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Sox2 Controls Periderm and Rugae Development to Inhibit Oral Adhesions

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Abstract

In humans, ankyloglossia and cleft palate are common congenital craniofacial anomalies, and these are regulated by a complex gene regulatory network. Understanding the genetic underpinnings of ankyloglossia and cleft palate will be an important step toward rational treatment of these complex anomalies. We inactivated the Sry (sex-determining region Y)–box 2 (*Sox2*) gene in the developing oral epithelium, including the periderm, a transient structure that prevents abnormal oral adhesions during development. This resulted in ankyloglossia and cleft palate with 100% penetrance in embryos examined after embryonic day 14.5. In *Sox2* conditional knockout embryos, the oral epithelium failed to differentiate, as demonstrated by the lack of keratin 6, a marker of the periderm. Further examination revealed that the adhesion of the tongue and mandible expressed the epithelial markers *E-Cad* and *P63*. The expanded epithelia are Sox9-, Pitx2-, and Tbx1-positive cells, which are markers of the dental epithelium; thus, the dental epithelium contributes to the development of oral adhesions. Furthermore, we found that *Sox2* is required for palatal shelf extension, as well as for the formation of palatal rugae, which are signaling centers that regulate palatogenesis. In conclusion, the deletion of *Sox2* in oral epithelium disrupts palatal shelf extension, palatal rugae formation, tooth development, and periderm formation. The periderm is required to inhibit oral adhesions and ankyloglossia, which is regulated by *Sox2*. In addition, oral adhesions occur through an expanded dental epithelial layer that inhibits epithelial invagination and incisor development. This process may contribute to dental anomalies due to ankyloglossia.

Keywords: ankyloglossia, cleft palate, tooth agenesis, palate rugae, craniofacial anomaly, oral epithelium

Introduction

Ankyloglossia is a common condition affecting up to 10% of newborns (Chandrashekar et al. 2014; Veyssiere et al. 2015; Yoon et al. 2017). The condition presents with an inappropriate fusion of the tongue to the alveolar ridge or floor of the mouth and can have several debilitating consequences, including difficulty with breastfeeding and problems with speech, swallowing, and gaining weight (Chandrashekar et al. 2014; Ferrés-Amat et al. 2016). Although the condition is readily treatable in human patients, the genetic underpinnings of ankyloglossia remain largely uninvestigated.

Clefting is one of the most common congenital defects observed in humans and has, in some cases, been shown to arise from oral adhesion (Richardson et al. 2009; Paul et al. 2017). Another defect observed in humans associated with ankyloglossia is tooth abnormalities, as reported in several families (Chandrashekar et al. 2014; Lenormand et al. 2018).

During craniofacial development, the oral cavity is usually protected from inappropriate oral adhesions by the formation of a transient structure called the *periderm*. The periderm is derived from the basal layer of the oral mucosa and lines the oral cavity. These flat Keratin 6 (K6)–, Keratin 17–positive cells have been shown to provide a barrier function in the developing oral cavity and skin. Several genes have been implicated in the development of the periderm, including *Irf6, Grhl3, Ikka*, and *Sfn*, and the genetic ablation of any of these

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genes in the oral ectoderm results in oral adhesions leading to cleft palate (Richardson et al. 2014; Kousa et al. 2017; Hammond et al. 2019). These previous studies indicate that ankyloglossia may be related with the abnormal formation of periderm during development.

We recently reported that the inactivation of *Sox2* in the oral epithelium with the $Pitx2^{Cre}$ resulted in tooth agenesis and defective invagination of developing tooth buds, which remained at the surface of the oral epithelial layer (Sun et al. 2016). Further examination of these embryos revealed that they also had a cleft palate and ankyloglossia. We found that the periderm was not well formed and poorly organized in this mouse model, resulting in ankyloglossia. Additionally, we show that the oral adhesion is composed of the inappropriate adhesion of the tongue and dental epithelia, linking ankyloglossia with defects in dental development.

Materials and Methods

Mouse Lines and Embryonic Staging

Mouse experiments were performed in accordance with rules provided by the Office of Animal Resources at the University of Iowa. The $Sox2^{Flox/Flox}$, $Pitx2^{Cre}$, and Shh^{Cre} were described in a previous publication (Liu et al. 2003; Taranova et al. 2006; Sun et al. 2016). The $Pitx2^{Cre}/Sox2^{F/F}$ mice are termed $Sox2^{CKO}$.

Genotype Primers. Sox2 WT:

Forward: 5′ GCTCTGTTATTGGAATCAGGCTGC 3′
Reverse: 5′ CTGCTCAGGGAAGGAGGGG 3′

Sox2 CKO:

Forward: 5′ CAGCAGCCTCTGTTCCACATACAC 3′
Reverse: 5′ CAACGCATTTCAGTTCCCCG 3′

Tissue Fixation and Slide Preparation

Embryos were fixed in 4% PFA (ChemCruz) and embedded in paraffin; samples were sectioned at 7 µm with Thermo (HM325) microtome per previous reports (Sun et al. 2016).

Hematoxylin and Eosin Staining

Sections were stained by hematoxylin and eosin as previously described (Sun et al. 2016).

Immunofluorescence Staining and Confocal Imaging

Slides were subjected to citric acid antigen retrieval, washed 2 times with 1X PBS for 5 min, and blocked with 20% donkey serum in PBS for 30 min at room temperature. Slides were then incubated with primary antibody (Pitx2 antibody, 1:50, AF7388 [R&D Systems]; Sox2 antibody, 1:50, AF2018 [R&D Systems]; P63 antibody, 1:50 [Biocare Medical]; Loricrin, 1:500, 905101 [BioLegend]; Keratin 6A, 1:500, 905702 [BioLegend]; Desmoplakin, 1:50 [Bio-Rad]) overnight at 4 °C. Slides were washed with 1X PBS, and secondary antibody (Life Technologies) was applied for 30 min, incubated with DAPI solution (Thermo Scientific), and mounted with cover slips. Confocal pictures were taken with a ZEISS 700 confocal microscope and Zen imaging software.

IdU/CIdU Labeling Assay

CIdU was injected 24 h prior to harvesting embryonic day 15.5 (E15.5) embryos (i.e., E14.5), and IdU was injected 1 h prior to harvesting embryos. Both analogues were injected at 100-µg/g body weight of the pregnant female mouse. Embryos were then harvested and embedded into paraffin with the approach described previously. Staining for IdU/CIdU was performed in accordance with a previous study (Tuttle et al. 2010; Sun et al. 2016).

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNAs were extracted with the RNeasy Mini Kit from Qiagen and reverse transcribed into cDNA with PrimeScript RT Reagent Kit (TaKaRa).

