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Cleft Palate in a Mouse Model of *SOX2* Haploinsufficiency

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Abstract

Objective—While SEX-determining region Y-Box 2 (*SOX2*) mutations are typically recognized as yielding ocular and central nervous system abnormalities, they have also been associated with other craniofacial defects. To elucidate the genesis of the latter, *Sox2* hypomorphic ($Sox2^{HYP}$) mice were examined, with particular attention to secondary palatal development.

Results—Clefts of the secondary palate were found to be highly penetrant in $Sox2^{HYP}$ mice. The palatal clefting occurred in the absence of mandibular hypoplasia and resulted from delayed or failed shelf elevation.

Conclusions—*Sox2* hypomorphism can result in clefting of the secondary palate, an effect that appears to be independent of mandibular hypoplasia and is thus expected to result from an abnormality that is inherent to the palatal shelves and/or their progenitor tissues. Further clinical attention relative to *SOX2* mutations as a basis for secondary palatal clefts appears warranted.

Keywords

abnormal elevation; cleft secondary palate; mouse model; SOX2 haploinsufficiency; unilateral

Sex-determining region Y-Box 2 (*SOX2*) is an high mobility group box domain-containing transcription factor that is widely expressed in the developing nervous system and which is involved in a wide array of developmental processes (Kamachi et al., 1998; Schneider et al., 2008; Sarkar and Hochedlinger, 2013). Humans with *SOX2* mutations/haploinsufficiency commonly exhibit severe ocular and central nervous system defects, hormone deficiencies, as well as craniofacial abnormalities, with retrognathia and facial asymmetry being reported (Fantes et al., 2003; Kelberman et al., 2006; Zenteno et al., 2006; Schneider et al., 2009). Notably, cleft palate has also been reported, but in only one case (Male et al., 2002). *Sox2* hypomorphic ($Sox2^{HYP}$) mice were generated to model human *SOX2* haploinsufficiency

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(Taranova et al., 2006). These mice express from 20% to 40% of wild type (WT) *SOX2* protein levels and have been previously reported to develop neural tube and ocular defects that are consistent with those observed in *SOX2* haploinsufficient humans (Taranova et al., 2006; Langer et al., 2012). The present study characterizes the craniofacial defects in this mouse model.

Materials and Methods

Animals

The generation of $Sox2^{IR}$ and $Sox2^{EGFP}$ alleles was previously described (Ellis et al., 2004; Taranova et al., 2006). For the current study, $Sox2^{IR/+}$ females were bred to $Sox2^{EGFP/+}$ males, generating $Sox2^{EGFP/IR}$ ($Sox2^{HYP}$), $Sox2^{+/IR}$, and $Sox2^{+/+}$ embryos and fetuses. Mice with the latter two genotypes are phenotypically indistinguishable and were considered controls ($Sox2^{CONT}$) (Taranova et al., 2006). Genotyping was performed as described in Langer et al. (2012). All analyses were performed on a CD1 background. The morning of vaginal plug detection was considered embryonic day (E) 0.5, and embryo/fetus staging was based on limb morphology (Kaufman, 1992). Embryos and fetuses were harvested on E13.5 to E16.5 following maternal sacrifice. The described animal work was performed with Institutional Animal Care and Use Committee approval and in accordance with the University of North Carolina at Chapel Hill Division of Laboratory Animal Medicine and the American Psychological Association animal care guidelines.

Tissue Preparation for Whole Mount Palate Analyses

Immediately following collection, the E13.5 to E16.5 embryos/fetuses were fixed in 2.5% glutaraldehyde at 4°C. The mandibles were removed from the heads to permit visualization of the palatal primordium/palate. The remaining portion of the head was incubated for 5 minutes in a solution of 1:750 ethidium bromide and imaged under green fluorescent light.

