Short stature homeobox 2 (SHOX2) regulates osteogenic differentiation and pattern formation during hard palate development in mice

Jue Xu1,2,3, Linyan Wang1,3, Hua Li3,4, Tianfang Yang3, Yanding Zhang4, Tao Hu1*, Zhen Huang4*, and YiPing Chen3*

1State Key Laboratory of Oral Diseases, Department of Preventive Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, P.R. China
2West China School of Public Health and West China Fourth Hospital, Sichuan University, Chengdu, Sichuan, P.R. China
3Department of Cell and Molecular Biology, Tulane University, New Orleans, LA, USA
4Southern Center for Biomedical Research and Fujian Key Laboratory of Developmental and Neural Biology, College of Life Sciences, Fujian Normal University, Fuzhou, Fujian, P.R. China

* Corresponding authors: ychen@tulane.edu (YC); zhuang@fjnu.edu.cn (ZH); hutao@scu.edu.cn (TH).

Running title: SHOX2 regulates osteogenesis and pattern of hard palate

Keywords: short stature homeobox 2 (SHOX2), hard palate, osteogenesis, patterning, lineage-specific chromatin accessibility, craniofacial development, gene regulation, epigenetics, transcription factor, cell differentiation

ABSTRACT

During mammalian palatogenesis, cranial neural crest–derived mesenchymal cells undergo osteogenic differentiation and form the hard palate, which is divided into palatine process of the maxilla and the palatine. However, it remains unknown whether these bony structures originate from the same cell lineage and how the hard palate is patterned at the molecular level. Using mice, here we report that deficiency in short stature homeobox 2 (Shox2), a transcriptional regulator whose expression is restricted to the anterior palatal mesenchyme, leads to a defective palatine process of the maxilla, but does not affect the palatine. Shox2 overexpression in palatal mesenchyme resulted in a hyperplastic palatine process of the maxilla and a hypoplastic palatine. RNA-Seq and assay for transposase-accessible chromatin (ATAC)-Seq analyses revealed that SHOX2 controls the expression of pattern-specification and skeletogenic genes associated with accessible chromatin in the anterior palate. This highlighted a lineage-autonomous function of SHOX2 in patterning and osteogenesis of the hard palate. H3K27ac ChIP-Seq and transient transgenic enhancer assays revealed that SHOX2 binds distal-acting cis-regulatory elements in an anterior palate–specific manner. Our results suggest that the palatine process of the maxilla and palatine arise from different cell lineages and differ in ossification mechanisms. SHOX2 evidently controls osteogenesis of a cell lineage and contributes to the palatine process of the maxilla by interacting with distal cis-regulatory elements to regulate skeletogenic gene expression and to pattern the hard palate. Genome-wide SHOX2 occupancy in the developing palate may provide a marker for identifying active anterior palate–specific gene enhancers.
INTRODUCTION

The palate in humans and other mammals is the roof of mouth that separates oral cavity from nasal cavity and is also responsible for assisting swallowing and speech. The palate is divided into the anterior bony hard palate and the posterior muscular soft palate (1). The hard palate consists of the anteriorly positioned palatine process of the maxilla and the posteriorly located palatine, both being differentiated directly from cranial neural crest-derived osteogenic cells (2, 3). The hard palate is thus specifically patterned during development. Disruptions in patterning and differentiation of the cranial neural crest-derived palatal mesenchymal cells lead to palate abnormalities that are usually represented as cleft palate. Specifically, malformation of the palatal bone and/or the abnormality of the soft palate are classified as a rare subset of cleft palate, submucosal cleft palate, which can also cause obstacles in speech as well as swallowing (4, 5). Thus far, most genetically modified mouse lines for studying cleft palate are complete cleft palate models, which do not allow for studying palatal bone development and patterning. There are limited transgenic mouse models for studying submucosal cleft palate. Tbx22−/− mice exhibited dramatically reduced palatine formation (6). Conditional inactivation of Bmpr1a by Osr2-IresCre in mice resulted in palatal bone defects both in the palatine process of the maxilla and palatine (7). Inhibition of TGFβ signaling as well as constitutive activation of ACVR1 in the palatal epithelium led to submucosal cleft and medial edge epithelium resistance (8-10). However, the molecular mechanisms that intrinsically pattern the hard palate and lay the ground for osteogenesis remain unknown.

It is generally accepted that tissue-specific enhancers, which are distant-acting and cis-regulatory sequences, bind to transcriptional co-activators and regulate gene expression in tissue specific manners (11-13). Recent studies on non-coding sequences especially the distal-acting enhancers have raised the significant role of these sequences in pattern formation of embryonic organs and tissues including craniofacial skeletons (14). Shox2 is a homeobox gene that is essential for the development of multiple organs including the heart, limb, and palate (15-19). We have shown previously that Shox2 plays a unique role in regulating osteogenesis and patterning the stylopodial element of the mouse developing limb by genome-wide co-occupancy with Hox-TALE factors as a direct regulator of functional distal tissue-specific enhancers (19). In the developing palate, Shox2 is specifically expressed in the anterior palatal mesenchymal cells, and Shox2 loss-of-function mutants present rare anterior clefting of the palate (15). The dramatic reduction in palatal bone formation in mice carrying conditional Shox2 inactivation in cranial neural crest cells indicates a regulatory role of Shox2 in palatal osteogenesis (16). The fact that Shox2 exhibits a genome-wide preferential co-occupation with TALE factors in the Hox-free palatal shelves suggests a patterning role for Shox2 in hard palate development (19).

In our recent study, we used Shox2Cre;R26RtmTmG alleles to trace Shox2+ cell lineage and its relationship with osteoblasts in the hard palate and found a complete absence of Shox2+ cell in the palatine (20). Interestingly, we have found previously that Shox2+ cells express Runx2 in the anterior part of the palatine process of the maxilla (named as ma-a), but are presented in a complementary manner to Runx2+ osteogenic cells in the posterior part of the palatine process of the maxilla (named as ma-p) (20). These observations suggest that the hard palate, even the palatine process of the maxilla, forms by different cell lineages and is patterned differentially. However, the underlying mechanisms of osteogenesis and patterning of the hard palate regulated by Shox2 remain to be addressed.

In this study, we took loss-of- and gain-of-function approaches to study Shox2’s effect on specific cell lineages during osteogenesis in the hard palate. Our phenotype analysis, gene expression assay, lineage-specific chromatin accessibility assay, and chromatin landscape
analysis revealed differential responses of the different portions of the hard palate to Shox2 expression and supported an essential role for Shox2 and involvement of Shox2-binding sites in the patterning of the hard palate and palatal osteogenesis during palate development.

