. 7G). To confirm that p21 expression is regulated by p63, we analyzed the mouse p21 promoter region (2.5 kb upstream and 2.5 kb downstream of the TSS), and found two potential p63-binding sites in the mouse genomic region of p21 (Fig. 7H). We performed ChIP analysis to test whether p63 could bind to the genomic region of p21, and found that the p21 genomic region immunoprecipitated with the p63 antibody at p63-binding site 1 (+727 bp to +746 bp), but not at p63-binding site 2 (~436 bp to ~455 bp) (Fig. 7I). These results indicate that p21 expression is likely to be regulated through the TGFβ/IRF6/ΔNp63 signaling cascade.

Finally, we investigated the functional significance of the Irf6/p63/p21 signaling cascade using an in 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 Tgfrbr2, Irf6 or control siRNA (Fig. 8G). After siRNA knockdown for p63 expression of p63 was increased in Irf6R84C/R84C keratinocytes (Moretti et al., 2010). Therefore, we investigated whether p63 expression was altered in Irf6R84C/R84C palates (Thomason et al., 2010) and siRNA knockdown for Irf6 results in upregulated p63 expression in human keratinocytes (Moretti et al., 2010). Therefore, we investigated whether p63 expression was increased in Irf6R84C/R84C palates, consistent with previous findings (Fig. 8H,J). Collectively, these data suggest that p21 expression is regulated through a TGFβ/IRF6/ΔNp63 signaling cascade (Fig. 8J).

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., 2012; 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 signaling in MEE cells results in submucous cleft palate (Xu et al., 2006). Because of the failure of palatal shelf elevation in mice, we were previously not able to investigate the role and function of Irf6 during palatal fusion fully. In this study, using an ex vivo organ culture system, we show for the first time that TGFβ signaling 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 (Andrabi 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 **SMAD4** mutations have normal palates, one copy of the R84C mutant mouse 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.

**Fig. 7. Increased ΔNp63 expression in Tgfbr2fl/fl;K14-Cre mice.** (A-F) Immunohistochemical analyses of ΔNp63 expression in the palates of Tgfbr2fl/fl control (A,D), Tgfbr2fl/fl;K14-Cre (B,E) and Tgfbr2fl/fl;K14-Cre;Irf6fl/fl;K14-Cre;Irf6R84C (C,F) mice at E14.5 (A-C) and E15.5 (D-F). Brown, positive signal. Nuclei were counterstained with 0.03% Methylene Blue. Scale bars: 50 μm. (G) Immunoblotting analysis of p63 in the MEE of E14.5 Tgfbr2fl/fl control (lane 1), Tgfbr2fl/fl;K14-Cre (lane 2) and Tgfbr2fl/fl;K14-Cre;Irf6fl/fl (lane 3) mice. GAPDH was used as a loading control. Bar graph (below) shows the ratio of p63 to GAPDH following quantitative densitometry analysis of immunoblotting data. Three samples were analyzed. (H) Schematic of the upstream region of the mouse p21 gene (not to scale), showing locations of putative p63-binding sites tested in ChIP assays. Putative p63-binding sequences are shown below. Arrowheads indicate the position of primers used in ChIP analysis. (I) ChIP analysis of DNA fragments immunoprecipitated with a p63-specific antibody or with an isotype-specific control antibody. Immunoprecipitates were PCR amplified with primers flanking the putative p63-binding region. Input lane shows PCR amplification of the sonicated chromatin before immunoprecipitation. No amplification of target sites was detected when an isotype-specific control antibody was used. Ab, antibody; BS, binding site.

(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 Tgfbr2fl/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.
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 Tgbr2<sup>−/−</sup>;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 Tgbr2<sup>−/−</sup>;K14-Cre mice.

Cleft soft palate in Tgbr2<sup>−/−</sup>;K14-Cre mice was not rescued in Irf6<sup>−/−</sup>ΔNp63<sup>−/−</sup> 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 Irf6<sup>R84C/R84C</sup> 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 Tgfβ 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 Tgbr2<sup>−/−</sup>;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.