Polymerase Chain Reaction Primers. SOX2:

Forward: 5′ GCCGAGTGGAAACTTTTGTCG 3′
Reverse: 5′ GGCAGCGTGTACTTATCCTTCT 3′

KRT6A:

Forward: 5′ TGCTGCCTACATGAACAAGG 3′
Reverse: 5′ TGTCTGAGATGTGGGTCTGC 3′

ChIP

The chromatin immunoprecipitation (ChIP) assay was performed on GMSMK cells with a ChIP assay kit (17-295; Millipore) and Sox2 Ab (R&D systems). ChIP primers were selected around the predicted Sox2 binding site and a control region, and regions were amplified by polymerase chain reaction.

ChIP Primers. Negative control:

Forward: 5′ CTTCTTCCAAATATGCCCGTCAGTG 3′
Reverse: 5′ CACATTGAGTTTGACGCATGTTC 3′

SOX2 BS:

Forward: 5′ CCAAATGTTGGAGAAATGGGACTG 3′
Reverse: 5′ GTTTACAGAGGAATGAGCTTCACTTCTCC 3′

Statistical Analysis for Experiments

Each experiment was repeated 3 times with at least 3 mutant and control embryos for mouse studies. The results are shown as the mean ± standard error of the mean (SEM), and the analyses were performed with an independent 2-tailed *t* test.

Results

Sox2^{CKO} *Embryos Develop Ankyloglossia due to Defects in Periderm Formation*

We examined the E13.5, E14.5, E15.5, and E18.5 developmental stages of murine embryos with sagittal sections of control (*Pitx2^{Cre}*) and *Pitx2^{Cre}/Sox2$^{F/F}$* (*Sox2^{CKO}*) embryos. Interestingly, a delay was noted in the invagination of the dental epithelium in the *Sox2^{CKO}* embryos (Fig. 1A, B). By E14.5, the delay in dental epithelial invagination may have caused the fusion of the tip of the tongue to the mandible (Fig. 1D, arrow). The apparent fusion of the dental and tongue epithelium results in a further disruption of incisor dental epithelial cell invagination. This ankyloglossia phenotype was present at E15.5 and E18.5 as compared with the control embryos (Fig. 1F, F′, H, H′). The ankyloglossia phenotype was also observed in the *Shh^{Cre}-Sox2$^{F/F}$* embryos, demonstrating that 2 independent murine *Cre* lines created similar *Sox2^{CKO}* phenotypes (Fig. 4E′).

To determine the nature of the tissue connecting the tongue and the mandible, we performed immunostaining for markers of different cell types found in the developing oral mucosa, and we determined that the adhesion tissue connecting the mandible epithelium to the tongue epithelium in *Sox2^{CKO}*embryos expressed E-cadherin (Fig. 2A, B). We next stained for K6, a protein expressed by the transient periderm layer. While the E15.5 control embryos had a well-formed layer of K6$^+$ periderm cells on the surface of the developing mandible (Fig. 2C, C′), this layer was poorly formed in *Sox2^{CKO}*embryos (Fig. 2D, D′), and only a few K6$^+$ cells were identified in the anterior mandibular oral epithelium. Interestingly, the K6$^+$ periderm layer identified in the controls at E15.5 also expressed *Sox2*, suggesting a potential role for *Sox2* in the formation of the periderm. To verify the results of the K6 staining, we next stained for Grhl3, a reported marker of the periderm (Fig. 2E, F). In *Sox2^{CKO}* embryos, Grhl3 protein levels were significantly reduced in regions where Sox2 is expressed as compared with the control.

Previous work has demonstrated that cell adhesion complexes, including desmosomes, are localized between basal cells but are not present on the apical surface of the epidermis, thus preventing the abnormal adhesion of opposing epithelial layers in the developing skin (Richardson et al. 2014). To determine if ectopic apical organization of desmosomes contributed to the ankyloglossia phenotype that we observed in the *Sox2^{CKO}* embryos, we stained for Desmoplakin, a component of desmosomes. In control embryos, staining for Desmoplakin revealed that it was not localized apically on either the mandibular or tongue epithelia (Fig. 2G). Interestingly, in *Sox2^{CKO}* embryos, Desmoplakin expression was no longer restricted, and the tongue and mandibular epithelial layers were connected by cells with unpolarized Desmoplakin localization (Fig. 2H). These data demonstrate that the malformation of the periderm resulting from the inactivation of *Sox2* allows for the mislocalization of desmosomes, resulting in epithelial adhesion.

Costaining of P63 and Sox2 in control E15.5 embryos (Fig. 2I, I′) revealed a well-organized single layer of P63$^+$, Sox2$^-$ basal cells and a suprabasal Sox2$^+$, P63$^+$ layer. The periderm layer separates the suprabasal layer from the oral cavity. Interestingly, in *Sox2^{CKO}* embryos, the P63$^+$ basal layer was poorly organized. The disorganized P63$^+$ cells did not give rise to the K6$^+$ periderm layer observed in control embryos but instead expanded and became attached to the tongue epithelium (Fig. 2J, J′).

Deletion of Sox2 *Results in Increased Proliferation of the Dental Epithelium within the Oral Adhesion*

We examined E15.5 control and *Sox2^{CKO}* embryos for differences in proliferation using injections of the 2 thymidine triphosphate analogues, CIdU (24 h prior to sacrifice) and IdU (1 h prior to sacrifice), coupled with immunofluorescence staining to label proliferating cells (Fig. 2K, L). Neither the control nor the *Sox2^{CKO}* oral epithelial region had large numbers of proliferating cells 1 h prior to sacrifice (green), although the *Sox2^{CKO}* embryo had a much greater number of CIdU$^+$ cells (red), indicating that the oral epithelium was proliferating 24 h prior to harvesting the embryos (quantification, Fig. 2M).

Loricrin is a differentiation marker expressed in mammalian stratified epithelia that contributes to barrier function (Nithya et al. 2015). Interestingly, while the control embryos had a coherent layer of Loricrin protein marking the superior layer of the oral and dental epithelium, *Sox2^{CKO}* embryos failed to develop the Loricrin$^+$ cell layer in the region of the mandible fused to the tongue (Fig. 2N, O), the same adhesion region previously shown to have greater numbers of CIdU$^+$ cells. Thus, we concluded that the oral adhesion disrupts the formation of the Loricrin+ layer specifically at the adhesion region of the oral mucosa.