RESULTS

Shox2 deficiency leads to a unique loss of the palatine process of the maxilla

Our recent study on Shox2 function in sinoatrial node development showed that the early embryonic lethality of Shox2 null mutants can be rescued by simultaneous deletion of Nkx2.5 in Shox2+ cell linages by compounding a Shox2-Cre knock-in allele with a Shox2-null allele and the Nkx2.5 floxed alleles (19), allowing for analysis of osteogenesis in the palate in Shox2-null background at the newborn stage (mutants die at birth). To rule out the possibility of Nkx2.5 contributing to the developing palate, we examined Nkx2.5 expression by immunostaining at E13.5 and traced Nkx2.5+ cell linages of the Nkx2.5Cre/+;R26RmTmG mice at E18.5, respectively. Our results showed that Nkx2.5 is never expressed in the palate, at least until E18.5 (Fig. S1, A-F), and Shox2Cre/-;Nkx2.5f/f mice have normal palate and survive to adulthood (data not shown), indicating that Nkx2.5 deletion in Shox2+ cell linages would not cause any abnormality in the palate. In Shox2Cre/-;Nkx2.5f/f mice, which presented a complete absence of Shox2 expression in the palate (Fig. S2), whole mount skeletal staining and histological examination revealed a very specific bone deficiency in the hard palate: a complete loss of the anterior part of the palatine process of the maxilla (ma-a) (Fig. 1), consistent with the expression of Shox2 in the osteogenic centers in this domain (20). In the posterior part of the palatine process of the maxilla (ma-p) where Shox2+ lineages exhibit complementary localization to the ossification centers, ossification was barely affected (Fig. 1, A’, B’, D’). The palatine of the Shox2Cre/++;Nkx2.5f/f and Shox2Cre/-;Nkx2.5f/f mice formed comparably, likely due to the absent Shox2 expression in this region (Fig. 1, A-B’, E-E’).

We then examined the expression of osteogenic differentiation markers, Runx2 and its downstream target Sp7/Osterix, in the Shox2+ palatine process of the maxilla of E15.5 Shox2Cre/++;Nkx2.5f/f and Shox2Cre/-;Nkx2.5f/f mice. In controls, Runx2 and Sp7/Osterix were expressed in the ossification centers that would become the palatine process of the maxilla (Fig. 2, A-D). Consistent with the complete loss of the ma-a of the Shox2Cre/-;Nkx2.5f/f palate, Runx2 expression was inhibited significantly and Sp7/Osterix expression was lost completely in the ma-a part (Fig. 2, A’, B’). However, in the ma-p region, the levels of Runx2 and Sp7/Osterix expression appeared similarly to the controls, although there was a slightly delayed expression at the ossification fronts towards the midline (Fig. 2, C’, D’). These observations demonstrate that Shox2 is essential for osteogenic fate and differentiation of palatal mesenchymal cells in the ma-a portion of the palate process of the maxilla. The slightly delayed osteoblastic differentiation at the ossification fronts could be attributed to the non-cell autonomous effect of Shox2+ lineage cells in the ma-p region.

In order to profile genome-wide effects of Shox2 in regulating osteogenesis in the developing palate, we further performed RNA-Seq on the anterior palatal tissues from E14.5 Shox2Cre/++;Nkx2.5f/f embryos and controls, respectively. Gene Ontology (GO) analysis revealed 531 down-regulated genes in the mutant palate (Fig. 2E). Among them are genes involved in ossification and skeletal system morphogenesis. In addition, Gene Set Enrichment Analysis (GSEA) indicated significant enrichment of down-regulated genes associated with ossification and skeletal system morphogenesis in the Shox2Cre/-;Nkx2.5f/f anterior palate (Fig. 2F, upper panel). The osteogenesis correlated genes Wnt5a, Dkk1, Shox2, Runx2 and Sp7 are involved in the ossification group, meanwhile Fgf4, Shox2, Runx2, Msx1 and Coll11a2 are included in the group of skeletal
system morphogenesis (Fig. 2F, lower panel). To confirm RNA-Seq results, we performed quantitative reverse transcription polymerase chain reaction (RT-qPCR) on several selected genes (Shox2, Dkk1, Runx2, Sp7, Wnt5a, Bmp2, Bmp4, Mx1, Smad3) that are associated with osteogenic differentiation and indeed found significantly reduced expression levels of these genes in Shox2Cre\/-;Nkx2.5F/F hard palate compared to controls (Fig. S3). These results further support an important role for Shox2 in the osteogenesis of the developing hard palate.

**Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 mice present both complete and incomplete cleft palate phenotypes**

The phenotypic and RNA-Seq analyses support a positive role for Shox2 in regulating osteogenesis in cranial neural crest-derived palatal mesenchyme. To determine if such Shox2-regulated osteogenesis is region specific, we activated a conditional Rosa26-Shox2 knock-in allele (R26\textsubscript{R}Shox2) by the Wnt1\textsuperscript{Cre} allele persistently in the entire palatal mesenchyme including the posterior palate that forms the muscular soft palate later after invasion of myogenic precursor cells, leading to ectopic Shox2 expression in the posterior palatal shelves and overexpressed Shox2 in the anterior palate, respectively (Fig. 3, A’, B’). Gross phenotypic analysis of Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 mice at P0 revealed the presence of two types of cleft palate defect at 100% penetrance (128/128), with incomplete cleft palate (ICP) (40.6%) and complete cleft palate (CCP) (59.4%), respectively. The ICP often appeared as fused anterior portion of the palate including the hard palate with a hole in the posterior end of the palate (Fig. 3D). All mutant mice, no matter with ICP or CCP, died soon after birth because of the velopharyngeal insufficiency caused feeding problem (Fig. 3, C-E, Fig. S4). In addition, micrognathia was seen in both ICP and CCP mutant mice (Fig. 3F). Tongue defects including malposition and abnormally patterned muscles were also observed in ICP and CCP mice (Fig. 3G). Since micrognathia and tongue malposition can mechanically interfere the elevation of palatal shelves and cause cleft palate (Pierre Robin sequence in humans), we wondered if these malformations were causative of CCP in Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 mice. Embryonic heads from E13.5 Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 and control mice (\(N = 6\) for each group), after removal of the mandible including the tongue, were placed in rotational culture for 3 days and subjected to gross examination. The results showed that the palatal shelves of Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 mice became elevated and partially fused, similar to the controls (Fig. 3H), indicating that Shox2 overexpression in the palatal mesenchyme does not represent an intrinsic factor causing CCP in Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 mice.