Analysis of Relative Mandible Length

E16.5 $Sox2^{CONT}$ and $Sox2^{HYP}$ fetuses were dissected in chilled phosphate-buffered saline (PBS), cut below the forelimbs, and fixed in room-temperature 10% phosphate-buffered formalin for 2 weeks. The fixed fetuses were placed in a premade mold, enabling the consistent positioning of the sample. The vertical midpoint of the ear was established, and the nasomaxillary and mandibular lengths were measured from this point. To control for slight deviations in the orientation of the fetus and within-litter size variations, the mandibular to nasomaxillary length ratio, rather than absolute measurements, of the two groups were compared. Nine measurements for each metric were averaged, and these averages were used to determine the mandibular to nasomaxillary length ratios. The measurements were performed with ImageJ v. 1.43u software.

Tissue Preparation for In Situ Hybridization

E13.5 to E16.5 embryos and fetuses were fixed at 4°C in a solution of PBS and 4% paraformaldehyde. Following three PBS washes, the embryos were cryoprotected in a sucrose gradient and mounted in optimum cutting temperature mounting medium (O.C.T., Tissue-Tek). For *in situ* analyses, 20-μm frontal sections were incubated with digoxigenin-

labeled probes and visualized using enzymatic detection, following the manufacturer's protocol (Roche). A probe against *Sox2* (a kind gift from Dr. Lovell-Badge) was used to show *Sox2* expression, and a probe against *Tbx2* was used to mark the palatal shelves for the morphologic analyses (Pontecorvi et al., 2008).

Statistical Analyses

The proportions of *Sox2*^{HYP} embryos that exhibited defects of either the left or the right palate were analyzed using a two-tailed chi-square test. The ratios of the mandibular to nasomaxillary lengths of E16.5 *Sox2*^{HYP} and *Sox2*^{CONT} fetuses were compared using Student's *t* test. The data are given as the mean ± SEM. Significance was defined as $P < .05$.

Results

SOX2 Expression in the Palatal Primordium

In situ staining for *Sox2* in the secondary palatal primordium of E13.5 to E16.5 *Sox2*^{CONT} mice reveals its expression throughout the secondary palatal epithelium, with stronger expression laterally prior to elevation and ventrally following elevation (arrows in Fig. 1A, B, and ventral palate in C).

Gross Morphologic Analysis of the Secondary Palate in Sox2^{HYP} Mice

As expected, the examined embryos exhibited ocular defects that grossly manifested as variably severe microphthalmia. As was observed in whole mounts, the developing secondary palatal shelves of *Sox2*^{HYP} embryos were indistinguishable from those of the controls at E13.5 (Fig. 1D versus G). At E14.75, however, when *Sox2*^{CONT} palatal shelves have elevated and initiated fusion, the shelves of *Sox2*^{HYP} mice are abnormally separated, with consistently unilateral defects in the anterior third of the palatal shelf (Fig. 1E versus H). By E16.5, when fusion is complete in *Sox2*^{CONT} fetuses, a broad cleft is apparent in *Sox2*^{HYP} embryos (Fig. 1F versus I). In newborns, 67% (8/12) of *Sox2*^{HYP} mice exhibit secondary palatal clefting.

Histologic Analysis of the Sox2^{HYP} Secondary Palate

Frontal (coronal) histologic sections of *Sox2*^{HYP} mice also illustrate that at E13.5, the *Sox2*^{HYP} palatal shelves are similar to those of *Sox2*^{CONT} embryos (Fig. 2A,C versus B,D). At E14.75, all of the mutants that exhibited clefting (5/5) also exhibited a unilateral failure of shelf elevation, either specifically in the anterior region (4/5) or along the length of the secondary palate (1/5), such that the shelf was oriented vertically (Fig. 2E versus F). At E16.5, most of the affected *Sox2*^{HYP} embryos exhibited bilaterally elevated palatal shelves (Fig. 2I,K versus J,L). No significant bias was observed with respect to the laterality of the elevation defect (three left, eight right, $\chi^2 = 2.3$, $P = .13$).