Oral Adhesion Is Composed of Dental Epithelial Cells

A major unanswered question concerning oral adhesions is the tissue type that directly interacts with the tongue epithelium. We stained for known dental epithelial marker expression in the oral adhesion because the oral adhesion connecting the tongue and the mandible occurs at the lower incisor region comprising the oral and dental epithelium. Other groups have demonstrated that pathogenic oral adhesions resulting in cleft can occur at the molar tooth buds (Richardson et al. 2006; Richardson et al. 2014; Paul et al. 2017). To determine if the dental epithelial tissue might participate in the formation of adhesions, we stained for Sox9, which is specifically expressed in the enamel organ of the developing lower incisor in control

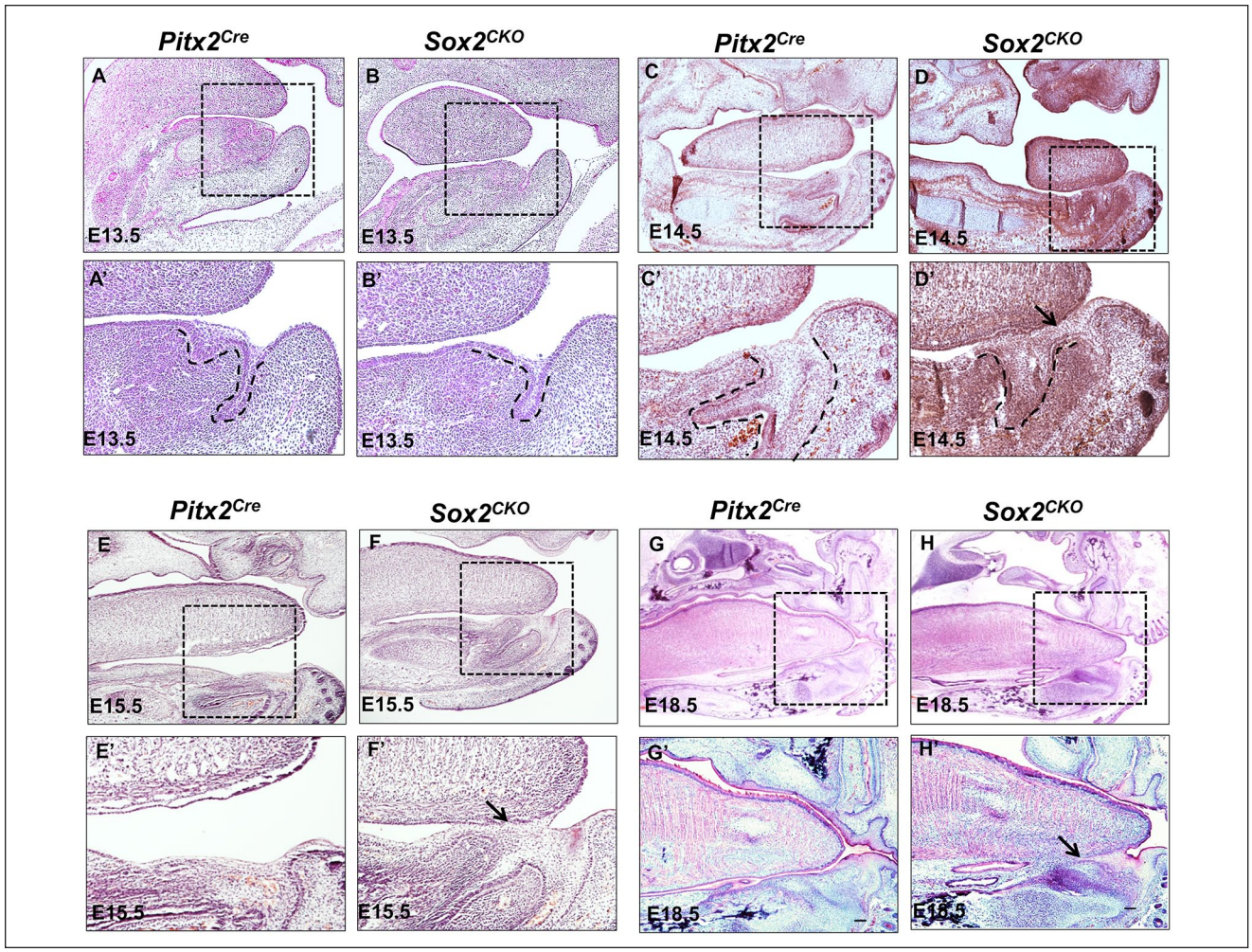

Figure 1. *Sox2^{CKO}* embryos exhibit ankyloglossia beginning at embryonic day 14.5 (E14.5). (**A**, **A′**) Sagittal sections and hematoxylin and eosin staining of E13.5 embryos reveal normal development of the tongue in control (*Pitx2^{Cre}*) embryos. The tongue is connected to the mandible in the posterior region of the mandible. (**B**, **B′**) In *Sox2^{CKO}* embryos, the tongue shows no signs of fusion in the anterior region at E13.5. (**C**, **C′**) Hematoxylin and eosin staining reveals that in control embryos, the tongue and the mandible are separated in the anterior region at E14.5. (**D**, **D′**) In *Sox2^{CKO}* embryos, the anterior portion of the tongue is fused to the mandible with an oral adhesion (black arrow). (**E**, **E′**) At E15.5, the tongue is elongated and clearly separated from the mandible in control embryos. (**F**, **F′**) Fusion of the tongue to the mandible persists in *Sox2^{CKO}* embryos. The fusion is located at the anterior region of the tongue above the tooth bud region. (**G**, **G′**) In control E18.5 embryos, tongue development proceeds, and the anterior tongue is separated from the mandible. (**H**, **H′**) In *Sox2^{CKO}* embryos, ankyloglossia persists at E18.5 (black arrow). Dotted lines in A′-D′ represent the developing dental epithelium, which is smaller and fails to invaginate in *Sox2^{CKO}* embryos. F′ Arrows denote ankyloglossia. Scale bar = 100 µm.

embryos (Fig. 3A, A′). Indeed, we demonstrate that many of the oral adhesion cells express the Sox9 protein (Fig. 3B, B′).

We stained for Pitx2 and Tbx1, which are both markers of the dental and oral epithelial tissue in control embryos (Fig. 3C, C′, E). Interestingly, the cells composing the oral adhesion expressed these markers associated with the dental epithelium (Fig. 3D, D′, E′). Thus, we conclude that the oral adhesion is composed of expanded dental epithelial cells.

Sox2 *Regulates the Palate Rugae Signaling Center and Palatogenesis*

We sectioned the heads of E15.5 control and *Sox2^{CKO}* embryos in the coronal orientation and found that whereas control E15.5 embryos had a well-formed, completely fused palate (Fig. 4A–C), palate closure had not occurred in *Sox2^{CKO}* embryos. Shelf extension, the process by which the 2 palate shelves meet at the midline at E15.5, had not occurred, nor did it occur at later stages (Fig. 4A′–C′). Furthermore, the *Shh$^{Cre\text{-}GFP}$/Sox2$^{fl/fl}$* E18.5 embryos had a cleft palate (Fig. 4D, D′) and ankyloglossia (Fig. 4E, E′). In addition, these embryos lacked palate rugae. Thus, using the *Pitx2^{Cre}* and *Shh^{Cre}* lines resulted in similar *Sox2^{CKO}* phenotypes.