**Fig. 8.** Altered p63-p21 cascade results in persistence of the MEE in Tgbr2<sup>−/−</sup>,K14-Cre mice. (A-C) H&E staining of palate explants from Tgbr2<sup>−/−</sup> (WT) mice and Tgbr2<sup>−/−</sup>;K14-Cre (CKO) mice treated with p63 (C) or control (B) siRNA for 72 hours. n=5 per group. (D-F) H&E staining of palate explants from Tgbr2<sup>−/−</sup> (WT) mice and Tgbr2<sup>−/−</sup>;K14-Cre (CKO) mice treated with p21 overexpression vector (F) or control (E) for 72 hours. n=5 per group. (G) Quantitative RT-PCR analyses of Irf6, p21 and ΔNp63 expression in primary mouse keratinocytes isolated from back skin of newborn wild-type mice after treatment with control (blue bars), Tgbr2 (red bars) or Irf6 (green bars) siRNA. Antisense siRNA was used as control. (H) Quantitative RT-PCR analyses of Irf6 and ΔNp63 expression in primary mouse keratinocytes isolated from newborn Irf6<sup>R84C/R84C</sup> (red bars) and littermate control (blue bars) back skin. (I) Immunoblotting analysis of Irf6 and p21 in the MEE of control (+/+) and Irf6<sup>R84C/R84C</sup> (R84C/R84C) mice. GAPDH was used as a loading control. (J) Schematic depicting our model of the mechanism of TGFβ-mediated Irf6-ΔNp63-p21 gene regulation in Tgbr2<sup>−/−</sup>;K14-Cre palates. Irf6 expression is regulated through both SMAD and p38 MAPK pathways. In the absence of TGFβ receptor type II (TβRII), Irf6 expression decreases, ΔNp63 expression increases in the absence of TβRII resulting in reduced p21 expression in the palatal epithelium. Altered TGFβ-IRF6-ΔNp63-p21 activity results in the persistence of the MEE. *P, phosphorylated. Error bars represent s.d. *P<0.05; **P<0.01. Scale bars: 50 μm.
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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