**Shox2 over/ectopic expression in cranial neural crest cells causes region specific malformation of the palatal bone**

Mice with complete clefting of the second palate often manifest severe hypoplastic palatal shelf residuals, preventing studies on palatal bone formation and patterning. The Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 mice with ICP, which have fused anterior domain of the palate including the hard palate, provided an excellent model to investigate the effect of Shox2 over/ectopic expression on osteogenesis and patterning of the hard palate.

Whole mount skeletal staining revealed severely inhibited osteogenesis in the palatine but morphologically unaltered palatine process of the maxilla in the ICP mice at E16 and E18.5, as compared to controls (Fig. 4, A-B’). Histological analysis showed that the palatal shelf of the palatine process of the maxilla (both the ma-a and ma-p portions) became aberrantly thickened and the amount of bone was dramatically increased as well in E18.5 Wnt1\textsuperscript{Cre};R26\textsubscript{R}Shox2 ICP mice, as compared to controls (Fig. 4, C-E’). The significantly elevated levels of cell proliferation in the mesenchyme of the palatine process of the maxilla but not in the palatine domain, as assayed by Ki67 immunostaining, explain the region-specific thickening of the palatal shelf (Fig. 4, H-K). Although the levels and patterns of Runx2...
and Sp7/Osterix expression appeared comparable in the palate process of the maxilla of Wnt1\textsuperscript{Cre};R26R\textsuperscript{Shox2} and control mice at E14.5 (Fig. S5, A-D'), the expression of these two osteogenic genes was dramatically enhanced in this domain at E16.5 (Fig. S5, E-H'), contributing to the increased bone formation. However, consistent with the presence of the hypoplastic palate, the expression Runx2 and Sp7/Osterix in this region was highly reduced at E14.5 and E16.5 (Fig. 4, F-G', Fig. S5, I-J'). These observations demonstrate that mesenchymal cells in the different domains of the hard palate appear heterogeneously and respond completely differently to over/ectopic expressed Shox2, suggesting involvement of different cell lineages and osteogenic mechanisms in bone formation in different portions of the hard palate.

**Shox2 is necessary for lineage-specific chromatin accessibility in the anterior palate**

Being a transcription factor, Shox2 binds to specific DNA sites to regulate gene expression via modulating chromatin status. To investigate the impact of Shox2 on the chromatin accessible landscape in the anterior hard palate, we subjected FACS-sorted Shox2\textsuperscript{+} cells from the palate of E14.5 Shox2\textsuperscript{Cre+};Nkx2.5\textsuperscript{F/F};R26R\textsuperscript{mTmG} and Shox2\textsuperscript{Cre-};Nkx2.5\textsuperscript{F/F};R26R\textsuperscript{mTmG} mice to ATAC-Seq analysis. The palate cells lacking Shox2 had some accessible landscape changes, with reduced accessibility at 530 regions and increased accessibility at 1368 regions compared to the control (Fig. 5A). These were indeed reproducible features, but there were far more sites that appear nearly as significant. Therefore, the low overlap of ATAC-Seq and RNA-Seq may represent noise levels. Heatmap plots of accessible chromatin landscape of the control and Shox2 deficient palatal cells also showed significantly distinct chromatin accessibility (Fig. 5B, left, >2 fold). Notably, the transcription factor binding sites of Meis2 and HOXA1 are similar in the closed regions, and CTCF, NRF1, Zfx and TFDP1 are similar in the open regions by de novo motif discovery (Fig. 5B, right). To further understand the impact of Shox2-associated accessible chromatin on differentially expressed genes, we comprehensively analyzed and integrated the RNA-Seq and ATAC-Seq data on the palate lacking Shox2 and controls. Indeed, 387 associated genes were found by using Venn analysis from differentially expressed genes and ATAC-Seq peak representing genes (Fig. 5C). To determine if the resulting sites we obtained represent the regulatory elements that are related to the pattern formation of the palate or skeletal development, we conducted GO analysis of the clusters’ representing genes. The results revealed a great number of skeletal system as well as pattern specification associated genes (Fig. 5D, Fig. S6). Furthermore, the ATAC-Seq peaks were present with high-level profile of osteogenic related genes Smad3, Bmp4 and Bmp2 in the control group while the accessible chromatin in the palatal cells lacking Shox2 was dramatically reduced (Fig. 5E). Thus, the ATAC-Seq results indicate that Shox2 may function as a critical transcriptional factor to recruit chromatin-binding factors. Together, these results suggest that Shox2 pioneers chromatin opening at a subset of sites that are probably associated with pattern formation and osteogenesis during hard palate development.