Analysis of the Relative Mandibular Length in Sox2^{HYP} Embryos

SOX2 haploinsufficient humans exhibit retrognathia (Zenteno et al., 2006). Because this defect has been associated with delayed palatal shelf elevation, an analysis was performed of the relative length of the mandible to the nasomaxillary complex, an important metric in the

context of the mandibular influence on palatal development (Latham, 1966; Diewert, 1979). The mandibular/maxillary length ratios of *Sox2*^{HYP} embryos were observed to be higher than those of *Sox2*^{CONT} embryos, indicating that the mandible is not shortened in *Sox2*^{HYP} embryos (*Sox2*^{CONT}: 0.913 ± 0.004; *Sox2*^{HYP}: 0.927 ± 0.004; $P = .03$) (Fig. 3A versus B). In fact, the data indicate that the mandible is relatively longer in *Sox2*^{HYP} embryos, indicating a shortened maxilla or a lengthened mandible. Analysis of the raw mandibular and maxillary length measurements did not reveal significant differences between the *Sox2*^{HYP} and the *Sox2*^{CONT} embryos with respect to the absolute lengths of these facial structures (data not shown); it can therefore not be concluded whether a shortened maxilla, an elongated mandible, or both, underlies this effect. However, these data indicate that the cleft palate that is observed in *Sox2*^{HYP} embryos is unlikely to be due to retrognathia.

Discussion

Mutations in the gene for the transcription factor *SOX2* result in abnormal development of the brain, eye, gut, and certain craniofacial structures (Kelberman et al., 2006; Zenteno et al., 2006; Schneider et al., 2009). The results of this study indicate that *Sox2*^{HYP} mice develop cleft palate at a high penetrance. *Sox2*^{HYP} mice have been previously demonstrated to variably exhibit ocular and hypothalamic defects that are consistent with those observed in *SOX2* haploinsufficient humans, indicating the faithfulness of these lines as models of the human disorder (Taranova et al., 2006; Langer et al., 2012). Moreover, the human and mouse *SOX2* amino acid sequences are highly similar, being identical in the DNA-binding domain (Gubbay et al., 1990; Collignon, 1993; Stevanovic et al., 1994). Although cleft palate has only rarely been reported in association with *SOX2* mutations in humans, dental anomalies, including both widely spaced and supernumerary teeth, have also been reported, indicating this gene's involvement in multiple aspects of normal oral development (Male et al., 2002; Ragge et al., 2005; Numakura et al., 2010).

That few human cases have been observed to exhibit cleft palate may be due to a combination of two factors: (1) the high variability and incomplete penetrance of the phenotypes that are associated with *SOX2* mutations, and (2) that cleft palate is not classically associated with this condition (Zenteno et al., 2006; Zhou et al., 2008). *SOX2* haploinsufficient humans who exhibit cleft palate in the absence of classically recognized phenotypes would therefore likely remain unidentified as mutation carriers.

Additional support for the role of *SOX2* in human palatal development is provided by the presence of overlapping phenotypes associated with *SOX2* haploinsufficiency and the CHARGE association, the latter of which includes cleft palate and is most commonly caused by mutations in the gene that encodes CHD7 (Kelberman et al., 2006; Schneider et al., 2009; Zentner et al., 2010). Notably, CHD7 physically interacts with SOX2 to regulate the expression of other genes that are mutated in several human syndromes (Engelen et al., 2011).

For these reasons, the present results support the premise that direct or indirect interference with *SOX2* activity should be considered important relative to the genesis of craniofacial malformations, including clefting. Importantly, the addition of palatal clefting to the list of

phenotypes that are suggestive of *SOX2* mutation may lead to the identification of neural or ocular defects or hormonal deficiencies that may otherwise be overlooked in such patients and which would provide important information with regard to the treatment and/or genetic counseling of these individuals.

