Odd-skipped related-1 controls neural crest chondrogenesis during tongue development

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The tongue is a critical element of the feeding system in tetrapod animals for their successful adaptation to terrestrial life. Whereas the oral part of the mammalian tongue contains soft tissues only, the avian tongue has an internal skeleton extending to the anterior tip. The mechanisms underlying the evolutionary divergence in tongue skeleton formation are completely unknown. We show here that the odd-skipped related-1 (Osr1) transcription factor is expressed throughout the neural crest-derived tongue mesenchyme in mouse, but not in chick, embryos during early tongue morphogenesis. Neural crest-specific inactivation of *Osr1* resulted in formation of an ectopic cartilage in the mouse tongue, reminiscent in shape and developmental ontogeny of the anterior tongue cartilage in chick. SRY-box containing gene-9 (*Sox9*), the master regulator of chondrogenesis, is widely expressed in the nascent tongue mesenchyme at the onset of tongue morphogenesis but its expression is dramatically downregulated concomitant with activation of *Osr1* expression in the developing mouse tongue. In *Osr1* mutant mouse embryos, expression of *Sox9* persisted in the developing tongue mesenchyme where chondrogenesis is subsequently activated to form the ectopic cartilage. Furthermore, we show that Osr1 binds to the *Sox9* gene promoter and that overexpression of Osr1 suppressed expression of endogenous *Sox9* mRNAs and *Sox9* promoter-driven reporter. These data indicate that Osr1 normally prevents chondrogenesis in the mammalian tongue through repression of *Sox9* expression and suggest that changes in regulation of *Osr1* expression in the neural crest-derived tongue mesenchyme underlie the evolutionary divergence of birds from other vertebrates in tongue morphogenesis.

craniofacial | cell fate | differentiation | tongue-tie

The evolutionary transition of vertebrates from aquatic to terrestrial life required major changes in the feeding mechanism. The development of a muscular tongue, together with other organs within and near the oral cavity, enabled the animals to feed efficiently in their new environment and played an important role in their success of adaptation to the dry-land habitat (1). Structural variations of the tongue also play important roles in the feeding behavior of different species. In mammals, the body of the tongue consists mainly of striated muscle, with the anterior two-thirds located in the oral cavity and the caudal pharyngeal part anchored to the basihyal of the hyoid skeleton (2). The tongue is responsible for mixing and manipulating food within and transporting food through the oral cavity and for pushing the food bolus into the esophagus. In birds, however, the hyoid skeleton underpins the entire tongue, with an entoglossum located anteriorly in the body of the tongue and articulating caudally with the basihyal (2, 3). Thus, most birds use a "slide-and-glue" feeding mechanism in which a small food item, grasped between the tips of the beaks, is glued to the moist tip of the tongue and lodged into the pharyngeal cavity through back-and-forth movements of the hyoid skeleton (2).

In mammals as well as in birds, the oral part of the tongue arises from lingual swellings of the mandibular arch, consisting of oral ectoderm covering mesenchyme derived from the neural crest cells from the embryonic midbrain and the rostral first and second rhombomeres of the hindbrain (4–7). During early tongue development, myoblasts derived from occipital somites migrate into the tongue primordium, proliferate, and differentiate to make the tongue a muscular organ (3). Fate mapping studies using chick-quail chimeras demonstrated that the entoglossum and anterior part of the basihyal skeleton are derived from the neural crest-derived mandibular arch mesenchyme. Thus, the differences in formation of the tongue skeleton in mammals and birds are most likely due to differences in the molecular program regulating neural crest mesenchyme differentiation during tongue development. However, nothing is known about the molecular mechanism underlying the differences in tongue skeleton formation in birds and mammals.

The odd-skipped family of zinc finger transcription factors plays critical roles in embryogenesis and organogenesis in *Drosophila* as well as in vertebrates. Vertebrates have two odd-skipped–related genes, *Osr1* and *Osr2*, which have partially overlapping expression patterns in the developing heart, kidney, limb, and craniofacial structures (8–14). Analyses of $Osr1^{-/-}$ mutant mice have shown that Osr1 is required for heart and kidney development (15, 16), whereas $Osr2^{-/-}$ mutant mice exhibit cleft palate and supernumerary tooth formation (17, 18). *Osr1* and *Osr2* have also been shown to regulate kidney and foregut development in *Xenopus* (14, 19). In addition, Osr1 and Osr2 have been shown to function partially redundantly in regulating synovial joint formation during limb skeleton development in mice (20). In this study, we found that Osr1 is strongly expressed in the neural crest-derived mesenchyme in the developing tongue in mouse embryos but not in chicken embryos. Tissue-specific inactivation

Significance

Development of the tongue is a major vertebrate adaptation to terrestrial life. Interestingly, although the tongues of birds and mammals initially develop similarly, the bird tongue is underpinned by an extensive internal skeleton, whereas the oral part of the mammalian tongue is boneless, which is critical not only for feeding but also for phonetic articulation and oral health. This paper shows that the mouse embryonic tongue, but not that in chick, expresses high levels of the odd-skipped related-1 (Osr1) transcription factor, which represses expression of SRY-box containing gene-9 (Sox9), the master regulator of cartilage formation. Mice lacking Osr1 function develop a cartilage in the anterior tongue reminiscent of that in chick. These data provide unique insights into the mechanisms of tongue development and evolution.