**Shox2 binds to anterior palate-specific distal acting elements**

Our previous ChIP-Seq experiments using a HA-tagged Shox2 knock-in allele (Shox2\textsuperscript{HA-DsRed}) and anti-HA antibody on the Shox2\textsuperscript{2} domain of the developing limb and palate showed its distal enhancer binding property and high relevance to skeletogenesis, although the top peak signals of the limb and palate do not overlap (19). The ATAC-Seq analysis in this study also suggests that Shox2 pioneered chromatin opening at a subset of sites that are probably associated with pattern formation during palate development. To characterize the anterior palate-specific chromatin landscape of the distal Shox2-binding sites, we conducted Shox2 ChIP-Seq again in parallel with H3K27ac ChIP-Seq on
the Shox2\(^+\) domain anterior palate and the Shox2\(^-\) posterior palate from E12.5 embryos. We first confirmed that the majority of Shox2-binding peaks are located distally from the transcription starting sites (Fig. 6A). Integrative analysis of the Shox2 and H3K27ac ChIP-Seq datasets and aggregate plots of binding signals of H3K27ac in the anterior and posterior palate, respectively, versus the summits of Shox2 binding sites showed close association of Shox2 binding peak with enriched H3K27ac in the anterior palate but not the posterior palate, indicate that Shox2 occupancy is highly related to active enhancer elements (Fig. 6B). To determine if these Shox2-binding sites are anterior palate-specific distal active elements, we further analyzed the distal Shox2-binding peaks on the palate and limb along with the H3K27ac ChIP-Seq datasets on the anterior and posterior palate from several known anterior or posterior palate-specific genes. Indeed, the Shox2 binding sites exhibit a diverse combination of active enhancers in the anterior palate. Shox2-binding peaks in the anterior palatal tissues are highly associated with H3K27ac-enriched peaks in the anterior palate clustering around the Msx1 and Wnt5a loci that are specifically expressed in the anterior palatal mesenchyme (21) (Fig. 6C; Fig. S7). However, no obvious Shox2-binding peaks and H3K27ac peaks from the anterior palate were found around loci of the posterior palate genes Barx1 and Meox2 where H3K27ac mark in the posterior palate is enriched (Fig. 6C; Fig. S7). To further confirm binding of Shox2 to distal enhancers, we also performed Hi-C assay on the anterior and posterior palatal tissues from E12.5 embryos. As presented in Fig. S8, the preliminary results of Hi-C assay showed a preferential interaction of Shox2 with distal enhancers of a few selected Shox2 targeted genes such as Msx1, Bmp4, Meis1, and Bmp2, suggesting that Shox2 binds to distal active enhancers to regulate the osteogenic genes through long-range chromatin loop. Lastly, we further intersected the ATAC-Seq and ChIP-Seq data around the loci of those differentially expressed genes (Bmp2, Bmp4 and Smad3) that were highlighted in Fig. 5E. The results showed clearly the co-occupancy of Shox2-binding peaks with enriched H3K27ac and highly open chromatin in the wild type anterior palate but closed chromatin in the absence of Shox2 (Fig. S9).

A Shox2-bound site exhibits palate-specific enhancer activity

Our recent study on the role of Shox2 in limb development identified a number of Shox2-bound limb specific distal enhancers, including one for the Meis1 gene (19). Our Shox2 ChIP-Seq on the palate also identified a distinct Shox2-bound potential enhancer for Meis1 (Fig. 7A, named as Shox2-enhancer2), which is about 1-Kb apart from the limb specific Shox2-enhancer. To define if Shox2 binding peaks in the palate are indeed active palate-specific enhancers, we decided to start with analysis of the Shox2-bound potential Meis1 enhancer (Shox2-enhancer2), because the Shox2-bound Meis1 enhancer in the limb has been shown to exhibit activity specifically in the proximal limb domain (19). Intersections of H3K27ac ChIP-Seq data from the anterior palate with Shox2 binding peaks on Meis1 locus revealed co-occupancy of enriched H3K27ac with Shox2 peak in the palate but not in the limb (Fig. 7A). Indeed, transient transgenic enhancer-reporter assay manifested anterior palate specific activity of this enhancer (Shox2-enhancer2). Among the four transgenic embryos harvested, two showed positive LacZ staining in the anterior palate at E12.5 (Fig. 7B).

DISCUSSION

In this study, we provide evidence that Shox2\(^+\) cell population is necessary for osteogenesis in the anterior hard palate. Shox2 is a specific marker in the developing anterior palate before fusion in mice (15), and was considered as the boundary of the anterior palate because Shox2 expresses in the mesenchyme anterior to the boundary ruga (22) that is also regarded as the boundary of the hard palate (23). However, we
recently identified a unique Shox2\textsuperscript{+} mesenchymal cell lineage in the anterior hard palate (palatine process of the maxilla) (20). Here, we showed that the palatine appeared intact in Shox2-null mice, as demonstrated by skeleton preparation and histology assessment, indicating that Shox2 is irrelevant to the posterior hard palate. In line with the palatal phenotype of bone deficiency seen in Shox2\textsuperscript{Cre}\textsuperscript{-};Nkx2.\textsuperscript{3\textsuperscript{F/F}} mice, our RNA-Seq analysis revealed a significant downregulation of genes involved in osteogenesis and skeletal system morphogenesis in the palatine process of the maxilla lacking Shox2.

We showed previously that Shox2\textsuperscript{+} cells are overlapped with the ossifying centers in the anterior part of the palatine process of the maxilla but are present in a complementary manner to the ossifying sites in the posterior domain of the palatine process of the maxilla (20). Shox2\textsuperscript{+} lineage cells in the anterior hard palate could be therefore divided into two populations. In the current study, we showed that in the absence of Shox2, bone formation in the anterior part of the palatine process of the maxilla is completely inhibited, but the ossification in the posterior part of the palatine process of the maxilla, despite of slight delay before birth, appears identical as compared to controls. Shox2 apparently functions distinctly in these two cell populations.

We conclude that expression of Shox2 is important for the patterning of the anterior hard palate. Consistent with the ability of this transcription factor to drive an osteogenic program in these cells, Shox2 overexpression in the Shox2\textsuperscript{+} palatine process of the maxilla led to excessive proliferation leading to abnormal thickening of the anterior hard palate, as well as over-ossification of the bone. This observation is consistent with the decrease in cell proliferation in the anterior palate in Shox2\textsuperscript{-/-} mice (15). Notably, ectopic Shox2 expression in the posterior hard palate (palatine), where Shox2 is not normally expressed, results in a specific inhibition of bone formation without affecting cell proliferation (Fig. 4), further highlighting a direct modulating role for Shox2 in osteogenic differentiation and patterning of the hard palate. The role of Shox2 in pattern formation of the skeleton is also supported by the fact that Shox2 inactivation in Shox2\textsuperscript{-/-};Runx2\textsuperscript{+} osteogenic precursors causes a mis-patterned stylopod (19).