Further investigation with sagittal sections taken from the midline of control (Fig. 4F, G) and *Sox2^{CKO}* embryos (Fig. 4F′, G′) revealed that the mutant embryos lacked well-defined palate rugae (denoted with arrows in control embryos), which are sources of signaling molecules during palatogenesis (Welsh and O'Brien 2009; Lin et al. 2011). *Lef-1*, which is specifically expressed in the palate rugae of E14.5 control embryos (Fig.

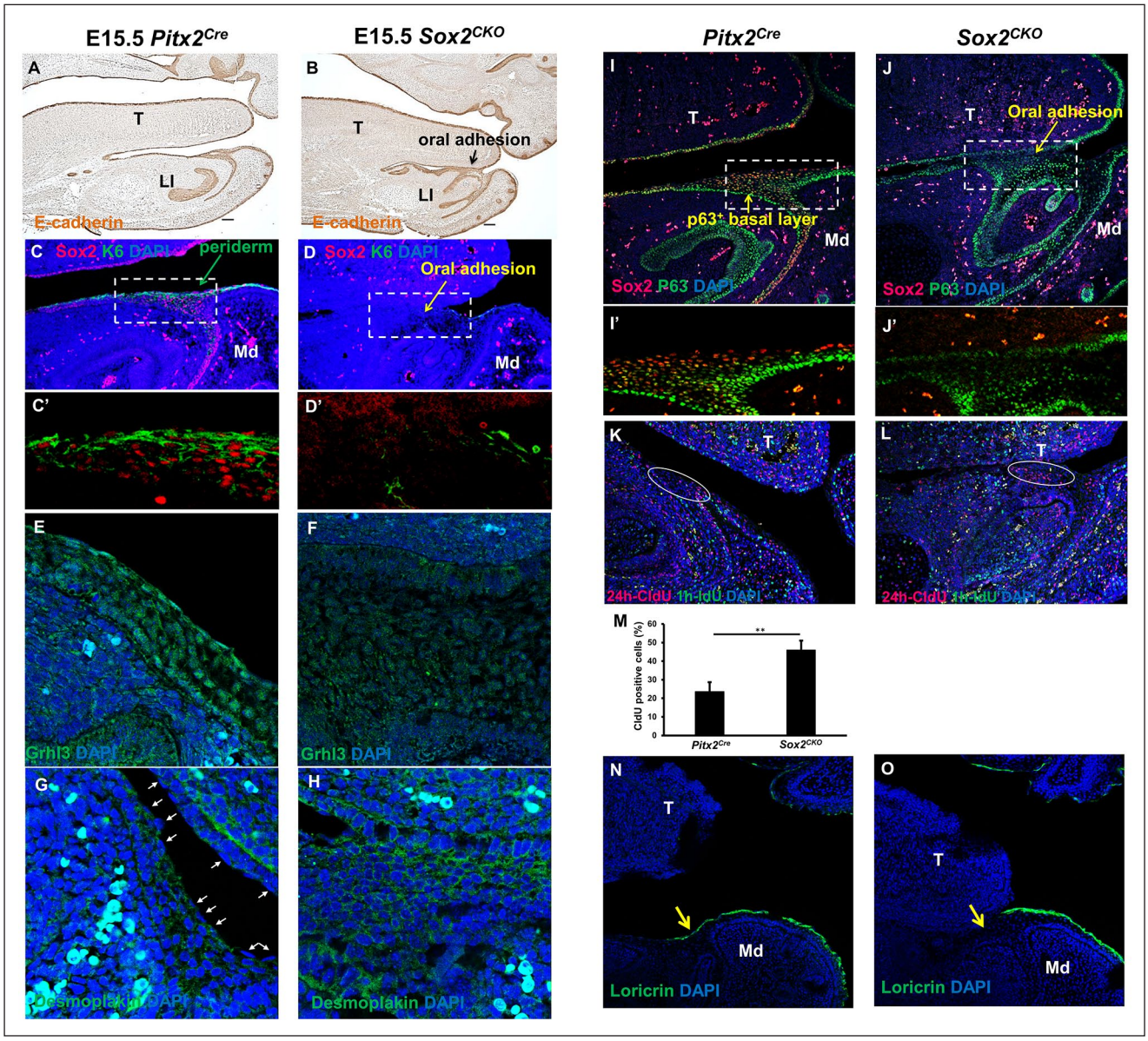

Figure 2. Loss of *Sox2* results in oral adhesion and impaired formation of the periderm. (**A**) In control embryos (embryonic day 15.5 [E15.5]), staining for E-cadherin labels the tongue, oral, and dental epithelial tissues. (**B**) When *Sox2* is ablated, E-cadherin labels the same structures, including the lower incisor, which fails to invaginate in these embryos. The oral adhesion, which is indicated with a black arrow, is also clearly expressing E-cadherin, demonstrating that the adhesion is epithelial tissue. (**C, C′**) In control embryos, Sox2 protein is labeled in the tongue and oral epithelial tissues, including the vestibule lamina. Staining for Keratin 6 also labels the periderm, as indicated by the green arrow. (**D, D′**) When *Sox2* is ablated with $Pitx2^{Cre}$, Sox2 protein is no longer labeled in the tongue and oral epithelial tissues. Additionally, the Keratin 6–positive periderm structure is almost completely ablated. (**E**) Grhl3 protein is expressed in multiple oral epithelia layers and highly expressed in the periderm of control embryos. (**F**) In $Sox2^{CKO}$ embryos, Grhl3 expression was diminished. (**G**) In control embryos, Desmoplakin protein is not expressed on the apical side of the most superficial oral epithelia. (**H**) In $Sox2^{CKO}$ embryos, Desmoplakin is ectopically expressed on the apical layer of the tongue and oral epithelia and within the oral adhesion connecting the tongue and mandible. (**I, I′**) P63 and Sox2 double staining reveals a well-ordered $P63^{+}$ basal layer (arrow). Superior to the basal layer are Sox2 and P63 double-positive cells. Finally, the periderm, the most superficial layer, contains only Sox2 protein. (**J, J′**) When *Sox2* is ablated, the well-ordered basal layer is disrupted. The oral adhesion is P63 positive. (**K**) Pregnant mice timed to E14.5 were injected with CldU, were injected with IdU 23 h later, and sacrificed 1 h later. (**L**) In $Sox2^{CKO}$ littermates, the oral adhesion is CldU positive, indicating that the oral epithelial layer was actively proliferating at E14.5. (**M**) The number of CldU+ cells is significantly higher in $Sox2^{CKO}$ embryos as compared with the control. $^{**}P < 0.01$. Values are presented as mean ± SEM. (**N**) Loricrin is expressed in the space separating the tongue and the mandible at E15.5 (yellow arrow). (**O**) In $Sox2^{CKO}$ embryos, Loricrin staining is lost the oral adhesion (yellow arrow). CldU positive cells were quantified in the oval region. Ll- Lower incisor, Md- Mandible, T- Tongue. Scale bar = 100 µm.