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of *Osr1* in the neural crest lineage caused formation of an ectopic cartilage in the mutant mouse tongue that is reminiscent of the entoglossum in the chick tongue. Further studies revealed that Osr1 prevents chondrogenesis from the neural crest mesenchyme in the developing tongue by repressing *Sox9* expression. These studies reveal a unique role for Osr1 in vertebrate development and evolution.

Results and Discussion

Differential Expression of *Osr1* in the Developing Tongue Mesenchyme in Mouse and Chicken Embryos. We previously showed that *Osr1* and *Osr2* exhibit distinct expression patterns during palate development and that expression of *Osr1* from the *Osr2* locus rescued cleft palate defects in the $Osr2^{-/-}$ mutant mice (17, 21). To investigate the roles of Osr1 in craniofacial development, we systematically compared the spatiotemporal expression patterns of Osr1 and Osr2 and found that they exhibit distinct expression patterns during tongue development. At embryonic day 12.5 (E12.5), when the tongue primordium was composed mostly of neural crest-derived mesenchyme (5), *Osr1* was strongly expressed throughout the tongue mesenchyme (Fig. 1*A*). In contrast, expression of *Osr2*, which was robust in the developing palate mesenchyme, was restricted to the peripheral tongue mesenchyme cells immediately underneath the epithelium (Fig. 1*D*). As myoblasts invaded the tongue mesenchyme, expanded and differentiated to tongue muscles from E12.5 to E14.5 (5), high levels of *Osr1* expression persisted in the neural crest-derived tongue mesenchyme (Fig. 1 *B* and *C*), whereas *Osr2* expression remained restricted to the peripheral tongue mesenchymal cells (Fig. 1 *E* and *F*). The distinct expression patterns suggest that Osr1 and Osr2 may play distinct roles in tongue morphogenesis.

Because birds, but not mammals, develop skeletal structures from the neural crest-derived mesenchyme in the anterior tongue, and because recent studies suggest that Osr1 and Osr2 may repress chondrogenesis during limb skeletal development (20, 22), we examined *Osr1* and *Osr2* expression during tongue development in chicken embryos. At embryonic day 4 (D4), when the chicken embryonic tongue was composed mainly of neural crest-derived mesenchyme, *Osr2* was expressed in the peripheral tongue mesenchyme (Fig. 1*J*), but little *Osr1* expression was detected in the developing tongue although strong *Osr1* mRNA expression was detected in the developing palatal mesenchyme on the same sections (Fig. 1*G*). At this stage, the core mesenchyme of the chicken embryonic tongue expressed high levels of *Sox9* mRNAs, indicating initiation of chondrogenesis (Fig. 1*M*). From D5 to D7.5 of chicken embryonic development, the *Sox9*-expressing tongue mesenchyme further condensed and formed cartilage in the center of the developing tongue (Fig. 1 *N* and *O*). No significant *Osr1* mRNA expression was detected in the chick tongue mesenchyme at D5 (Fig. 1*H*) but moderate levels of *Osr1* mRNA expression were detected in a subset of tongue mesenchyme cells adjacent to the entoglossal cartilage by D7.5 (Fig. 1*I*). At these developmental stages, *Osr2* expression remained in the peripheral tongue mesenchyme in the chicken embryos (Fig. 1 *K* and *L*).

To investigate the functional significance of the differences in *Osr1* expression in chick and mouse tongue development, we used the replication-competent avian retrovirus system (RCAS) (23) to ectopically express *Osr1* in the early developing chicken mandible and found that *Osr1* misexpression significantly impaired development of both the Meckel's and the tongue cartilages, particularly in the distal region (Fig. S1). These results indicate that Osr1 negatively regulates cartilage formation from the neural crest-derived mandibular and tongue mesenchyme. Stricker et al. (22) recently showed that overexpression of *Osr1* in embryonic chicken limbs using the RCAS viral expression vector also resulted in thinner cartilage elements. Moreover, RNAi knockdown of *Osr1* in chick limb mesenchyme led to significantly increased cartilage differentiation (22). Thus, the robust expression of *Osr1* in the tongue mesenchyme in the mouse embryos and its absence in the developing tongue in chicken embryos suggest that Osr1 may play an important role in mammalian tongue development.