Epigenetic regulation, especially the chromatin structure and composition, is crucial for gene expression and the subsequent cellular phenotypes (24). Studies have showed that enhancers are involved in defining face and skull morphology during development (13-14). In addition, Rahimov et al. reported association of the disruption of an AP-2\textalpha bound IRF6 enhancer with cleft lip (25). A recent study demonstrated that three transposable elements cooperatively function as a distal enhancer for Wnt5a expression during development of the secondary palate (26). Our previous study found that Shox2 not only binds to distal-acting enhancers in the limb but also can bind to the palate-specific distal elements (19). Here, we took advantage of ATAC-Seq technology to precisely analyze the Shox2-accessible chromatin in Shox2\textsuperscript{+} cell lineage in the developing palate. We found that a multitude of accessible chromatin sites were closed in Shox2 deficient palatal cells. Given that HoxA1 and Meis2 are highly related to pattern formation during embryogenesis, the presence of the binding motifs of these two transcriptional factors in the Shox2-accessible sites suggests that Shox2-accessible chromatin is associated with the patterning of the anterior palate. Furthermore, changes of pattern specification and ossification associated genes were found in the GO analyses from RNA-Seq and ATAC-Seq data between Shox2 mutants and controls. All these results support the involvement of Shox2-accessible sites in the regulation of palate patterning and osteogenesis during development. On the other hand, CTCF, which functions to block the communication between promotors and enhancers as an insulator protein, a repressor or a transcriptional activator (27), is activated in the mutants. It suggests a repressive function of Shox2-accessible sites on the transcription activity of the enhancers. Through
comprehensive analyses of ATAC-Seq and RNA-Seq, we found that the Shox2-regulatory chromatin opening sites represent the ossification feature for the hard palate, as the osteogenic related genes Smad3, Bmp4 and Bmp2 show a low-level profile in Shox2 mutants compared to the control group. Thus, Shox2 probably acts as the switch of accessible chromatin in regulating osteogenesis of the hard palate. To characterize anterior palate specific Shox2-bound regulatory elements and palate specific enhancer, we performed H3K27ac ChIP-Seq on the anterior and posterior palatal tissues along with Shox2 ChIP-Seq on the palatal tissues, as well as transgenic enhancer-reporter assay. We validated that Shox2 binds to the anterior palate-specific distal enhancer elements by computational and comparative analyses on Shox2 ChIP-Seq data as well as H3K27ac ChIP-Seq data of the anterior and posterior palate. This notion is further supported by Hi-C assay on the anterior palatal tissues and by the specific activity of Shox2-bound Meis1 enhancer in the anterior palate in transient transgenic enhancer-reporter assay. Therefore, the Shox2-occupied elements that are unique to the anterior palate were extracted and validated, further supporting that Shox2-bound sites are highly associated with the anterior palate pattern specification.

In sum, this study reveals that Shox2 is necessary in the skeleton formation of the anterior hard palate and regulates osteogenesis and palate pattern formation through the genome wide Shox2-occupied anterior-palate specific regulatory elements.

**EXPERIMENTAL PROCEDURES**

**Mouse Models**

Animals used in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) of Tulane University and by the Animal Use Committee of Fujian Normal University. The generation and genotyping of Shox2\textsuperscript{Cre}, Nkx2.5\textsuperscript{F/F}, Nkx2.5\textsuperscript{Cre}, R26R\textsuperscript{InlG}, Wnt1\textsuperscript{Cre}, R26R\textsuperscript{Shox2}, Shox2\textsuperscript{HA-DsRed} mice have been described previously (28-34).

**Histology, Immunofluorescence, and Skeletal Staining**

Embryos were collected in ice-cold PBS from timed-pregnant female mice and fixed in 4% paraformaldehyde at 4°C, then subsequently dehydrated in graded ethanol and embedded in paraffin. Paraffin-embedded samples were sectioned at 8 μm for Azon red/Aniline blue and immunofluorescent staining according to previously described protocols (16, 18). The primary antibodies used in this study were as follows: anti-Shox2 (homemade, 1:1000), anti-Nkx2.5 (Santa Cruz, sc-8697, 1:300), anti-GFP (Novus Biologicals, NB100-1614, 1:1000), anti-Runx2 (Santa Cruz, sc-390351, 1:300), anti-Sp7/Osterix (Abcam, ab22552, 1:500), anti-MF20 (1:25), anti-Ki67 (Abcam, ab15580, 1:500). The secondary antibodies including donkey-anti mouse, donkey-anti rabbit, donkey-anti-goat and goat-anti chicken were all used at 1:1000 and purchased from Life Technologies. Whole mount Alcian Blue/Alizarin Red staining of animals was performed as described previously (17). Each experiment was performed at least 3 times. For cell proliferation assay, Ki67\textsuperscript{+} cells were counted and analyzed using the protocols reported previously (20, 35). ANOVA statistical analysis was used and the data were represented as mean ± SD. *P<0.05 is considered as significant statistically.

**Rotational Culture of Embryonic Heads**

Embryos at E13.5 from timed-pregnant female mice were sacrificed (N = 6 for both controls and mutants). Embryonic heads with removal of the jaw were placed in a sterile 50ml bottle containing 10ml BGJb medium (Gibco) with 50% fetal bovine serum. A gas mixture of 50% oxygen, 45% nitrogen and 5% carbon dioxide was used to flush the bottles for two minutes. Bottles containing samples were cultured in a rotary apparatus at the speed of 20rpm at 37 °C for 3 days and re-flushed every day. Samples were then fixed and processed according to the procedures described previously (36).
**RNA-Seq and Analysis**

The palatal tissues that would form the future anterior hard palate were dissected out from E14.5 Shox2\(^{Cre/++;Nkx2.5^{F/F}}\) and Shox2\(^{Cre/-;Nkx2.5^{F/F}}\) mice (N = 3 for each group), respectively. Dissected tissues were subjected to RNA extraction under the manufacturer’s instructions (RNeasy Micro Kit, Qiagen). Libraries were subsequently generated for RNA-Sequencing (RNA-Seq) analysis as described (20). The RNA-Seq data were deposited in the Gene Expression Omnibus (GEO) database with accession number GSE129821.

**ChIP-Seq and ATAC-Seq**

The Shox2-expressing anterior palatal tissues and non-Shox2 expressing posterior palate shelves were dissected out under a fluorescence dissecting scope from Shox2\(^{HA-DsRed}\) embryos. Pooled palatal tissues were subjected to chromatin immunoprecipitation (ChIP) using anti-HA (Abcam, ab9110) or anti-H3K27ac antibodies (Abcam, ab4729) following the manufacturer’s protocol with minor modifications according to the published researches (13, 19). Three independent ChIPs were conducted for library generation for each group. The libraries were then sequenced on the Illumina HiSeq 2000 platform using 50-bp single-end configuration. HA-enriched or H3K27ac-enriched genomic regions of the Shox2-expressing anterior palate and the posterior palate, and the Shox2-binding peaks of the developing palate and limb from our previously deposited data (GEO with accession number GSE81897) were analyzed and intersected comprehensively. The GFP-positive palatal mesenchymal cells from E14.5 Shox2\(^{Cre/++;Nkx2.5^{F/F};R26R_{mTmG}}\) and Shox2\(^{Cre/-;Nkx2.5^{F/F};R26R_{mTmG}}\) embryos were sorted by FACS following our published protocol (19, 20). Sorted cells were counted and pooled (N = 50,000 for each group) and prepared for ATAC-Seq and analysis as described (37, 38). The ATAC-Seq data were deposited in the GEO database with accession number GSE129538. ATAC-Seq data were aligned and filtered as previously described (39). TBtools (40) was used to visualize the chromatin accessible regions. The ChIP-Seq data deposited in GEO database with accession number GSE138721.