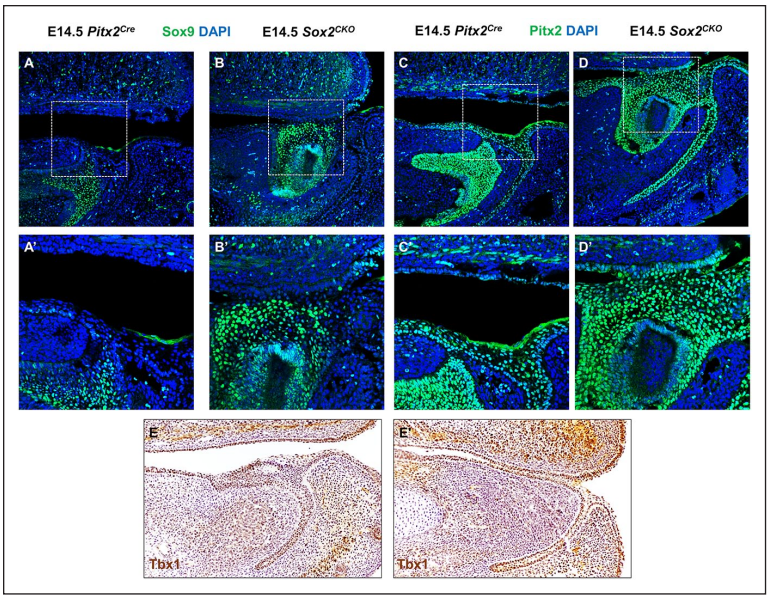

Figure 3. Oral adhesions are composed of dental epithelial cells. (**A**, **A′**) Staining for *Sox9* marks dental epithelial cells in control lower incisors. The white boxed region is shown in higher magnification in A′. (**B**) In *Sox2*^{CKO} embryos, the cells composing the oral adhesion are also *Sox9*+, indicating that they are derived from dental epithelial tissue. The white boxed region is magnified in B′. (**C**, **C′**) In control embryos, *Pitx2* is strongly expressed by the oral and dental epithelial tissue, including the developing lower incisor. (**D**, **D′**) In the *Sox2*^{CKO} embryos, oral adhesion cells are also *Pitx2*+. (**E**) The tongue epithelium expresses *Tbx1*, which is also expressed by dental epithelial cells. (**E′**) In the *Sox2*^{CKO} embryo, the oral adhesion is composed by *Tbx1*+ cells.

4H), distinguishes the rugae from the surrounding *Sox2*$^{+}$ oral epithelium. In *Sox2*^{CKO} embryos, Sox2 and Lef-1 proteins were undetectable in the oral epithelium, and the rugae failed to form (Fig. 4H′).

Sox2 *Is Required for Palate Periderm Formation*

To test if Sox2 regulated periderm differentiation of the palate as well, we examined the oral mucosa by staining for P63, Sox2, and K6. In control embryos, we found that the oral mucosa was composed of a layer of P63^{+} basal cells and that a subset of these cells expressed Sox2. On the apical side of the basal cell layer, we identified a population of P63^{-}, Sox2^{+} cells (Fig. 4I). This outermost layer also expressed K6, demonstrating that the periderm of the palate expresses Sox2 (Fig. 4J, K). In *Sox2*^{CKO} embryos, we found that the P63^{+} basal layer was severely disrupted (Fig. 4I′) and that K6 expression was almost completely undetectable (Fig. 4J′, K′). This demonstrates that *Sox2* is required for the differentiation of periderm in the palate and has a role in basal cell maintenance. We confirmed that periderm formation was decreased in *Sox2*^{CKO} palates by staining for Grhl3, which is strongly expressed in the periderm of control embryos (Fig. 4L, L′).

Sox2 *Regulates K6 Expression*

Because K6 was reduced in *Sox2*^{CKO} embryos (Figs. 2D and 4I′, J′), we reasoned that the role for *Sox2* in periderm differentiation might include promoting the expression of periderm-specific genes, so we tested the ability of *Sox2* to regulate *K6* expression. In GMSM-K cells overexpressing *Sox2*, we observed a ~10-fold increase in *K6* transcripts, indicating that *Sox2* does have a role in regulating *K6* expression (Fig. 5A, B). We further confirmed that K6 was upregulated at the protein level by Western blotting (Fig. 5C).

To determine if *SOX2* directly regulates *K6* expression, we examined the *K6* promoter for the *Sox2* binding sequence previously identified by our group (Sun et al. 2016) and noted a match 1.2 kb upstream of the transcription start site (Fig. 5D). To test if endogenous SOX2 protein interacts with the binding site, we performed a ChIP assay using a Sox2 antibody and tested for enrichment with primers flanking the SOX2 binding site, as well as a pair of negative control primers that did not flank a *SOX2* site. The specific and negative control primers amplified the correct product from an input sample, and neither amplified a product when the pull-down was performed with a nonspecific IgG. Only the primer pair specific for the SOX2 binding site was found to amplify a product when the specific Sox2 antibody was used, demonstrating that SOX2 protein indeed binds the *K6* promoter. To confirm the direct regulation of *K6* by SOX2, we cloned the *K6* promoter sequence upstream of a luciferase reporter vector, as well as a promoter reporter with a mutated SOX2 binding motif. The overexpression of SOX2 significantly increased the luciferase signal of the WT *K6* promoter reporter as compared with the mutated K6 reporter, demonstrating that SOX2 activates the *K6* promoter (Fig. 5E).

Discussion

Ankyloglossia, the fusion of the mandible to the tongue, occurred after E13.5 and persisted until birth in the *Sox*^{CKO} embryos. Our report describes how Sox2 regulates periderm formation and oral adhesions due to expanded surface dental epithelium and delayed incisor epithelial cell invagination and associated tooth agenesis. Furthermore, Sox2 regulates development of the palate rugae, and a loss of this palate signaling center may contribute to clefting.