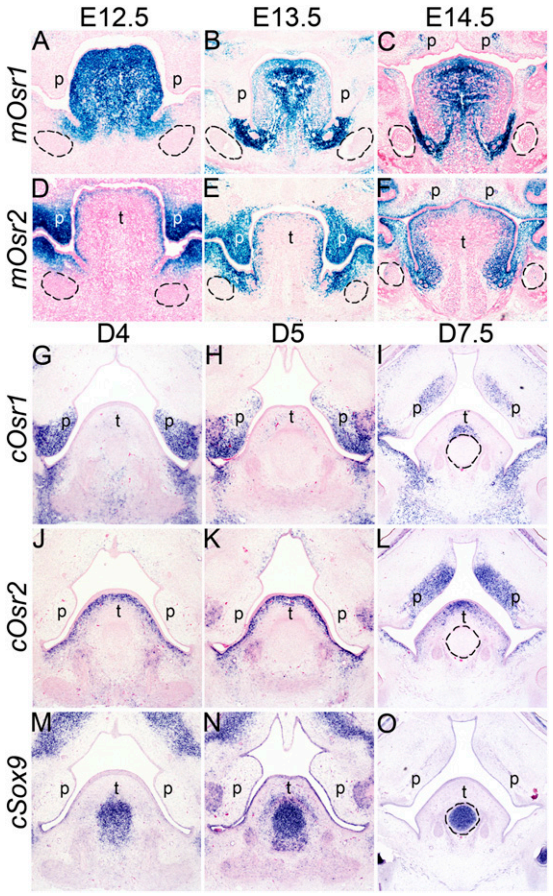

Fig. 1. Patterns of expression of *Osr1* and *Osr2* in the developing tongue in the mouse and chicken embryos. (*A*–*C*) X-Gal staining of frontal sections through the developing tongue in $Osr1^{tm1Jian/+}$ embryos detected strong *Osr1* expression in neural crest-derived mouse tongue mesenchyme from E12.5 to E14.5. The Meckel's cartilage primordia are outlined using dashed circles. (*D*–*F*) X-Gal staining of frontal sections through the developing tongue in $Osr2^{tm1Jian/+}$ embryos showed that *Osr2* expression was restricted in the peripheral tongue mesenchyme underneath the epithelium in the mouse embryos from E12.5 to E14.5. *Osr2* is also strongly expressed in the developing palatal mesenchyme at E12.5 (*D*) and E13.5 (*E*). (*G* and *H*) *Osr1* mRNA was hardly detectable in the early tongue mesenchyme in chicken embryos at D4 (*G*) and D5 (*H*), although strong *Osr1* mRNA expression was detected in the developing palatal mesenchyme. (*I*) At D7.5, *Osr1* mRNA expression was observed in a domain of the tongue mesenchyme above the entoglossal cartilage. The cartilage is outlined using dashed circle. (*J*–*L*) *Osr2* was expressed in the peripheral tongue mesenchyme underneath the epithelium in chicken embryos from D4 (*J*) to D5 (*K*) and D7.5 (*L*). (*M*–*O*) *Sox9* was strongly expressed in the central region of the early tongue mesenchyme that condensed from D4 (*M*) to D5 (*N*) and developed to cartilage by D7.5 (*O*) in chicken embryos. p, palate; t, tongue.

Aberrant Cartilage Formation in the Tongue in Mice with Neural Crest-Specific Inactivation of the *Osr1* Gene. Because *Osr1* null mouse embryos died before E12, due to embryonic heart failure (15), we investigated the role of Osr1 in tongue development through generation and analysis of mice with neural crest-specific *Osr1* gene inactivation. The *Wnt1Cre* transgenic mice express Cre recombinase transiently in premigratory neural crest cells and have been shown to efficiently delete loxP-flanked DNA sequences

from neural crest-derived craniofacial mesenchyme (24–26). We crossed *Wnt1Cre* mice to *Osr1*$^{+/-}$ mice and subsequently crossed the *Osr1*$^{+/-}$*; Wnt1Cre* mice to *Osr1*$^{f/f}$ mice, which contains two directly repeating loxP sites flanking exon 2 of the *Osr1* gene (27), to generate *Osr1*$^{f/-}$*; Wnt1Cre* tissue-specific mutant embryos. The *Osr1*$^{f/-}$*; Wnt1Cre* mutant pups developed to term but none survived postnatally. Histological analysis of neonatal *Osr1*$^{f/-}$*; Wnt1Cre* pups detected an ectopic cartilage in the anterior tongue that connected and anchored the distal part of the tongue to the symphysis of the Meckel's cartilage in the mandible (Fig. 2 *B* and *D*). Skeletal preparations of neonatal mice showed the presence of an arrow-shaped cartilage in the anterior tongue (Fig. 2*F*). The location and shape of this ectopic tongue cartilage in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant mice resemble the entoglossal cartilage in the anterior chick tongue (3, 4, 6), but no similar structure has ever been described in mammals.

Although both the Meckel's cartilages and the tongue cartilages are derived from cranial neural crest mesenchyme, the tongue entoglossum cartilage is not directly connected to the mandibular symphysis in chick (3, 4, 6). We thus investigated the relationship between the formation of the Meckel's cartilages and the ectopic tongue cartilage in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant embryos. At E12.5, when mandibular mesenchyme condensations of the Meckel's cartilage primordia were first visible in the control embryos (Fig. 3*A*), *Osr1*$^{f/-}$*; Wnt1Cre* mutant embryos exhibited a distinct mesenchymal condensation at the base of the anterior tongue region in addition to the Meckel's cartilage primordia in the mandible (Fig. 3*B*). Because *Osr1* is strongly expressed throughout the neural crest-derived tongue mesenchyme but not in the mandibular mesenchyme that gives rise to the Meckel's cartilages (Fig. 1 *A*–*C*), we examined β-galactosidase expression from the *Osr1*$^{-}$ (*Osr1*^{tm1Jian}) allele, which contains an in-frame insertion of the modified bacterial