**Transient Transgenic Enhancer-reporter Assay**

Based on our ChIP-Seq data, a specific Shox2 enhancer sequence on the palate (named as Shox2-enhancer2) was identified and the transgenic enhancer assay was then performed. The Shox2-enhancer2 sequences (chr11:18, 614, 903-18, 615, 615; Primer: Forward: AAATAATGTATATGCTAGGA; Reverse: TGTGGAACAGACATCGTTGACA) were amplified and cloned into Hsp68-LacZ plasmid, following by the pronuclear injection and X-gal staining of the embryos using standard protocols.

**Author Contributions**: J.X., Z.H. and Y.C. designed the study. J.X. performed most of the experiments. Z.H. analyzed the RNA-Seq, ChIP-Seq and ATAC-Seq data, and conducted Hi-C assay and transient transgenic enhancer-reporter assay. L.W. and H. L. performed the qPCR. L.W., H. L. and T. Y. helped with harvesting samples for RNA-Seq, ChIP-Seq, and ATAC-Seq analyses, histology, and co-immunoprecipitation assay. J.X. wrote the manuscript. Z.Y. helped with revision of the manuscript. Z.H., T.H. and Y.P.C. contributed to conception, insight, and revision of the manuscript.

**Conflict of Interest**: All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**: We acknowledge financial support by the National Natural Science Foundation of China to J. X. (81900966), to Y.Z. (81870739), to Z.H (81700933), and a grant (R01 DE026482) from the National Institutes of Health to Y.C. Z.H is financially supported by the funding of College of Life Sciences, Fujian
Normal University (FZSKG2018015). We thank all members of the Chen laboratory at the Department of Cell and Molecular Biology, Tulane University, New Orleans, USA for their scientific inputs during the studies. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

REFERENCES

1. Bush, J. O., and Jiang, R. (2012). Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. Development, 139, 231-243.
2. Ho, T. V., Iwata, J., Ho, H. A., Grimes, W. C., Park, S., Sanchez-Lara, P. A., and Chai, Y. (2015). Integration of comprehensive 3D microCT and signaling analysis reveals differential regulatory mechanisms of craniofacial bone development. Developmental biology, 400, 180-190.
3. Martínez-Abadías, N., Holmes, G., Pankratz, T., Wang, Y., Zhou, X., Jabs, E. W., and Richtsmeier, J. T. (2013). From shape to cells: mouse models reveal mechanisms altering palate development in Apert syndrome. Disease models & mechanisms, 6, 768-779.
4. Funato, N., Nakamura, M., and Yanagisawa, H. (2015). Molecular basis of cleft palates in mice. World journal of biological chemistry, 6, 121.
5. Kaplan, E. N. (1975). The occult submucous cleft palate. The Cleft palate journal, 12, 356-368.
6. Pauws, Erwin, Aya Hoshino, Lucy Bentley, Suresh Prajapati, Charles Keller, Peter Hammond, Juan-Pedro Martinez-Barbera, Gudrun E. Moore, and Philip Stanier. (2009). Tbx22 null mice have a submucous cleft palate due to reduced palatal bone formation and also display ankyloglossia and choanal atresia phenotypes. Human molecular genetics, 18, 4171-4179.
7. Baek, J. A., Lan, Y., Liu, H., Maltby, K. M., Mishina, Y., and Jiang, R. (2011). Bmpr1a signaling plays critical roles in palatal shelf growth and palatal bone formation. Developmental biology, 350, 520-531.
8. Dudas, M., Kim, J., Li, W.Y., Nagy, A., Larsson, J., Karlsson, S., Chai, Y. and Kaartinen, V. (2006). Epithelial and ectomesenchymal role of the type I TGF-β receptor ALK5 during facial morphogenesis and palatal fusion. Developmental biology, 296, 298-314.
9. Lane, J., Yumoto, K., Azhar, M., Ninomiya-Tsuji, J., Inagaki, M., Hu, Y., Deng, C.X., Kim, J., Mishina, Y. and Kaartinen, V. (2015). Tak1, Smad4 and Trim33 redundantly mediate TGF-β3 signaling during palate development. Developmental biology, 398, 231-241.
10. Noda, K., Mishina, Y., and Komatsu, Y. (2016). Constitutively active mutation of ACVR1 in oral epithelium causes submucous cleft palate in mice. Developmental biology, 415, 306-313.
11. Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F. and Afzal, V. (2009). ChiP-seq accurately predicts tissuespecific activity of enhancers. Nature, 457, 854.
12. Minoux, M., Holwerda, S., Vitobello, A., Kitazawa, T., Kohler, H., Stadler, M. B., and Rijli, F. M. (2017). Gene bivalency at Polycomb domains regulates cranial neural crest positional identity. Science, 355, eaal2913.
13. Malt, A. L., Cesario, J. M., Tang, Z., Brown, S., and Jeong, J. (2014). Identification of a face enhancer reveals direct regulation of LIM homeobox 8 (Lhx8) by wingless-int (WNT)/β-catenin signaling. Journal of Biological Chemistry, 289, 30289-30301.
14. Attanasio, C., Nord, A.S., Zhu, Y., Blow, M.J., Li, Z., Liberton, D.K., Morrison, H., Plajzer-Frick, I., Holt, A., Hosseini, R. and...
Phouanenavong, S. (2013). Fine tuning of craniofacial morphology by distant-acting enhancers. *Science, 342*, 1241006.

15. Yu, L., Gu, S., Alappat, S., Song, Y., Yan, M., Zhang, X., Zhang, G., Jiang, Y., Zhang, Z., Zhang, Y. and Chen, Y. (2005). Shox2-deficient mice exhibit a rare type of incomplete clefting of the secondary palate. *Development, 132*, 4397-4406.

16. Gu, S., Wei, N., Yu, X., Jiang, Y., Fei, J., and Chen, Y. (2008). Mice with an anterior cleft of the palate survive neonatal lethality. *Developmental dynamics: an official publication of the American Association of Anatomists, 237*, 1509-1516.