Sox2 *Is Required for the Formation of the Periderm Layer by Regulating K6 Expression*

It is well known that *Sox2* is a dental epithelial stem cell marker (Juuri et al. 2012; Li et al. 2015; Sun et al. 2016), and in this report, we show that it functions to promote the differentiation of the periderm structure. *Sox2* is also expressed in the tongue epithelium (Arnold et al. 2011). *Sox2* was expressed in progenitor cells superior to the basal cell layer in the mandible of WT embryos superior to the tooth bud region, as well as in the periderm. In *Sox2*^{CKO} embryos, which lacked

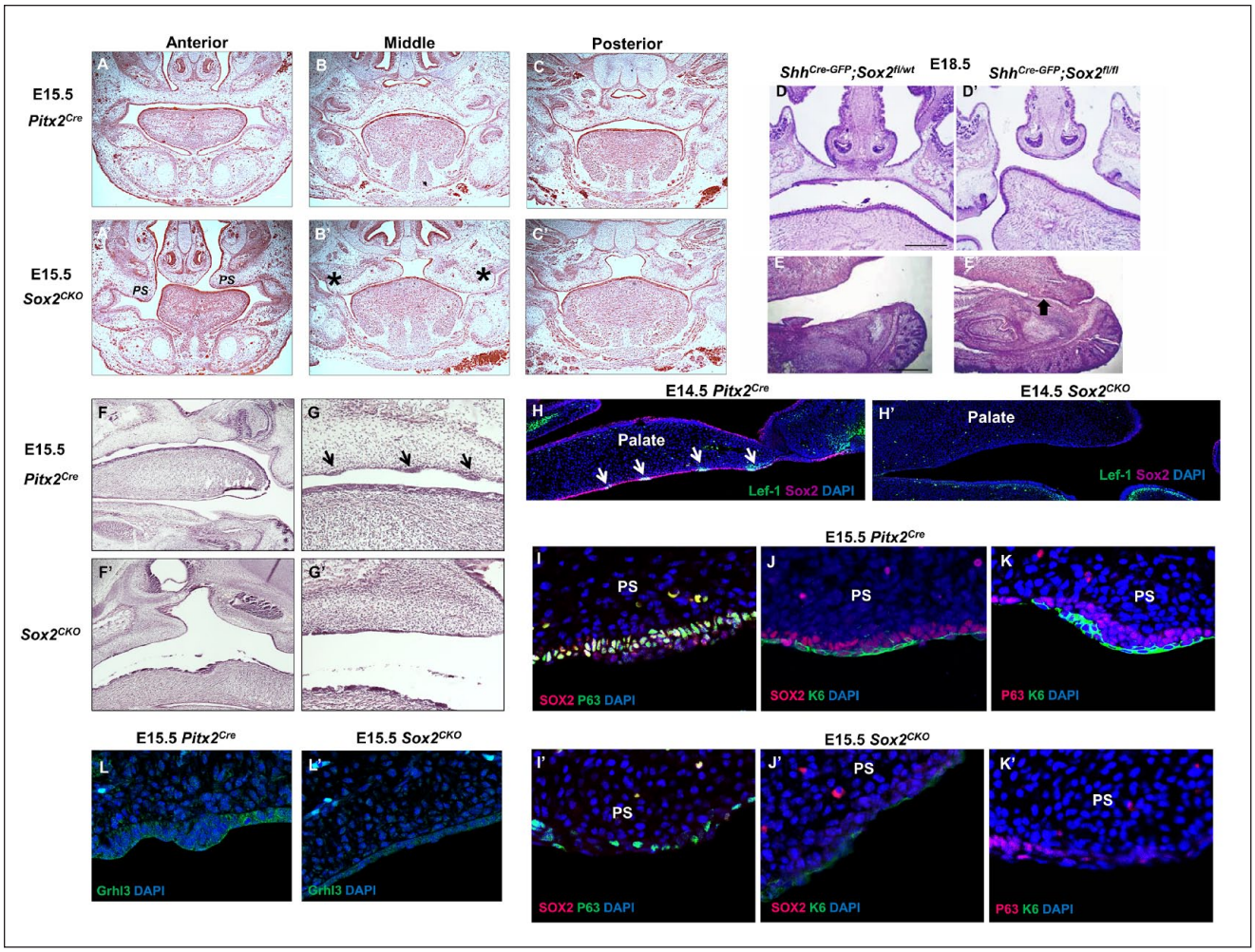

Figure 4. Ablating *Sox2* in the oral epithelium results in cleft palate. (**A–C**) Coronal sectioning of a control embryonic day 15.5 (E15.5) embryo in the anterior, middle, and posterior of the palate reveals complete fusion of the shelves at this stage. (**A′–C′**) Coronal sectioning of the *Sox2*^{CKO} E15.5 embryos reveals cleft palate. The asterisks in panel B′ designate molar tooth buds, which fail to develop in *Sox2*^{CKO} embryos. (**D**, **D′**, **E**, **E′**) *Shh*^{CreGFP}/*Sox2*$^{fl/fl}$ embryos have a cleft phenotype and ankyloglossia (black arrow denotes the oral adhesion), respectively. (**F**, **G**) Sagittal sectioning of an E15.5 control embryo and an enlargement of the palate. Palatal rugae are signified by black arrows. (**F′**, **G′**) A similar plane of section is shown for *Sox2*^{CKO} E15.5 embryos, which demonstrates clefting of the palate. An enlargement of the palate reveals that no rugae are formed at this stage. (**H**) Staining for Lef-1 and Sox2 in E14.5 control embryos. *Sox2* is a marker for the oral epithelial tissue, and *Lef-1* marks the developing rugae. (**H′**) Immunofluorescence staining for Sox2 and Lef-1 in the E14.5 *Sox2*^{CKO} embryos reveals the loss of *Sox2* expression in the oral epithelial tissue as well as the loss of Lef-1 expression in the palate rugae. (**I**) P63/Sox2 double staining of the control embryos shows that P63 is expressing on palatal epithelium at E15.5. The most outer layer of the epithelium is expressing Sox2 but not P63. (**J**) Staining of the control for Sox2 and the periderm marker K6 reveals that the outer-most epithelial layer is periderm and that the periderm expresses Sox2. (**K**) Double staining for P63 and K6 demonstrates that the periderm lacks P63 expression. (**I′**) Double staining for P63 and Sox2 in *Sox2*^{CKO} embryos reveals that *Sox2* has been successfully ablated from the palatal epithelium and that ablating *Sox2* negatively effects the organization of the P63^{+} cells. (**J′**) Double staining for Sox2 and K6 demonstrates that periderm formation is greatly decreased in *Sox2*^{CKO} embryos. (**K′**) Double staining for P63 and K6 in *Sox2*^{CKO} embryos demonstrates defects in periderm formation in the *Sox2*^{CKO} embryos. (**L**, **L′**) Grhl3 protein is highly expressed in the periderm of control embryos and greatly reduced in *Sox2*^{CKO} embryos. PS, Palate Shelf. Scale bar = 400 µm.

a periderm layer, the mandibular basal cell layer was disorganized, and P63-positive cells are expanded (Fig. 2) due to Sox2-regulating K6 expression and periderm formation (Fig. 5).