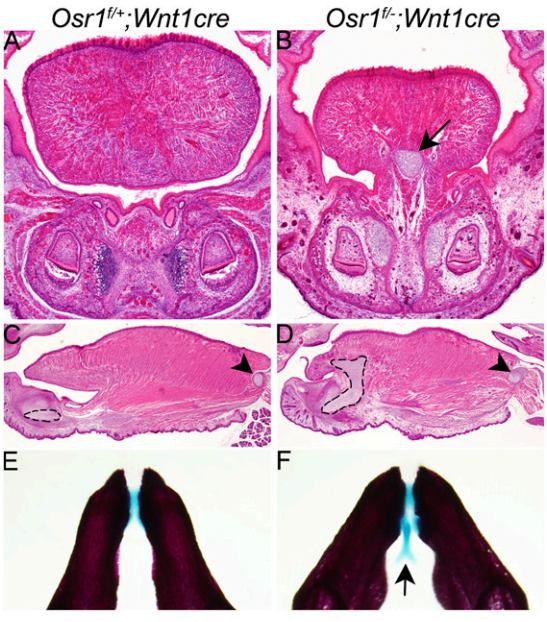

Fig. 2. *Osr1*$^{f/-}$*; Wnt1Cre* mutant mice exhibit ectopic cartilage in the anterior tongue. (*A* and *B*) Frontal sections through the anterior tongue of P0 control (*A*) and *Osr1*$^{f/-}$*; Wnt1Cre* mutant (*B*) littermates. Arrow in *B* points to an ectopic cartilage. (*C* and *D*) Sagittal sections through the midline of the tongue of P0 control (*C*) and mutant (*D*) littermates. The ectopic cartilage in the tongue fused with the symphysis of the Meckel's cartilage (outlined by black dashed line). Arrowhead points to the basihyal cartilage in the posterior region of the tongue in both control and mutants. (*E* and *F*) Skeletal preparations of the P0 control (*E*) and *Osr1*$^{f/-}$*; Wnt1Cre* mutant (*F*) mandibles. Arrow in *F* points to the ectopic tongue cartilage.

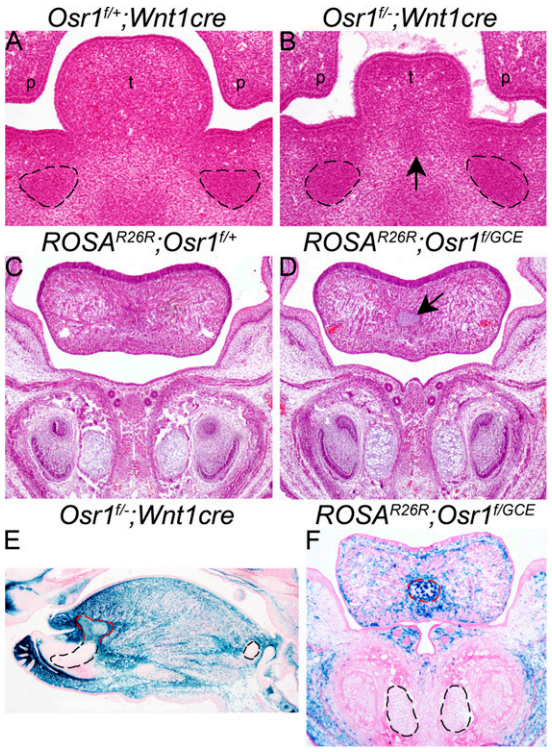

Fig. 3. Cell autonomous requirement for Osr1 in preventing chondrogenesis in the developing mouse tongue mesenchyme. (*A* and *B*) At E12.5, *Osr1*$^{f/-}$*; Wnt1Cre* mutant embryos exhibit ectopic mesenchymal cell condensation (arrow in *B*) at the base of the developing tongue. The condensed mesenchyme of the Meckel's cartilage primordia in both the control (*A*) and *Osr1*$^{f/-}$*; Wnt1Cre* mutant (*B*) are outlined using dashed lines. (*C* and *D*) Tamoxifen induction of Cre activity in the early tongue mesenchyme expressed from the *Osr1* locus in the *Osr1*$^{f/GCE}$ embryos also resulted in ectopic cartilage formation in the anterior tongue (arrow in *D*). (*E* and *F*). X-Gal staining showed that the ectopic cartilage (outlined using red dashed line) in the *Osr1*$^{f/-}$*; Wnt1Cre* (*E*) and *R26R; Osr1*$^{f/GCE}$ mice developed from *Osr1*-expressing tongue mesenchyme cells, whereas the basihyal and Meckel's cartilages developed from cells that do not express *Osr1*. p, palate; t, tongue.

β-galactosidase gene (lacZ) in the first coding exon (15), to investigate whether the ectopic tongue cartilage in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant embryos was derived from ingrowth of the Meckel's cartilage or from aberrant differentiation of the tongue mesenchyme. As shown in Fig. 3*E*, although the ectopic cartilage was directly connected to the symphysis of the Meckel's cartilages, only the ectopic cartilage cells expressed lacZ, whereas the Meckel's cartilage did not. To further verify that the ectopic cartilage in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant mice was derived from the neural crested-derived tongue mesenchyme, we crossed *Osr1*$^{GCE/+}$ male mice, in which a cDNA cassette encoding the eGFP-Cre-ERt2 fusion protein was inserted next to the translation initiation codon of the *Osr1* gene to disrupt *Osr1* gene function and to allow spatially and temporally regulated Cre activity in *Osr1*-expressing cells (28), to *Osr1*$^{f/f}$ female mice and injected the pregnant females intraperitonially with tamoxifen from E10.5 to E12.5 to activate Cre-mediated inactivation of the *Osr1*^{f} allele in the *Osr1*-expressing tongue mesenchyme cells. As shown in Fig. 3*D*, the *Osr1*$^{f/GCE}$ mice developed ectopic cartilage in the anterior part of the tongue. Lineage tracing using Cre-activated lacZ expression from the *R26R* reporter allele (29) showed that the ectopic cartilage cells were derived from the Cre-expressing cells (Fig. 3*F*). The ectopic cartilage contained some lacZ-negative cells, which were possibly derived from incomplete Cre-mediated recombination at the *R26R* locus or from ingrowth of