Although it is clear from this study that *Sox2* hypomorphism-induced palatal clefting can occur in the absence of micro/retrognathia (i.e., is not a result of tongue obstruction as is considered likely in the Pierre Robin anomaly), the developmental basis for the clefting remains unknown. However, considering that *Sox2* is expressed in the epithelium of the palatal primordia, failure of the secondary palatal shelves to unite appears likely to be an inherent abnormality of the palatal shelves. Additional experiments to explore this premise are warranted.

Acknowledgments

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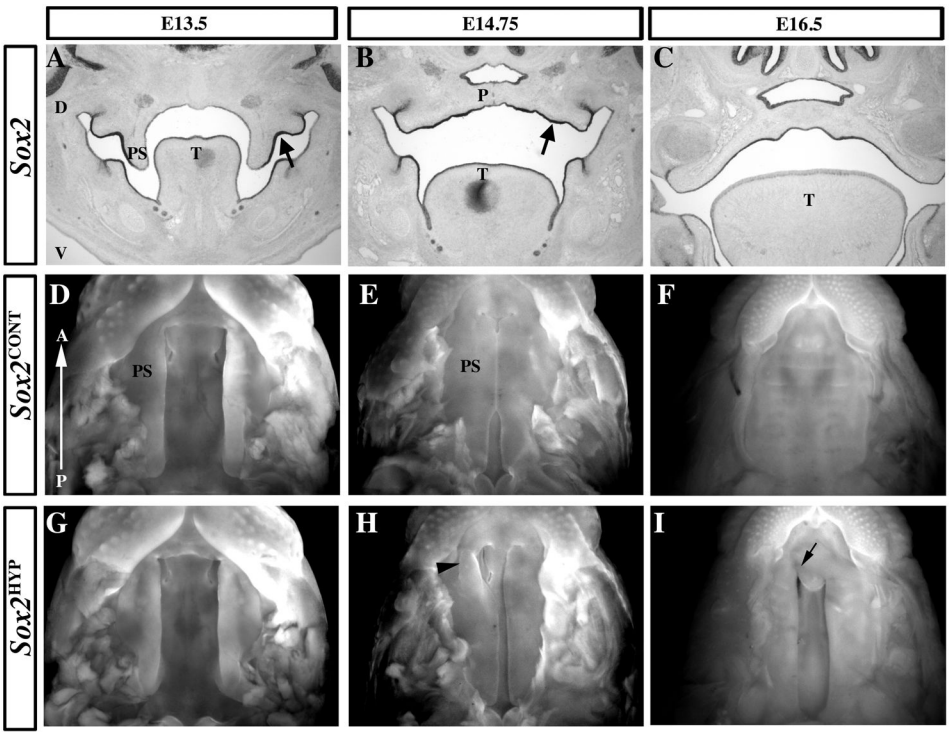

FIGURE 1.

SOX2 expression in the palatal epithelium and the gross secondary palatal morphology of *Sox2*^{CONT} and *Sox2*^{HYP} embryos. A–C: *Sox2* expression in the palatal epithelium of E13.5 (A), E14.75 (B), and E16.5 (C) *Sox2*^{CONT} embryos. The sections were taken at the mid optic level. *SOX2* is expressed throughout the palatal epithelium at all of the examined stages (black arrows). D–F: Morphology of the *Sox2*^{CONT} palate at E13.5 (D), E14.75 (E), and E16.5 (F). G–I: Morphology of the *Sox2*^{HYP} palate at E13.5 (G), E14.75 (H), and E16.5 (I). At E13.5 (D,G), no clear difference can be observed between *Sox2*^{HYP} and *Sox2*^{CONT} secondary palates. By E14.75 (H), the palate is cleft and there is a clear defect in the anterior third of the *Sox2*^{HYP} palate (red arrowhead). At E16.5 (I), the cleft is broad and asymmetry of the palatal shelves can be observed in a subset of *Sox2*^{HYP} embryos (red arrow).

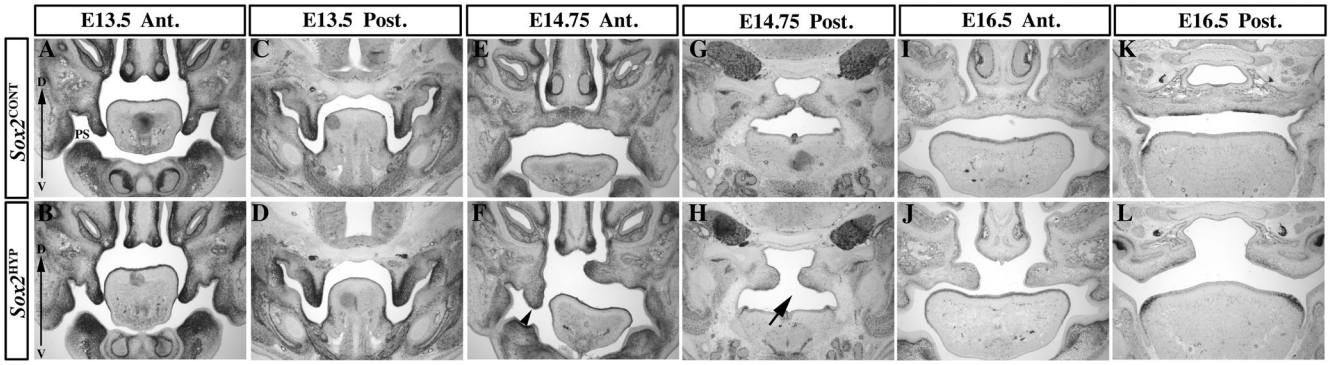

FIGURE 2.

Histologic analysis of palatal morphology of *Sox2*^{CONT} and *Sox2*^{HYP} embryos. A–D: Anterior and posterior frontal sections of E13.5 *Sox2*^{CONT} embryos (A,C) and *Sox2*^{HYP} embryos (B,D). No clear differences were observed between *Sox2*^{HYP} and *Sox2*^{CONT} palates at this stage. E–H: Anterior and posterior frontal sections of E14.5 *Sox2*^{CONT} embryos (E,G) and *Sox2*^{HYP} embryos (F,H). In affected *Sox2*^{HYP} embryos, one of the palatal shelves is consistently unelevated at this stage (arrowhead in F), and the shelves fail to extend in more posterior regions (arrow in H). I–L: Anterior and posterior frontal sections of E16.5 *Sox2*^{CONT} embryos (I,K) and *Sox2*^{HYP} embryos (J,L). Both palatal shelves have elevated in *Sox2*^{HYP} embryos, but fusion has not occurred. All of the sections were stained with an *in situ* probe against *Tbx2*.

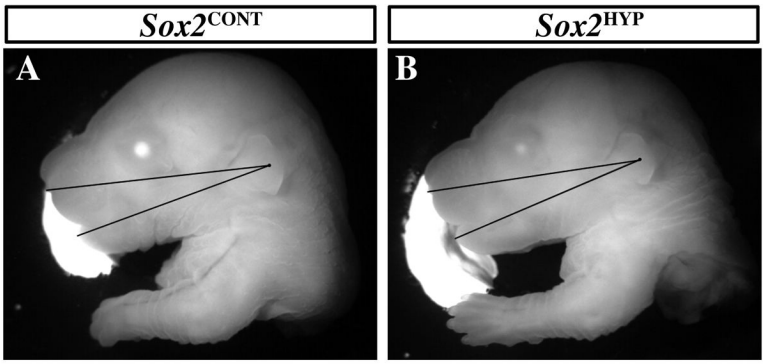

FIGURE 3.

Analysis of relative mandible lengths in *Sox2*^{HYP} embryos. A,B: E16.5 *Sox2*^{CONT} (A) and *Sox2*^{HYP} (B) embryos with lines indicating the lengths of the maxilla (top line) and the mandible (bottom line), from which relative measures of mandibular length were calculated. No retrognathia is observed in the *Sox2*^{HYP} fetuses.