References


Fig. S1. Periderm disappearance is unaffected in Tgfb2<sup>fl/fl</sup>;K14-Cre mice. (A-H) H&E staining of Tgfb2<sup>fl/fl</sup> control (A-D) and Tgfb2<sup>fl/fl</sup>;K14-Cre (E-H) mice at the indicated developmental stages. Boxed areas are shown magnified. Arrows indicate peridermal cells. (I-L) Staining of the periderm with anti-Lex/SSEA1 antibody in Tgfb2<sup>fl/fl</sup> control (I-K) and Tgfb2<sup>fl/fl</sup>;K14-Cre (L) mice. Arrows indicate positive signal.
Fig. S2. Loss of IRF6 activity causes MEE persistence. (A-C) H&E staining in palatal explants of control (A), Irf6^{+/R84C} (B) and Irf6^{R84C/R84C} (C) mice after 3 days culture. (D) Immunostaining of phosphorylated histone H3 (pH3) in Irf6^{R84C/R84C} palatal shelf explants after 3 days culture. Dashed lines indicate the MEE. Arrows indicate positive signal (green). Nuclei were counterstained with DAPI (blue). Scale bars: 100 μm.
Fig. S3. Haploinsufficiency of Irf6 in Smad4<sup>fl/fl</sup>;K14-Cre mice causes syndactyly. (A-C) Limb morphologies of newborn control (A), Smad4<sup>fl/fl</sup>;K14-Cre (B) and Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> (C) mice. Open arrow indicates abnormal nails in Smad4<sup>fl/fl</sup>;K14-Cre mice. Arrows indicate extra digits in Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> mice. (D-F) Whole-mount Alcian Blue-Alizarin Red skeletal staining of newborn control (D), Smad4<sup>fl/fl</sup>;K14-Cre (E) and Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> (F) mice. Open arrow indicates abnormal nails. Arrow indicates extra digit. (G-I) microCT images of the hind-limb in newborn control (G), Smad4<sup>fl/fl</sup>;K14-Cre (H) and Smad4<sup>fl/fl</sup>;K14-Cre;Irf6<sup>+/R84C</sup> (I) mice. Arrow indicates extra digit.
Fig. S4. Morphologies of Irf6 transgenic mice. (A,B) Top panels show side views of wild-type (A) and Irf6\textsuperscript{Tg} (B) mice, bottom panels show frontal views. Approximately 22% of Irf6 transgenic embryos exhibit a defect in calvaria formation and open eye at E18.5. (C) SEM image of the palate of newborn Irf6\textsuperscript{Tg} mice. Scale bar: 500 μm. (D-H) H&E staining of the skin (D-F) and oral epithelium (G,H) of wild-type control (D,G) and Irf6\textsuperscript{Tg} (E,F,H) mice at E18.5. Boxed area in F is shown magnified in E. Scale bars: 50 μm. (I-N) Whole-mount Alcian Blue-Alizarin Red skeletal staining of wild-type control (I,L) and Irf6\textsuperscript{Tg} (J,K,M,N) newborn mice. Arrow indicates a defect in the calvaria. Open arrow indicates a defect in the palatine bone. Eighty percent of Irf6\textsuperscript{Tg} mice show normal craniofacial development (middle panels; J,M), but 20% of Irf6\textsuperscript{Tg} mice exhibit defects in the calvaria and the palatine bone (right panels; K,N).
Fig. S5. Cleft soft palate in Tgfbr2<sup>fl/fl</sup>;K14-Cre and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> mice. (A-H) H&E staining of Tgfbr2<sup>fl/fl</sup>;K14-Cre (A-D) and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> (E-H) mice at E18.5. Boxed areas are enlarged in panels to the right. Scale bars: 250 μm in left-hand panels; 100 μm in right-hand panels. (I-K) MicroCT analysis of the hard tissues of E18.5 control (I), Tgfbr2<sup>fl/fl</sup>;K14-Cre (J) and Tgfbr2<sup>fl/fl</sup>;K14-Cre;Irf6<sup>Tg</sup> (K) mice.
### Table S1. Conserved SMAD recognition sequences in the Irf6 gene

<table>
<thead>
<tr>
<th>Binding site position relative to TSS</th>
<th>Genomic context</th>
<th>Binding site sequence context*</th>
<th>Conservation of 4-bp site</th>
</tr>
</thead>
<tbody>
<tr>
<td>–2375 to –2372</td>
<td>Upstream</td>
<td>TGT[<strong>G</strong>TCTTG<strong>A</strong>]</td>
<td>All but rat</td>
</tr>
<tr>
<td>–1695 to –1692</td>
<td>Upstream</td>
<td>GAC[<strong>G</strong>TCTTCT]</td>
<td>All but dog/horse</td>
</tr>
<tr>
<td>–274 to –271</td>
<td>Upstream</td>
<td>CCAG[<strong>G</strong>TCTC<strong>A</strong>G]</td>
<td>All eight species</td>
</tr>
</tbody>
</table>

*Binding site motif is underlined in all cases. It is noted in red if conserved in all eight species, and in blue if conserved in all but the noted species in the rightmost column. The eight species include: human (Build 19), chimpanzee (Build 2.1.3), orangutan (Build 2.0.2), rhesus macaque (Build 1.0), mouse (Build 38), rat (Build 3.4), dog (Build 2) and horse (Build equCab2).
Table S2. Penetrance of abnormalities in *Irf6* transgenic mice

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Wild-type mice</th>
<th><em>Irf6</em> transgenic mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skull defect</td>
<td>1.28% (2/156)</td>
<td>21.92% (32/146)</td>
</tr>
<tr>
<td>Open eye</td>
<td>0% (0/156)</td>
<td>21.92% (32/146)</td>
</tr>
<tr>
<td>Cleft lip</td>
<td>0% (0/156)</td>
<td>2.74% (4/146)</td>
</tr>
</tbody>
</table>

Phenotype of wild-type littermate control and *Irf6* transgenic mice was scored at E18.5.