Osr1-nonexpressing prechondrogenic progenitor cells. Importantly, the Meckel's cartilages completely lacked lacZ staining (Fig. 3 *E* and *F*), indicating that *Osr1*-expressing cells in the developing mandibular mesenchyme do not give rise to cartilage. Together, these results suggest that Osr1 functions cell autonomously to suppress cartilage differentiation in the developing tongue mesenchyme.

Osr1 Suppresses *Sox9* Expression and Chondrogenic Differentiation of the Developing Mouse Tongue Mesenchyme. To investigate the molecular mechanism of ectopic cartilage formation in the *Osr1* mutant mouse tongue, we microdissected the E12 tongue primordium from *Osr1*$^{f/+}$*; Wnt1Cre* control and *Osr1*$^{f/-}$*; Wnt1Cre* mutant litter mates, respectively, and carried out whole transcriptome sequencing (RNA-Seq) analysis. We found that, whereas *Osr1* mRNAs were dramatically reduced as expected, expression of *Sox9*, *Sox5*, and a number of chondrocyte collagens were significantly up-regulated in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant tongue in comparison with the control littermates. We further verified these changes of gene expression profiles by using quantitative real-time RT-PCR and in situ hybridization assays (Fig. 4). At E11.5, when the tongue primordium first arose from the mandibular processes, *Osr1* was not expressed in the tongue mesenchyme (Fig. 4*B*). By E12.5, *Osr1* mRNA expression was strongly activated in the developing tongue mesenchyme (Fig. 4*C*). In contrast, whereas *Sox9* was strongly expressed in the nascent tongue mesenchyme at E11.5, this expression was dramatically down-regulated by E12.5 in control embryos (Fig. 4 *D* and *F*). In *Osr1*$^{f/-}$*; Wnt1Cre* mutant embryos, *Sox9* mRNAs were similarly expressed in the tongue mesenchyme as in control embryos at E11.5, but significant amounts of *Sox9* mRNA persisted in the central region of the developing tongue at E12.5 (Fig. 4 *E* and *G*). Sox5, which acts downstream of Sox9 and enhances Sox9-mediated transcriptional activation of chondrocyte differentiation (30, 31), was ectopically activated in the central domain of the developing tongue mesenchyme by E12.5 in *Osr1*$^{f/-}$*;Wnt1Cre* mutant embryos (Fig. 4 *H* and *I*). Expression of *Col2a1* and *Col9a1*, two downstream target genes of Sox9 and early markers of chondroblast differentiation, was absent in the E12.5 control tongue mesenchyme but highly activated in the central regions of the E12.5 *Osr1*$^{f/-}$*; Wnt1Cre* mutant tongue mesenchyme (Fig. 4 *J*–*M*).