17. Bobick, B. E., and Cobb, J. (2012). Shox2 regulates progression through chondrogenesis in the mouse proximal limb. *Journal of Cell Science, 125*, 6071-6083.

18. Ye, W., Wang, J., Song, Y., Yu, D., Sun, C., Liu, C., Chen, F., Zhang, Y., Wang, F., Harvey, R.P. and Schrader, L. (2015). A common Shox2-Nkx2-5 antagonistic mechanism primes the pacemaker cell fate in the pulmonary vein myocardium and sinoatrial node. *Development, 142*, 2521-2532.

19. Ye, W., Song, Y., Huang, Z., Osterwalder, M., Ljubojevic, A., Xu, J., Bobick, B., Abassah-Oppong, S., Ruan, N., Shamba, R. and Yu, D. Zhang, L., Cai, C., Visel, A., Zhang, Y., Cobb, J., Chen Y. (2016). A unique stylopod patterning mechanism by Shox2-controlled osteogenesis. *Development, 143*, 2548-2560.

20. Xu, J., Huang, Z., Wang, W., Tan, X., Li, H., Zhang, Y., Tian, W., Hu, T. and Chen, Y.P. (2018). FGF8 signaling alters the osteogenic cell fate in the hard palate. *Journal of dental research, 97*, 589-596.

21. Smith, T. M., Lozanoff, S., Iyyanar, P. P., and Nazarali, A. J. (2013). Molecular signaling along the anterior–posterior axis of early palate development. *Frontiers in physiology, 3*, 488.

22. Pantalacci, S., Prochazka, J., Martin, A., Rothova, M., Lambert, A., Bernard, L., Charles, C., Viriot, L., Peterkova, R. and Laudet, V. (2008). Patterning of palatal rugae through sequential addition reveals an anterior/posterior boundary in palatal development. *BMC developmental biology, 8*, 116.

23. Welsh, I. C., and O'Brien, T. P. (2009). Signaling integration in the rugae growth zone directs sequential SHH signaling center formation during the rostral outgrowth of the palate. *Developmental biology, 336*, 53-67.

24. Chen, T., and Dent, S. Y. (2014). Chromatin modifiers and remodellers: regulators of cellular differentiation. *Nature Reviews Genetics, 15*, 93.

25. Rahimov, F., Marazita, M.L., Visel, A., Cooper, M.E., Hitchler, M.J., Rubini, M., Domann, F.E., Govil, M., Christensen, K., Bille, C. and Melbye, M. (2008). Disruption of an AP-2α binding site in an IRF6 enhancer is associated with cleft lip. *Nature genetics, 40*, 1341.

26. Nishihara, H., Kobayashi, N., Kimura-Yoshida, C., Yan, K., Bornmuth, O., Ding, Q., Nakanishi, A., Sasaki, T., Hirakawa, M., Sumiyama, K. and Furuta, Y. (2016). Coordinately co-opted multiple transposable elements constitute an enhancer for wnt5a expression in the mammalian secondary palate. *PLoS genetics, 12*, e1006380.

27. Kim, S., Yu, N. K., and Kaang, B. K. (2015). CTCF as a multifunctional protein in genome regulation and gene expression. *Experimental & molecular medicine, 47*, e166.

28. Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K., and McMahon, A. P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Current biology, 8*, 1323-S2.

29. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *genesis, 45*, 593-605.

30. Pashmforoush, M., Lu, J.T., Chen, H., St Amand, T., Kondo, R., Pradervand, S., Evans,
S.M., Clark, B., Feramisco, J.R., Giles, W. and Ho, S.Y. (2004). Nkx2-5 pathways and congenital heart disease: loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. Cell, 117, 373-386.

31. Scott, A., Hasegawa, H., Sakurai, K., Yaron, A., Cobb, J., and Wang, F. (2011). Transcription factor short stature homeobox 2 is required for proper development of tropomyosin-related kinase B-expressing mechanosensory neurons. Journal of Neuroscience, 31, 6741-6749.

32. Sun, C., Zhang, T., Liu, C., Gu, S., and Chen, Y. (2013). Generation of Shox2-Cre allele for tissue specific manipulation of genes in the developing heart, palate, and limb. Genesis, 51, 515-522.

33. Wang, J., Bai, Y., Li, N., Ye, W., Zhang, M., Greene, S.B., Tao, Y., Chen, Y., Wehrens, X.H. and Martin, J.F. (2014). Pitx2-microRNA pathway that delimits sinoatrial node development and inhibits predisposition to atrial fibrillation. Proceedings of the National Academy of Sciences, 111, 9181-9186.

34. Moses, K. A., DeMayo, F., Braun, R. M., Reecy, J. L., and Schwartz, R. J. (2001). Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. genesis, 31, 176-180.

35. Liu, W., Lan, Y., Pauws, E., Meester-Smoor, M. A., Stanier, P., Zwarthoff, E. C., and Jiang, R. (2008). The Mn1 transcription factor acts upstream of Tbx22 and preferentially regulates posterior palate growth in mice. Development, 135, 3959-3968.

36. Parada, C., Han, D., Grimaldi, A., Sarrión, P., Park, S.S., Pelikan, R., Sanchez-Lara, P.A. and Chai, Y. (2015). Disruption of the ERK/MAPK pathway in neural crest cells as a potential cause of Pierre Robin sequence. Development, 142, 3734-3745.

37. Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y., and Greenleaf, W. J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nature methods, 10, 1213.

38. Mayran, A., Khetchoumian, K., Hariri, F., Pastinen, T., Gauthier, Y., Balsalobre, A., and Drouin, J. (2018). Pioneer factor Pax7 deploys a stable enhancer repertoire for specification of cell fate. Nature genetics, 50, 259.

39. Tao, H., Lin, H., Sun, Z., Pei, F., Zhang, J., Chen, S., Liu, H., Chen, Z. (2019). Klf4 promotes dentinogenesis and odontoblastic differentiation via modulation of TGF-β signaling pathway and interaction with histone acetylation. Journal of Bone and Mineral Research.

40. Chen, C., Xia, R., Chen, H. and He, Y. (2018). TBtools, a Toolkit for Biologists integrating various biological data handling tools with a user-friendly interface. BioRxiv, p.289660.