Oral Adhesions Associated with Ankyloglossia Form between an Expanded Dental Epithelium and Tongue Epithelium

Sox2 deletion in the developing mouse caused invagination defects in the developing lower incisor and, combined with a lack of the periderm structure, resulted in oral adhesions. In several other mouse models, oral adhesions occur near tooth buds of the molars (Richardson et al. 2006; Richardson et al. 2014; Paul et al. 2017). Taken together, these data suggest that the dental epithelium plays a role in the formation of oral adhesions. We determined that the oral adhesion tissue was dental epithelium and that the oral adhesion is formed by proliferating surface dental epithelium, due to a delayed invagination of the dental epithelium, which results in dental anomalies and tooth agenesis. Because ankyloglossia can affect up to 10% of newborns (Chandrashekar et al. 2014; Veyssiere et al. 2015; Yoon

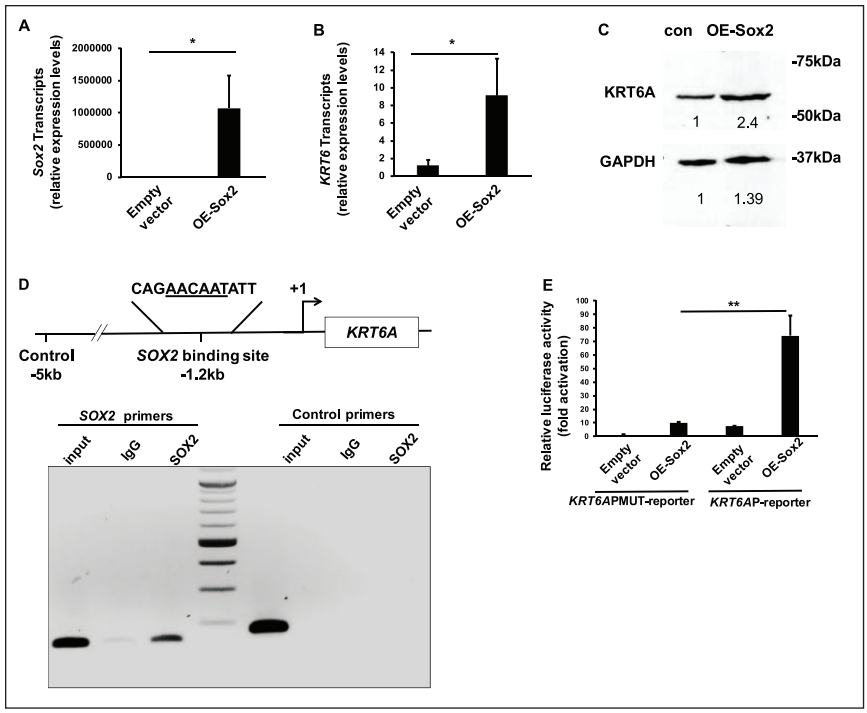

Figure 5. *SOX2* directly regulates *K6* expression in oral epithelial cells through promoter binding. (**A**) The GMSM-K cell line was transfected with empty vector and *Sox2* overexpression plasmid, and *Sox2* mRNA levels were detected through quantitative reverse transcription polymerase chain reaction (PCR). (**B**) From the same reverse transcription sample, levels of *KRT6* mRNA were detected with quantitative PCR. Cells overexpressing *Sox2* had a significant increase in *KRT6* mRNA. (**C**) Western blotting was used to determine the protein levels of empty vector and *Sox2*-transfected GMSMK cells. Cells overexpressing *Sox2* had higher K6 protein levels when compared with control. Bands were quantitated, and relative values are shown. (**D**) To determine if *SOX2* directly interacts with the *KRT6A* promoter, a ChIP assay was performed with nonspecific IgG and a *SOX2* antibody. Input samples and precipitated chromatin were then used in a PCR assay with primer flanking the SOX2 binding site (left) or a negative control region (right). Primers flanking the SOX2 binding site amplified a PCR product in input, and SOX2 antibody precipitated samples but not when chromatin was precipitated with IgG. Primers flanking the negative control region amplified the input sample but failed to amplify when the specific or nonspecific antibody was used. (**E**) The *KRT6A* promoter (3,000 bp prior to transcription start site) was cloned into TK luciferase reporter. Either the control reporter or *KRT6A* promoter luciferase reporter was cotransfected with empty vector or Sox2 overexpression plasmid into LS-8 cells. Overexpressing *Sox2* increased the normalized *KRT6A* promoter luciferase signal as compared with the control. Values are presented as mean ± SEM. *$P < 0.05$. **$P < 0.01$.

et al. 2017), these oral adhesions can also affect tooth development and may cause tooth anomalies in children.

Sox2 *Controls Formation of the Palate Rugae Signaling Center and Palatogenesis*

Sox2 played a related but slightly different role in the palate. In the basal cell layer, many of the palatal P63$^+$ cells coexpressed *Sox2* in control embryos, and ablating *Sox2* resulted in the disorganization of the basal cell layer. These data suggest that *Sox2* functions to promote periderm differentiation in the mandible but has a dual function of regulating basal cell maintenance in the palate. The periderm covering the palate was also poorly formed, demonstrating the conserved role of *Sox2* in periderm development. Interestingly, although the periderm covering the palate was defective, oral adhesions between the tongue and the palatal shelves did not occur due to the restricted ability of the tongue to contact the palate. Abnormal fusion between the palatal shelf and mandible has been reported in multiple mouse models (Xiong et al. 2009; Richardson et al. 2014; Kousa et al. 2017; Hammond et al. 2019).

While clefting in other mouse models with periderm defects usually resulted from oral adhesions between the palate and the tongue that prevent shelf elongation, this was not the case in the *Sox2^{CKO}* embryo. Instead, we found that the ablation of *Sox2* resulted in a loss of palatal rugae (Fig. 4). Others have shown that the rugae serve as *Shh* signaling centers during palate development (Welsh and O'Brien 2009; Lin et al. 2011). In a ß-catenin conditional knockout mouse, the palate rugae did not form, and a lack of *Lef-1, Pitx2*, and *Tcf-1* was shown (Lin et al. 2011). *Shh* expression, which is localized to the rugae, was also reduced, linking *Shh* and *Wnt* signaling in the formation of the rugae (Lin et al. 2011). The rugae provide morphogens such as *Shh* to ensure that the palate extends to the midline (Welsh and O'Brien 2009; Lin et al. 2011). The loss of *Sox2* prevented these structures from forming and deprived the developing palatal shelves from the signals driving cell proliferation and shelf elongation. While we show a lack of rugae formation and associated cleft palate in the *Sox2* mutant embryos, it is not clear how periderm formation affects palate formation. We speculate that the rugae may be required for periderm formation and proliferation of the palate epithelium. Conversely, the periderm layer may interact with the palate rugae during palatogenesis. Interestingly, we have shown that ablation of *Sox2* also results in a lack of *Lef-1* expression in the palate rugae, suggesting that *Sox2* is required to form and maintain the palate rugae.

This study is the first to report that the dental epithelial tissue participates in the formation of inappropriate oral adhesions and provides an explanation for defects in the permanent dentition observed in several families with severe hereditary ankyloglossia (Chandrashekar et al. 2014; Lenormand et al. 2018). Clinically, these results suggest that the presence of ankyloglossia can be used to diagnose potential defects in dental development.

Author Contributions

Y.Y. Sweat, M. Sweat, W. Yu, S. Eliason, B.A. Amendt, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; M. Sanz-Navarro, L. Zhang, Z. Sun, O.D.

Klein, F. Michon, Z. Chen, contributed to conception, design, and data analysis, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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References

Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N, Hochedlinger K. 2011. Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell. 9(4):317–329.

Chandrashekar L, Kashinath KR, Suhas S. 2014. Labial ankyloglossia associated with oligodontia: a case report. J Dent (Tehran). 11(4):481–484.

Ferrés-Amat E, Pastor-Vera T, Rodríguez-Alessi P, Ferrés-Amat E, Mareque-Bueno J, Ferrés-Padró E. 2016. Management of ankyloglossia and breastfeeding difficulties in the newborn: breastfeeding sessions, myofunctional therapy, and frenotomy. Case Rep Pediatr. 2016:3010594.

Hammond NL, Dixon J, Dixon MJ. 2019. Periderm: life-cycle and function during orofacial and epidermal development. Semin Cell Dev Biol. 91:75–83.

Juuri E, Saito K, Ahtiainen L, Seidel K, Tummers M, Hochedlinger K, Klein OD, Thesleff I, Michon F. 2012. Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. Dev Cell. 23(2):317–328.

Kousa YA, Roushangar R, Patel N, Walter A, Marangoni P, Krumlauf R, Klein OD, Schutte BC. 2017. IRF6 and SPRY4 signaling interact in periderm development. J Dent Res. 96(11):1306–1313.

Lenormand A, Khonsari R, Corre P, Perrin JP, Boscher C, Nizon M, Pichon O, David A, Le Caignec C, Bertin H, et al. 2018. Familial autosomal dominant severe ankyloglossia with tooth abnormalities. Am J Med Genet A. 176(7):1614–1617.

Li J, Feng J, Liu Y, Ho TV, Grimes W, Ho HA, Park S, Wang S, Chai Y. 2015. BMP-SHH signaling network controls epithelial stem cell fate via regulation of its niche in the developing tooth. Dev Cell. 33(2):125–135.

Lin C, Fisher AV, Yin Y, Maruyama T, Veith GM, Dhandha M, Huang GJ, Hsu W, Ma L. 2011. The inductive role of wnt-β-catenin signaling in the formation of oral apparatus. Dev Biol. 356(1):40–50.

Liu W, Selever J, Lu MF, Martin JF. 2003. Genetic dissection of *Pitx2* in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. Development. 130(25):6375–6385.

Nithya S, Radhika T, Jeddy N. 2015. Loricrin—an overview. J Oral Maxillofac Pathol. 19(1):64–68.

Paul BJ, Palmer K, Sharp JC, Pratt CH, Murray SA, Dunnwald M. 2017. Arhgap29 mutation is associated with abnormal oral epithelial adhesions. J Dent Res. 96(11):1298–1305.

Richardson RJ, Dixon J, Jiang R, Dixon MJ. 2009. Integration of IRF6 and Jagged2 signalling is essential for controlling palatal adhesion and fusion competence. Hum Mol Genet. 18(14):2632–2642.

Richardson RJ, Dixon J, Malhotra S, Hardman MJ, Knowles L, Boot-Handford RP, Shore P, Whitmarsh A, Dixon MJ. 2006. IRF6 is a key determinant of the keratinocyte proliferation-differentiation switch. Nat Genet. 38(11):1329–1334.

Richardson RJ, Hammond NL, Coulombe PA, Saloranta C, Nousiainen HO, Salonen R, Berry A, Hanley N, Headon D, Karikoski R, et al. 2014. Periderm prevents pathological epithelial adhesions during embryogenesis. J Clin Invest. 124(9):3891–3900.

Sun Z, Yu W, Navarro MS, Sweat M, Eliason S, Sharp T, Liu H, Seidel K, Zhang L, Moreno M, et al. 2016. Sox2 and Lef-1 interact with Pitx2 to regulate incisor development and stem cell renewal. Development. 143(22):4115–4126.

Taranova OV, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR, Pevny LH. 2006. Sox2 is a dose-dependent regualtor of retinal neural progenitor competence. Genes Dev. 20(9):1187–1202.

Tuttle AH, Rankin MM, Teta M, Sartori DJ, Stein GM, Kim GJ, Virgilio C, Granger A, Zhou D, Long SH, et al. 2010. Immunofluorescent detection of two thymidine analogues (CldU and IdU) in primary tissue. J Vis Exp. 46:2166.

Veyssiere A, Kun-Darbois JD, Paulus C, Chatellier A, Caillot A, Bénateau H. 2015. Diagnosis and management of ankyloglossia in young children. Rev Stomatol Chir Maxillofac Chir Orale. 116(4):215–220.

Welsh IC, O'Brien TP. 2009. Signaling integration in the rugae growth zone directs sequential Shh signaling center formation during the rostral outgrowth of the palate. Dev Biol. 336(1):53–67.

Xiong W, He F, Morikawa Y, Yu X, Zhang Z, Lan Y, Jiang R, Cserjesi P, Chen Y. 2009. Hand2 is required in the epithelium for palatogenesis in mice. Dev Biol. 330 (1):131–141.

Yoon AJ, Zaghi S, Ha S, Law CS, Guilleminault C, Liu SY. 2017. Ankyloglossia as a risk factor for maxillary hypoplasia and soft palate elongation: a functional-morphological study. Orthod Craniofac Res. 20(4):237–244.