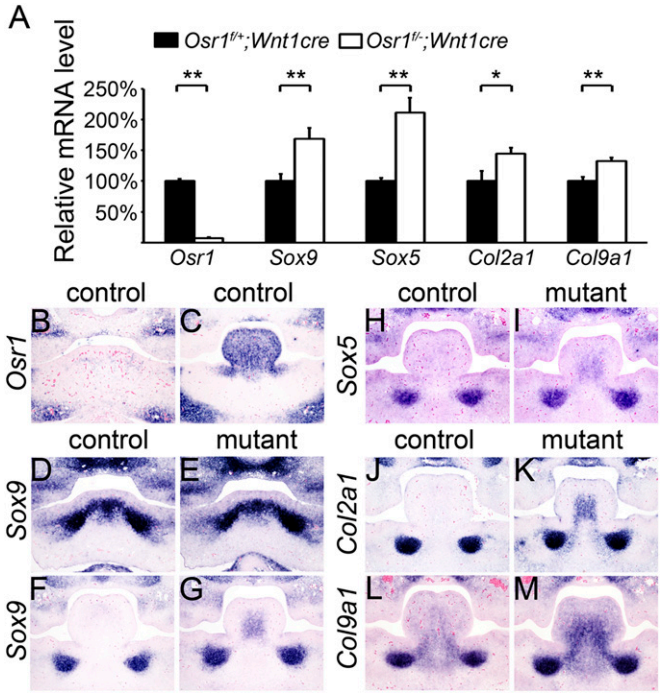

Fig. 4. Ectopic activation of chondrogenic gene expression in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant tongue mesenchyme. (*A*) Real-time RT-PCR analysis demonstrates significantly increased levels of expression of *Sox9*, *Sox5*, *Col2a1*, and *Col9a1* mRNAs in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant tongue mesenchyme in comparison with the control littermates at E12.5. **P* < 0.05; ***P* < 0.01. (*B* and *C*) *Osr1* expression in the nascent tongue mesenchyme was activated from E11.5 (*B*) to E12.5 (*C*). (*D* and *E*) *Sox9* mRNA was strongly expressed in the nascent tongue mesenchyme in both control (*D*) and mutant (*E*) embryos at E11.5. (*F* and *G*) *Sox9* mRNA expression in developing tongue mesenchyme was dramatically down-regulated in control embryos by E12.5 (*F*) but persisted at moderate levels in the mutant tongue (*G*). (*H*–*M*) Expression of *Sox5* (*H* and *I*), *Col2a1* (*J* and *K*), and *Col9a1* (*L* and *M*) was ectopically activated in the mutant tongue mesenchyme (*I*, *K*, and *M*) by E12.5.

Previous studies have demonstrated that Sox9 is a master regulator of chondrogenesis (30, 31). Tissue-specific inactivation of *Sox9* in developing early limb bud mesenchyme resulted in complete absence of mesenchymal condensation and of subsequent cartilage and bone formation in mice (32). Tissue-specific inactivation of *Sox9* in cranial neural crest cells also prevented chondrogenic condensations from neural crest-derived mesenchyme, resulting in complete absence of neural crest-derived cartilage (33). Moreover, misexpression of *Sox9* in developing limb bud mesenchyme caused ectopic chondrogenesis (34). These studies indicate that Sox9 is both necessary and sufficient for chondrocyte specification. We found that *Sox9* is highly expressed in the nascent tongue mesenchyme at the onset of tongue morphogenesis but its expression is dramatically down-regulated concomitant with activation of *Osr1* mRNA expression in the neural crest-derived tongue mesenchyme from E11.5 to E12.5. These data, together with the finding that *Sox9* mRNA expression persisted in the tongue mesenchyme, which subsequently condensed and developed ectopic cartilage in the *Osr1*$^{f/-}$*; Wnt1Cre* mutant mice, suggest that Osr1 may function to suppress *Sox9* expression in the tongue mesenchyme during normal mouse tongue development. To test this hypothesis, we transfected primary cultures of E11.5 embryonic mouse mandibular mesenchyme with an *Osr1* expression construct and found that overexpression of *Osr1* significantly suppressed endogenous *Sox9* mRNA expression (Fig. 5*C*). Because endogenous *Sox9* mRNA expression is naturally down-regulated in the developing tongue mesenchyme from E11.5 to E12.5, we also transfected the *Osr1* expression construct into primary cultures of E11.5 limb bud mesenchyme, which normally exhibit increased *Sox9* mRNA expression during early limb cartilage development (35), and found that overexpression of *Osr1* also suppressed endogenous *Sox9* mRNA expression in the limb bud mesenchyme (Fig. 5*C*).

Although the specific Osr1-binding DNA sequence has not been reported, a previous PCR-based DNA-binding sequence selection study suggested that the mouse Osr2 protein binds to a core DNA sequence similar to that bound by the *Drosophila* odd-skipped protein (36, 37). Osr1 and Osr2 share almost identical DNA-binding domains (11). We found that the region around 2,295 bp upstream of the mouse *Sox9* gene transcription start site contains a sequence with perfect match to the previously reported *Drosophila* odd-skipped binding core sequence, GCTACTG (37); Fig. 5*A*). To investigate whether Osr1 binds to this region of the endogenous *Sox9* gene promoter, we transfected primary cultured E12.5 mouse tongue mesenchyme cells with the FLAG-tagged *Osr1* expression construct and performed chromatin immunoprecipitation (ChIP) followed by PCR analyses. As shown in Fig. 5*B*, the Osr1 protein bound to this specific region of the endogenous *Sox9* gene promoter in the tongue mesenchyme cells. To further test whether Osr1 could directly repress *Sox9* gene promoter activity, we tested effects of *Osr1* overexpression on *Sox9* promoter driven luciferase reporter expression in both primary cultures of E11.5 mouse mandibular mesenchyme and of E11.5

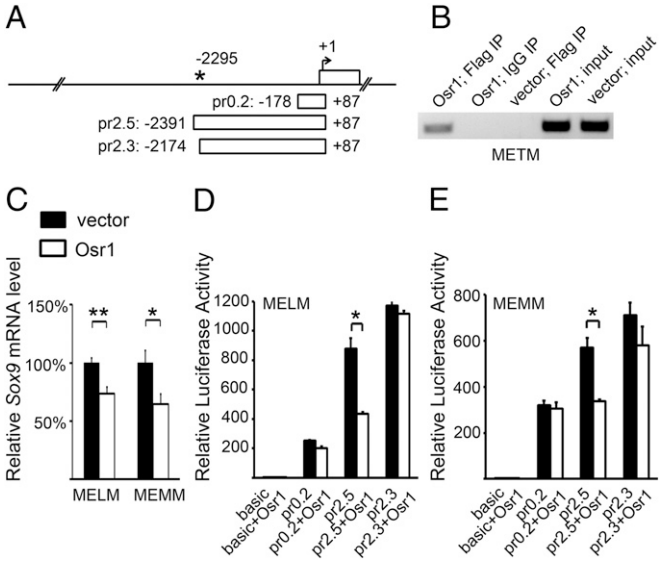

Fig. 5. Osr1 binds to the endogenous *Sox9* gene promoter and suppresses *Sox9* gene expression in the developing tongue mesenchyme. (*A*) Schematic presentation of the *Sox9* proximal promoter regions used for luciferase reporter constructs. A predicted Osr1 binding site located around 2,295 bp 5′ to the *Sox9* transcription initiation site (+1) is marked with an asterisk (*). DNA fragments corresponding to −178 to +87, −2,391 to +87, and −2,174 to +87, respectively, were used to drive luciferase reporter expression in cell transfection assays. (*B*) ChIP assay showed that Osr1 protein bound to the *Sox9* promoter region around the −2,295 site. (*C*) Overexpression of *Osr1* suppressed endogenous *Sox9* mRNA expression in primary cultures of E11.5 mouse embryonic limb mesenchyme (MELM) and mouse embryonic mandibular mesenchyme (MEMM) cells, respectively. (*D* and *E*) Osr1 specifically repressed activity of the 2.5-kb *Sox9* promoter, but not the 0.2-kb basal promoter or the 2.2-kb promoter (−2,174 to +87) that lacks the Osr1 binding sequence, in either MELM or MEMM cells. $*P < 0.05$; $**P < 0.01$.

mouse limb bud mesenchyme. Osr1 repressed the activity of the 2.5-kb promoter but not that of the 0.2-kb basal promoter of the *Sox9* gene in both cell types (Fig. 5 *D* and *E*). Moreover, deletion of the region containing the putative Osr1-binding sequence completely abolished Osr1-mediated suppression of the *Sox9* promoter activity (Fig. 5 *D* and *E*). Together, these data suggest that Osr1 prevents chondrogenesis of the neural crest-derived mouse tongue mesenchyme at least in part through direct repression of *Sox9* gene expression.

Our findings that neural crest-specific inactivation of *Osr1* results in formation of an ectopic cartilage in the developing mouse tongue reminiscent of the entoglossal cartilage in the chick tongue, together with the correlation of cartilage formation with absence of *Osr1* expression in the neural crest-derived chick tongue mesenchyme, strongly suggest that changes in regulation of *Osr1* gene expression underlie the evolutionary novelty of tongue skeleton formation in avians. Whereas recent studies have shown that Osr1 and Osr2 function as transcriptional repressors and that overexpression of either Osr1 or Osr2 in chick limb buds led to reduced cartilage formation (14, 22), our results identify *Sox9* as a likely direct target in Osr1-mediated repression of chondrogenesis in the neural crest-derived tongue mesenchyme. The fact that *Sox9* expression is restricted to the central domain of the neural crest-derived tongue mesenchyme in the $Osr1^{f/-}$*; Wnt1Cre* mutant embryos suggest that the activators of *Sox9* gene expression are spatiotemporally regulated. *Sox9* expression in the embryonic chick tongue is also restricted to the central domain (Fig. 1 *M–O*). Further studies are necessary to define the complete molecular mechanism regulating the domains of *Sox9* gene expression in the embryonic chicken tongue and to investigate whether the expression patterns of the activators of *Sox9* gene expression are conserved in mammals. On the other hand, the fact that ectopic expression of *Osr1* in the embryonic chick mandible caused significant reduction in, but not a complete inhibition of, cartilage formation in the mandible and tongue may be due to a combination of technical limitations related to the timing and dosage of exogenous gene expression and the possibility that the chicken tongue mesenchyme might have evolved additional robust mechanisms for localized activation of chondrogenic differentiation. Nevertheless, the finding of a requirement for *Osr1* in preventing chondrogenic differentiation during mammalian tongue development, together with our previous discovery that Osr2 antagonizes Msx1-mediated odontogenesis to pattern the mammalian teeth into a single row (18), indicates that the Odd-skipped family transcription factors play critical roles in patterning the vertebrate orofacial organs. Further investigation of the roles and regulation of the *Osr1* and *Osr2* genes during vertebrate embryogenesis will provide unique insights into the evolutionary mechanisms of craniofacial and skeletal patterning.

Materials and Methods

Mouse Strains. Research use of animals in this study was reviewed and approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee. The $Osr1^{tm1Jian}$, $Osr2^{tm1Jian}$, $Osr1^{f/f}$, and $Osr1^{\Delta E2/+}$ mice have been described previously (15, 17, 27). The *Wnt1Cre* transgenic mice (25), *R26R* (29), and $Osr1^{GCE/+}$ knock-in mice (28) were obtained from the Jackson Laboratory. *Wnt1Cre* mice were maintained in CD1 background, $Osr1^{f/f}$ and *R26R* mice were maintained by homozygous intercrossing (mixed C57BL/6J and 129 background), whereas all other mouse strains were maintained by backcrossing to C57BL/6J mice. To inactivate *Osr1* in the neural crest cell lineage, $Osr1^{tm1Jian/+}$ mice were first crossed with *Wnt1Cre* mice to generate $Osr1^{+/-}$*; Wnt1Cre* mice, which were then crossed with $Osr1^{f/f}$ mice to generate $Osr1^{f/-}$*; Wnt1Cre* mutant embryos. For inducible inactivation of *Osr1* in the developing tongue mesenchyme, $Osr1^{f/f}$ females were crossed with $Osr1^{GCE/+}$ males and the pregnant female mice were injected intraperitoneally with 1 mg of tamoxifen once daily from gestational days E10.5 to E12.5.

Histology and Skeletal Preparations. Embryos were dissected at desired stages, fixed in Bouin's fixative, dehydrated through ethanol series, and embedded in paraffin. Embryos of the selected genotypes were sectioned at 7-μm thickness and stained with hematoxylin and eosin. Skeletal preparations were performed as previously described (38).

Detection of β-Galactosidase Activity and in Situ Hybridization Assays. X-Gal staining of whole mount embryos and cryostat sections was performed as previously described (39). Sections were counterstained with eosin. For in situ hybridization, embryos were fixed in 4% (wt/vol) paraformaldehyde in PBS overnight at 4 °C, dehydrated through ethanol series, and embedded in paraffin. Serial sections of 7-μm thickness were used for in situ hybridization assays as described previously (40).

RNA-Seq Analysis. Tongue tissues were microdissected from embryos at E12 in cold diethylpyrocarbonate (DEPC)-treated PBS. Total RNAs were extracted from pooled tongue tissues from three wild-type and mutant embryos, respectively, using the Qiagen RNeasy Micro kit (Qiagen; 74004). Sequencing libraries were generated by using Illumina Nextera DNA Sample Prep kit and sequenced using Illumina HisEq 2000. Sequence reads were mapped to the reference mouse genome (mm9) using Bowtie. Single-end reads were aligned using Tophat. RNA-Seq data were then analyzed using Avadis NGS software, with the reads per kilobase exon per million mapped sequences value calculated for each RefSeq gene for relative levels of gene expression. For analyses of differential expression, the fold-change cutoff was set at 1.5 or higher and *P* value less than 0.01 from the Audic Claverie test was considered statistically significant, with Benjamini–Hochberg false discovery rate multiple testing correction.

Real-Time RT-PCR. Embryonic tissues or cultured cells were harvested in cold DEPC-treated PBS. Total RNAs were extracted using the Qiagen RNeasy Micro kit (Qiagen; 74004), and first-strand cDNA was synthesized using the SuperScript First-Strand Synthesis system (Invitrogen; 11904-018). Quantitative real-time

PCR was performed on the Bio-Rad CFX96 Real-Time system, using the SsoAdvanced SYBR Green Supermix according to manufacturer instructions. Experiments were replicated with six samples for each genotype. Each reaction was performed in triplicates. The quantity of each experimental sample is first determined using a standard curve based on the Ct values and then expressed relative to the internal control. All mRNA levels were normalized to that of *Hprt*. Sequence information for the PCR primers are listed in Table S1.

Primary Cells Culture, Transfection, and Luciferase Assays. Wild-type tongue, mandible, or limb bud tissues were microdissected from embryos at desired developmental stages in cold-aspirated HBSS. Under aseptic conditions, the solution was replaced with 500 μL of 0.25% trypsin (Invitrogen) and incubated at 4 °C for 5 min followed by 37 °C for 3 min. Cells were then dissociated by trituration in 0.25% trypsin. Trypsinization was halted by addition of 60 μL FBS, and the dissociated cells were seeded in a 10-cm dish. The culture medium consisted of DMEM/F12 (3:1 mix) (Invitrogen), 10% (vol/vol) FBS, and penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen). The luciferase reporter vectors (50 ng per well, the actual amounts were normalized to the equal molarity of Pr-0.2) and *Myc-Osr1* expression vectors were cotransfected into cells plated on 24-well plates using Lipofectamine LTX (Invitrogen) following the manufacturer's manual. Luciferase assays were performed 48 h after transfection with the Dual-Luciferase Reporter Assay System kit (Promega; E1960) following the manufacturer's instructions. Luciferase activity was measured using the GloMax 96 Microplate Luminometer with Dual Injectors (Promega). Transfection efficiency was normalized by the ratio of the firefly luciferase activity to the cotransfected Renilla luciferase activity. Data were collected from three independent replicate assays.

Retroviral Constructs and Chicken Embryo Manipulation. The *RCAS-GFP* plasmid vector has been described previously (41) and was obtained from Addgene. Full-length chicken and mouse *Osr1* cDNAs were amplified by RT-PCR using primers listed in Table S2. The PCR products were first cloned into the *pCS2* vector containing a 6x-Myc tag coding sequence. Both the *Myc-cOsr1* and *Myc-mOsr1* coding regions were subcloned into the *RCAS-GFP* vector via the ClaI site. Concentrated viral supernatants of the *RCAS-GFP* control and the *RCAS-cOsr1* and *RCAS-mOsr1* constructs were produced using the protocol of Logan and Tabin (23). Research-grade specific pathogen-free chicken eggs (Charles River) were used for all viral infection experiments. Viruses with titer of at least 10^8 IU/mL were used for injection into the right side of the embryonic chick mandible at embryonic day 3 (Hamburger–Hamilton stage 14–15). The injected embryos were harvested at embryonic day 7 and processed for alcian blue staining or histology analysis.

Statistical Analysis. All results were presented as mean ± SEM. Two-tailed Student *t* tests were used for comparisons between two groups. *P* value less than 0.05 was considered statistically significant.

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