FIGURE LEGENDS

Figure 1. **Shox2 deficiency leads to the loss of the anterior portion (ma-a) of the palate process of the maxilla.** (A–B’) Whole mount skeletal preparation shows the complete absence of the anterior portion (green dotted boxes) of the palate process of the maxilla, while the posterior part (ma-p, black dotted boxes) of the palate process of the maxilla and the palate were barely affected at E18.5 and P0, respectively. (C–E’) Histological analyses of control and Shox2-Cre/-;Nkx2.5^{Cre/-} palate at P0 demonstrate the complete loss of the ma-a (marked by asterisk) in the mutant. However, the ma-p and palate appeared identical both in the mutants and the controls (C–E’). Scale bars = 500 µm (A–B’) and 400 µm (C–E’).

Figure 2. **Inhibition of osteogenic differentiation in the palate process of the maxilla by Shox2 inactivation in the hard palate.** (A–B’) Osteogenic marker Runx2 was dramatically reduced while Sp7/Osterix was completely loss in the ma-a portion of the
mutants at E15.5. (C-D’) Runx2 expression in the ma-p domain was comparable in mutants and the controls, however, Sp7/Osterix in the ma-p of the mutants were slightly decreased in the midline of the palate (marked by arrows). (E) Gene ontology analysis from RNA-Seq data shows downregulated genes in the palate of Shox2Cre;Nkx2.5F/F embryos at E14.5 that are involved in ossification, skeletal system morphogenesis, bone development and bone morphogenesis. (F) Gene Set Enrichment Analysis of the RNA-Seq data shows the enrichment of the genes in the anterior palate tissues lacking Shox2 including osteogenesis related genes. Scale bar = 100 µm (A-D’).

Figure 3. Wnt1Cre;R26RShox2 mice exhibit incomplete cleft palate (ICP) or complete cleft palate (CCP). (A-B’) Immunostaining shows overexpressed Shox2 in the anterior palatal shelf and ectopic Shox2 expression in the posterior palatal shelf in an E13.5 Wnt1Cre;R26RShox2 mice compared to the controls. (C-E) Wnt1Cre;R26RShox2 mice presented either posterior clefting of the palate or the complete cleft palate. (F) Ventral views of whole mount heads (upper) and skeletons (lower) with removal of the mandible at P0 reveal micrornathia in Wnt1Cre;R26RShox2 mice with ICP or CCP. (G) Immunostaining of muscle marker MF20 reveals malposition and abnormal patterning of the intrinsic (light blue arrows) and extrinsic (yellow arrows) muscles in the tongue of E14.5 Wnt1Cre;R26RShox2 mice with ICP or CCP. (H) The palatal shelves of embryonic heads from E13.5 Wnt1Cre;R26RShox2 and control mice elevated and partially fused at the midline (white arrow indicated) after 3 days in rotational culture. Scale bars = 50 µm (A–B’), 1mm (C-F), 200 µm (G) and 1mm (H).

Figure 4. Shox2 ectopic/over expression in cranial neural crest cells of Wnt1Cre;R26RShox2 mice with ICP inhibits osteogenesis in the palate specifically. (A-B’) Whole mount skeleton preparations show palatal bone defects in the mutants. (C-E’) Histological assessments reveal aberrant thickening of the ma-a and ma-p (marked by arrows). However, the palatine formation was inhibited (marked by asterisk). (F-G’) The expression of osteogenic markers, Runx2 and Sp7/Osterix, was highly reduced in the presumptive palatine region of E14.5 Wnt1Cre;R26RShox2 mice. (H-K) Ki67 staining of the palate at E14.5 of the control and Wnt1Cre;R26RShox2 mice with ICP shows significantly elevated levels of cell proliferations in the ma-a and ma-p domains (*P<0.05), but a comparable level of Ki67+ cells in the palatine of the mutant, as compared to controls. Scale bars = 500 µm (A–B’), 400 µm (C-E’), 50 µm (F-J’).

Figure 5. Shox2 is necessary for lineage-specific chromatin accessibility in the anterior palate. (A) Venn analysis of the chromatin accessible regions in palatal cells lacking Shox2 indicates 530 closed regions and 1368 opened regions in the mutants (MT) as compared to that in the wildtype controls (WT). (B) ATAC-Seq analysis shows differential accessibility (>2 fold) between the mutants and the controls (left); De novo motif discovery indicates the transcription factor binding sites of Meis2 and HOXA1 are present in the closed regions, on the other hand, CTCF, NRF1, Zfx and TFPD1 are present in the open regions (right). (C) Venn analysis of differentially expressed genes from RNA-Seq data and ATAC-Seq peak representing genes identifies 387 associated genes. (D) GO analysis of genes associated with a transcription starting sites showing changes in pattern specification and ossification associated genes. (E) ATAC-Seq peaks (20-200kb from transcription starting sites) of osteogenic related genes Smad3, Bmp-4 and Bmp-2 were present with high-level profile in the control group while the accessible chromatin in the palatal cells lacking Shox2 was dramatically reduced.

Figure 6. Shox2 binding peaks fall in the vicinity of H3K27ac enriched domains in the anterior palate. (A) ChIP-Seq results reveal the majority of genome wide H3k27ac peaks in the
developing palate fall into 10-100 kb regions away from the transcription starting site, suggesting that these H3K27ac enriched sites are distal enhancers. (B) Aggregate plots on the genome wide H3K27ac enriched peaks and Shox2-binding peaks in the anterior palatal shelves show a close association, indicating that Shox2-binding sites are highly related to active enhancers. (C) Shox2-binding peaks in the anterior palatal tissues are highly associated with H3K27ac-enriched peaks in the anterior palate clustering around the *Msx1*, but no obvious peaks were found around loci of the posterior palate gene *Barx1*.

**Figure 7. Correction of Shox2 ChIP-Seq and H3K27ac ChIP-Seq profiles in the anterior palate.** (A) IGV genome browser tracks showing the binding peaks of Shox2 (palate and limb) and H3K27ac (anterior and posterior palate). Co-occupancy of Shox2-binding peaks and H3K27ac-binding peaks in the anterior palate is found around *Meis1* locus, but there are no obvious peaks in the Shox2-signals in limb and H3K27ac-signals in the posterior palate around *Meis1*. (B) Transient transgenic enhancer-reporter assay shows positive *LacZ* staining in the anterior palatal mesenchyme.
Short stature homeobox 2 (SHOX2) regulates osteogenic differentiation and pattern formation during hard palate development in mice
Jue Xu, Linyan Wang, Hua Li, Tianfang Yang, Yanding Zhang, Tao Hu, Zhen Huang and YiPing Chen

J. Biol. Chem. published online October 24, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.008801

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts