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Hand2 is required in the epithelium for palatogenesis in mice

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Summary

The basic helix-loop-helix (bHLH) transcription factor Hand2 has been implicated in the development of multiple organs, including craniofacial organs. Mice carrying *Hand2* hypomorphic alleles (*Hand2*$^{LoxP/-}$) display a cleft palate phenotype. A specific deletion of the *Hand2* branchial arch-specific enhancer also leads to a hypoplastic mandible and cleft palate formation in mice. However, the underlying mechanism of *Hand2* regulation of palate development remains unknown. Here we show that *Hand2* is expressed in both the epithelium and mesenchyme of the developing palate. While mesenchymal specific inactivation of *Hand2* has no impact on palate development, epithelial specific deletion of *Hand2* creates a cleft palate phenotype. *Hand2* appears to exert distinct roles in the anterior and posterior palate. In the anterior palate of *Hand2*$^{LoxP/-}$ mice, premature death of periderm cells and a down-regulation of *Shh* are observed in the medial edge epithelium (MEE), accompanied by a decreased level of cell proliferation in the palatal mesenchyme. In the posterior palate, a lower dose of *Hand2* causes aberrant periderm cell death on the surface of the epithelium, triggering abnormal fusion between the palatal shelf and mandible and preventing palatal shelf elevation. We further demonstrate that BMP activities are essential for the expression of *Hand2* in the palate. We conclude that *Hand2* is an intrinsic regulator in the epithelium and is required for palate development.

Keywords

Hand2; cleft palate; hypomorphic; craniofacial development

Introduction

Among the most frequent congenital disorders is a cleft palate. Palate development is a multistep morphogenetic process and is controlled by a highly coordinated genetic network. It forms by union of the primary palate of the frontonasal process and a pair of the secondary

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palatal shelves. In mammals, the secondary palatal shelves first arise from the paired maxillary process and grow vertically along the developing tongue. A rapid elevation positions the palatal shelves horizontally above the tongue. Following fusion of the palatal shelves and disappearance of the midline seam, an intact palatal shelf finally forms separating the nasal cavity from the oral cavity (Ferguson, 1988). A cleft palate can result from intrinsic or extrinsic disruptions at any of these steps. Intrinsic defects of palate development include palatal shelves outgrowth deficiency, failure in elevation, and persistence of a midline seam. For example, in *Shox2*$^{-/-}$ or *Msx1*$^{-/-}$ embryos, outgrowth defects result in shortened palatal shelves that fail to make contact at the midline (Zhang et al., 2002; Yu et al., 2005; Gu et al., 2008). However, in mice lacking *Fgf10*, *Jagged2*, or *Pax9*, the palatal shelves fail to elevate due to abnormal palatal shelf-mandible/tongue fusion or internal force deficiency (Jiang et al., 1998; Peters et al., 1998; Rice et al., 2004; Alappat et al., 2005; Casey et al., 2006). The persistence of the midline seam also represents a cause of cleft palate formation, such as in *Tgfβ3* mutants (Kaartinen et al., 1995; Proetzel et al., 1995; Taya et al., 1999). Malformation of other craniofacial structures could also cause cleft palate formation. In mice lacking *Ryk*, the deformed tongue physically obstructs the palatal shelves elevation leading to cleft palate formation (Halford et al., 2000).

Hand genes encode proteins belonging to the basic helix-loop-helix (bHLH) family of transcription factors (Massari and Murre, 2000). *Hand2* is expressed in a variety of cell types during embryogenesis and has been implicated in development of a number of tissues and organs including the peripheral nervous system, limb bud, heart and pharyngeal arches (Srivastava et al., 1995; 1997; Charite et al., 2000; Morikawa et al. 2005; 2007; Barbosa et al., 2007; D'Autreaux et al., 2007). For example, in the developing heart, *Hand2* is expressed in the right ventricle, and right ventricle apoptosis is seen in mice lacking *Hand2* (Srivastava et al., 1997). During limb development, *Hand2* is expressed in the posterior limb mesenchyme, overlapping with the *Shh* expression domain. Altered *Hand2* activity leads to limb bud defects accompanied by changes in *Shh* expression, indicating that *Hand2* acts as an upstream regulator of *Shh* (Charite et al., 2000; Fernandez-Teran et al., 2000; McFadden et al., 2002). During craniofacial development, *Hand2* is expressed in the cranial neural crest (CNC) derived mesenchyme of the first and second branchial arches (Clouthier et al., 2000). Mice carrying a specific deletion of the *Hand2* branchial arch-specific enhancer exhibit severe mandibular hypoplasia, which is thought to be causative of a cleft palate defect (Yanagisawa et al., 2003). We have recently reported that mice carrying a *Hand2*-null allele and a floxed *Hand2* allele (*Hand2*$^{\mathrm{LoxP}/-}$) exhibit complete cleft of the secondary palate with a 100% penetrance, despite normal mandible formation, suggesting a direct involvement of *Hand2* in the regulation of palate development (Morikawa et al., 2007).

In the present work, we analyzed the cellular and molecular etiology of the cleft palate in *Hand2*$^{\mathrm{LoxP}/-}$ mice, and investigated its tissue specific requirement during palatogenesis. We demonstrate that *Hand2* activity is required in the palatal epithelium where it controls cell proliferation via *Shh* and *Bmp2* in the anterior palate. Intriguingly, in the posterior palate, the *Hand2* hypomorphic condition leads to abnormal fusion of the palatal shelf and mandible. Hand2 therefore regulates palate development through two distinct mechanisms.

Materials and Methods

Animals

The generation and genotyping of *Hand2* floxed, *Hand2*$^{+/-}$, and *Osr2-Cre* animals have been described previously (Srivastava et al., 1997; Lan et al., 2007; Morikawa et al., 2007). *Wnt1-Cre* transgenic mice (Danielian et al., 1998) and the *R26R* conditional reporter line (Soriano, 1999) were obtained from Jackson Laboratories (Bar Harbor, ME). To create *Pitx2-Cre* transgenic animals, the 1.3-kb mouse *Pitx2* P1 promoter and the 17-kb first branchial arch enhancer fragments (*17-P1-ΔASE*) (kindly provided by Dr. Hiroshi Hamada of Osaka

University, Japan; Shiratori et al., 2001) were linked with the *Cre* gene. The transgenic construct (*Pitx2-Cre*) was released from the vector, gel-purified, and suspended in injection buffer, as described previously (Zhang et al., 2000). To generate *pMes-Nog* transgenic mice, the coding sequence of the mouse Noggin gene was cloned into *pMES-IRES-Egfp* vector in front of the *IRES-Egfp* sequence under the control of the chick β-actin promoter. A *STOP* cassette flanked by *LoxP* sequences was inserted between the β-actin promoter and the *Noggin* sequence. The transgenic construct is named *pMes-Nog*. Pronuclear injection and embryo transfer were performed according to a published protocol (Hogan et al., 1994). The integration of *Pitx2-Cre* or *pMes-Nog* transgene was determined by polymerase chain reaction (PCR) using genomic DNA extracted from the tail tip of founders. The specificity of the *Pitx2* promoter/enhancer was determined by *LacZ* activity in mice carrying the *Pitx2-Cre* transgene and the *R26R* conditional reporter allele (Chai et al., 2000). Embryos were collected from timed pregnant mice and fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for overnight. All the genetically engineered mice used in this study were maintained in B6/C57 background. All animal studies were approved by the Tulane University Institutional Animal Care and Use Committee.

Cell proliferation and TUNEL assays

Cell proliferation rate was monitored by BrdU incorpotation. BrdU was injected into the peritoneal of timed pregnant mice for one hour at a dose of 1.5 ml of labeling reagent/100 g body weight using the BrdU labeling and Detection Kit II from Roche. Samples were fixed in Carnoy's fixative, ethanol-dehydrated, paraffin-embedded, and sectioned at 5-μm. The sections were subjected to immunodetection according to the manufacturer's protocol. Three individual embryos of both wild type and mutant were subjected for cell proliferation assay, and three adjacent sections from each sample were counted for BrdU labeling. BrdU-labeled cells were counted and presented as percentage of total nuclei within arbitrarily defined areas, and Student's *t*-test was used to determine if significance of differences and *P* values were present in the mutant samples and wild type controls. The TUNEL assay was performed to detect cell apoptosis in the developing palate in paraffin sections as described previously (Alappat et al., 2005).

In vitro palatal shelf culture

Palatal shelf culture was carried out in Trowell type organ cultures with chemically defined medium, as described previously (Taya et al., 1999; Zhang et al., 2002). Briefly, paired secondary palatal shelves were isolated from individual E13.5 embryos obtained from the mating between *Hand2*$^{\mathrm{LoxP/LoxP}}$ and *Hand2*$^{+/-}$ mice, and placed in contact with the MEE of each palatal shelf facing the other in each organ culture. Body tissues from each embryo were subsequently subjected to genotyping. Samples were cultured for 3 days prior to being harvested.

Histology, in situ hybridization, and immunohistochemical staining

For histological analysis and in situ hybridization assays, samples fixed in 4% paraformaldehyde (PFA) at 4°C for overnight, dehydrated through a graded ethanol series, and then processed for paraffin sectioning. Serial sections were made at 10-μm and subjected to standard Hematoxylin/Eosin staining or to section in situ hybridization using non-radioactive riboprobes, as described previously (St. Amand et al., 2000). For immunohistochemical staining, samples were fixed in 4% PFA, washed with 30% sucrose/PBS and embedded in O.C.T. compound (Tissue-Tek), and cryo-sectioned. Sections were washed in PBS (containing 0.1% Triton-X-100) and blocked with 10% horse serum/0.1% BSA/0.1% Triton-X-100 in PBS for 1 hour prior to being incubated with Anti-Hand2 antibody (R&D systems) at 4°C overnight. For negative controls, the primary antibody was omitted. Biotinylated Anti-Goat IgG (H+L)

(Vector Laboratories) was used as secondary antibody. After extensive washing in PBS, samples were stained with DAPI (Invitrogen), mounted with VECTASHIELD Fluorescent Mounting Media (Vector Laboratories), and the results were examined under a fluorescent microscope and digitally recorded.

Real-time PCR

Paired palatal shelves isolated from individual E13.5 wild type, *Hand2*$^{LoxP/+}$, and *Hand2*$^{LoxP/-}$ embryos were subjected to RNA extraction with RNase Mini Kit (Qiagene) and reversely transcribed using SuperScript III First Strand Synthesis System (Invitrogen). *Hand2* transcripts were quantified by real-time PCR using the primers (upper: 5′-TACCAGCTACATCGCCTACCT-3′; and lower: 5′-CAGCTTCACTCCAGGCCACT-3′). *Gapdh* was used as a reference control. Real-time PCR was performed using the iCycleriQ™ Real-time Detection System (Bio-Rad).

Results

Hypomorphic *Hand2* mice exhibit complete cleft of secondary palate

Previous studies have shown that *Hand2* is involved in craniofacial development including palatogenesis, intrinsically or extrinsically (Ruest et al., 2003; Yanagisawa et al., 2003; Barbosa et al., 2007). We previously reported that mice bearing one *Hand2* null and one hypomorphic (*Hand2*$^{LoxP/-}$) alleles display a complete cleft secondary palate with 100% penetrance (Morikawa et al., 2007). To establish a role for *Hand2* in palate development, we began with a histological analysis of palatal defects in *Hand2*$^{LoxP/-}$ mice. Morphologically, the developing palatal shelves from E11.5 and E12.5 *and2*$^{LoxP/-}$ embryos were indiscernible from the wild type controls (data not shown). At E13.5 when the normal palatal shelves take a vertical position on both sides of the tongue, the mutant palatal shelves assumed the similar position but appeared slightly smaller as compared to the littermate controls (Fig. 1A, 1B). A pronounced aberration in the mutant palate was observed at E14.5 when the wild type palatal shelves have elevated to above the tongue and begun to fuse at the midline (Fig. 1C–F). In *Hand2*$^{LoxP/-}$ embryos, the palatal shelves failed to elevate and remained in a vertical position on the sides of the hypoplastic tongue (Fig. 1D, 1F). At E15.5, the mutant palate displayed distinct developmental defects along the anterior-posterior (A–P) axis. Though delayed, the anterior palatal shelves elevated to the horizontal level, but appeared short and misaligned (Fig. 1H). However, the posterior portion of the palate did not elevate (Fig. 1J). These observations suggest that *Hand2* may exert distinct roles along the A–P axis of the developing palate during palatogenesis.

Hand2 is expressed in the developing palate

Hand2 is expressed in the neural crest derived mesenchyme of first and second pharyngeal arches (Yanagisawa et al., 2003), however, its expression in the developing palatal shelf has not been examined. The cleft palate defect seen in mice lacking *Hand2* expression in branchial arches was attributed to the formation of a severely hypoplastic mandible (Yanagisawa et al., 2003). To differentiate if *Hand2* acts intrinsically or extrinsically to regulate palate development, we examined *Hand2* expression in the developing palatal shelf from E11.5 to E13.5 by in situ hybridization. Our results showed that *Hand2* is expressed in the developing palatal shelves from E11.5 through E13.5, exhibiting differential expression in the anterior and posterior palate. In the anterior palate, *Hand2* expression was detected in the epithelium as well as in the mesenchyme in a punctuate pattern (Fig. 2A–C). At E13.5, the expression in the palatal epithelium is mainly restricted in the medial edge epithelium (MEE) (Fig. 2C). The expression in the MEE was not detectable at E14.0 when the palatal shelves elevate to the dorsum of the tongue (data not shown). In contrast, in the posterior palate, we did not detect *Hand2* expression at E11.5 and E12.5 (Fig. 2D, 2E). However, at E13.5 a restricted expression

domain was observed in the epithelial cells of the lateral junction of the palatal shelf and mandible (Fig. 2F). These observations strongly suggest that *Hand2* plays a direct role in regulating palate development.

We previously demonstrated that the *LoxP* site insertion does not alter *Hand2* expression pattern in developing embryos including the craniofacial region but creates a hypomorphic allele (Morikawa et al., 2007). To confirm that the insertion of *LoxP* sites reduces *Hand2* expression in the developing palate, we performed quantitative RT-PCR to determine *Hand2* expression levels in the palatal shelves of E13.5 wild type, $Hand2^{LoxP/+}$, and $Hand2^{LoxP/-}$ embryos. *Hand2* expression levels were reduced by 16% (±6%) in the $Hand2^{LoxP/+}$ palatal shelves, and 66% (±6%) in the $Hand2^{LoxP/-}$ palates, as compared to wild type controls (data not shown). To further confirm a reduced Hand2 protein level in $Hand2^{LoxP/-}$ embryo, we performed immunohistochemical studies. We examined E13.5 mandible due to the known high expression of *Hand2* (Yanagisawa et al., 2003). The immunostaining results demonstrate a significant reduction of Hand2 protein in $Hand2^{LoxP/-}$ mandible as compared to that in $Hand2^{Loxp/+}$ sample (Fig. 3).

Hand2 function is required in the palatal epithelium

Since *Hand2* is expressed in both the epithelial and mesenchymal components of the developing palate, we determined the tissue requirement for *Hand2* during palate development. We took a tissue-specific gene deletion approach to inactivate *Hand2*. The palatal mesenchyme derives from CNC cells (Ito et al., 2003). We inactivated *Hand2* in the CNC cell-derived palatal mesenchyme using the *Wnt1-Cre* transgenic line. Although a hypoplastic mandible was observed in *Wnt1-Cre;*$Hand2^{LoxP/LoxP}$ mice, a cleft palate defect was not found (Fig. 4D). To confirm this observation, we used another Cre deleter mouse line, *Osr2-Cre*, which expresses Cre in the palatal mesenchyme from the beginning of palatogenesis (Lan et al., 2007). Mice carrying $Hand2^{LoxP/LoxP}$ and *Osr2-Cre* transgenic allele had a normal palate (Fig. 4F). We therefore conclude that *Hand2* expression in the palatal mesenchyme is dispensable for palate development. To determine the requirement of *Hand2* in the palatal epithelium, we generated a *Pitx2-Cre* transgenic line using the 1.3-kb mouse *Pitx2* P1 promoter and the 17-kb first branchial arch enhancer fragments (Shiratori et al., 2001) to drive *Cre* expression. *Pitx2-Cre* mice exhibit Cre activities in the palatal epithelium and dental epithelium at E11.5 and entire palatal epithelium at E13.5 (Fig. 4B, 4C). Upon inactivation of *Hand2* in the palatal epithelium, a wide open cleft palate defect was observed in *Pitx2-Cre;*$Hand2^{LoxP/LoxP}$ mice (Fig. 4E). It has been previously reported that Cre activity alone could cause dramatic developmental defects in certain transgenic lines, including up-regulated apoptosis in many embryonic tissues (Naiche and Papaioannou, 2007). None of the Cre expressing lines used in this study showed a cleft palate defect. We examined if Cre activity alone would cause any alternations in cell proliferation, apoptosis, and gene expression in the developing palate. In the mesenchyme of E13.5 *Wnt1-Cre* palatal shelves, we did not observe changes in cell proliferation or the gene expression changes including *Bmp2, Fgf10*, and *Sox9* (data not shown). We also did not observe aberrant apoptosis in the epithelium of E13.5 *Pitx2-Cre* palate (data not shown). We thus conclude that Cre activity does not exert detrimental effects to the developing palate and Hand2 is an essential factor required in the epithelial component to regulate palate development.

Hand2 functions through Shh signaling to regulate palate development

The fact that *Hand2* is expressed and required in the palatal epithelium prompted us to test if the reduced *Hand2* expression in the $Hand2^{LoxP/-}$ palatal shelves could cause a failure in palate fusion. We placed paired palatal shelves isolated from E13.5 $Hand2^{LoxP/+}$ and $Hand2^{LoxP/-}$ embryos in an in vitro organ culture with the MEE of the shelves in contact. Histological analyses of cultured samples demonstrated successful fusion of palatal shelves from either $Hand2^{LoxP/+}$ (10/12) or $Hand2^{LoxP/-}$ (10/13) mice, as determined by the disappearance of the

midline seam and the establishment of the mesenchymal continuity (data not shown). We therefore rule out the possibility that failure of fusion contributes to cleft palate defect in *Hand2*$^{LoxP/-}$ mice.

Shh signaling has been implicated in palatogenesis (Zhang et al., 2002; Rice et al., 2004; Gritli-Linde, 2007). In the anterior portion of developing palate, *Shh* is expressed in the MEE, overlapping with *Hand2* and acts to regulate palatal growth via Bmp2 (Zhang et al., 2002). Since *Hand2* is both sufficient and necessary for *Shh* expression in the developing limb (Charite et al., 2000; Fernandez-Teran et al., 2000; McFadden et al., 2002), we determined if *Hand2* also functions through the Shh signaling pathway to regulate palate development. Our in situ hybridization studies revealed a down-regulation, but not complete elimination, of *Shh* expression in the MEE of the anterior palate in *Hand2*$^{LoxP/-}$ mice (Fig. 5A, 5B). Interestingly, *Shh* expression in the future regal epithelium was not altered in the mutant embryos, indicating a specific regulation of *Shh* by Hand2 in the MEE. Consistent with the down-regulation of *Shh* in the MEE, the *Shh* downstream targets *Ptc* and *Bmp2* also exhibited attenuated expression in the palatal mesenchyme adjacent to the MEE (Fig. 5C–F).

The expression of *Shh* in the MEE of developing palatal shelves is known to be controlled by palatal mesenchyme expressed *Bmp4* and *Fgf10* (Zhang et al., 2002; Rice et al., 2004). To ensure that the down-regulation of *Shh* in the MEE of *Hand2*$^{Loxp/-}$ palate is a direct consequence of reduced Hand2 activity in the MEE, we examined the expression of *Bmp4* and *Fgf10* in E13.5 *Hand2*$^{Loxp/-}$ palatal shelves. The expression of these two genes is unaltered in the *Hand2*$^{LoxP/-}$ palate (data not shown), indicating that Hand2 functions as an upstream regulator of *Shh* in the MEE of developing palatal shelves.

Shh signaling has been demonstrated to regulate cell proliferation in the palatal mesenchyme via *Bmp2* (Zhang et al., 2002). Consistent with a down-regulation of *Shh* and *Bmp2*, cell proliferation level in the palatal mesenchyme of E13.5 *Hand2*$^{LoxP/-}$ mice was significantly reduced as compared to the wild type controls (Fig. 6C–E, $P<0.01$). However, cell proliferation rate was not changed in the palate of E12.5 *Hand2*$^{LoxP/-}$ embryo, consistent with an unaltered morphology at this developmental stage (data not shown). Since programmed cell death is thought to attribute to the cellular defects in the developing heart and branchial arch of *Hand2* null embryos, we examined the levels of apoptosis using TUNEL assays. Excess apoptotic cells in the MEE were found in the *Hand2*$^{LoxP/-}$ palate (Fig. 6A and B). A close examination revealed that apoptosis was restricted to the periderm cells, the surface layer of the epithelium, but not in the basal layer epithelial cells (Fig. 6B). Thus in the anterior palate, altered cell proliferation and apoptosis represent major cellular defects contributing to cleft palate formation in *Hand2*$^{LoxP/-}$ mice.

Abnormal fusion between lateral palatal shelf and mandible leads to failed elevation of the posterior palate in *Hand2* $^{LoxP/-}$ mice

In *Hand2*$^{LoxP/-}$ mice, the anterior palate was delayed in elevation, but the posterior palate never elevated (Fig. 1). These observations indicate distinct roles for Hand2 in regulating palatogenesis along the A–P axis. Since *Hand2* expression is restricted in the epithelial cells of the lateral junction of the palatal shelf and mandible at E13.5, a defect could occur in this specific region. An examination of serial histological sections revealed an abnormal adhesion/fusion of the posterior palate and mandible (Fig. 7A–F). The epithelia of the lateral junction of palatal shelf and mandible became adherent initially, followed by the disappearance of the adhesive epithelia, causing pathological fusion of the palatal shelf and mandible. We examined cell apoptosis in this specific fusion region in E13.5 embryo by TUNEL assay. As expected, at the lateral junction of the posterior palatal shelf and mandible of E13.5 *Hand2*$^{LoxP/-}$ embryos, apoptotic cells were found in the epithelia of the fusion region (Fig. 7G–I). Surprisingly, we identified a unique cell apoptotic pattern. Before the epithelial contact occurs

in the lateral region, apoptosis was restricted to the periderm cells, similar to what was observed in the MEE of the anterior palate (Fig. 7H). However, when the epithelia made contact, apoptosis was observed in the basal layer epithelial cells (Fig. 7I), leading to a fusion between the palatal shelf and mandible. This aberrant fusion apparently results in hindrance of the palatal shelf elevation and thus contributes to the cleft palate formation in *Hand2*$^{LoxP/-}$ mice. Abnormal palate-mandible fusion has been reported in *Jagged2*$^{-/-}$ and *Fgf10*$^{-/-}$ mice (Jiang et al., 1998;Alappat et al., 2005;Casey et al., 2006). In *Fgf10*$^{-/-}$ palate, altered expression of *Tgfβ3* and *Jagged2* was thought to contribute to the aberrant fusion phenotype (Alappat et al., 2005). We thus asked if similar molecular defects could exist in *Hand2*$^{LoxP/-}$ palate. In situ hybridization studies revealed an unaltered expression of *Tgfβ3* and *Jagged2* in the epithelia of the lateral palate shelf and mandible junction in *Hand2*$^{LoxP/-}$ embryos (data not shown), suggesting involvement of a different genetic pathway.

BMP activity is required for *Hand2* expression in the developing palate

Our previous studies demonstrated a regulation of *Shh* in the MEE by *Bmp4* in the anterior palate (Zhang et al., 2002). Since *Hand2* regulates *Shh* in the MEE, we examined if *Hand2* acts as a component in the *Bmp4-Shh* genetic pathway. To test this hypothesis, we took a loss-of-function approach by creating a conditional transgenic model which, upon crossing to a *Cre* line, expresses *Noggin* under the control of the chick β-actin promoter. The transgenic construct, named *pMes-Nog*, also contains an *IRES-Egfp* cassette following the *Noggin* transgene, to allow simultaneous expression of *Egfp* as a marker for the transgene expression. To ectopically express *Noggin* in the palatal mesenchyme, we generated binary transgenic embryos by compounding the *pMes-Nog* transgenic allele with *Osr2-Cre* allele. *Egfp* expression in the developing limbs and craniofacial region indicated successful activation and correct expression pattern of the transgene (Fig. 8A, 8B; Lan et al., 2007). In the developing palatal shelf, *Noggin* is not expressed in most regions of the palate with the exception of a small region in the anterior extremity (Fig. 8C, and unpublished data). In the palatal shelves of *Osr2-Cre;pMes-Nog* mice, abundant *Noggin* transcripts were detected (Fig. 8D). Overexpression of *Noggin* to the palatal mesenchyme caused retarded palatal growth (Fig. 8F) and eventually led to a complete clefting of the secondary palate (Xiong, W. and Chen, Y.P., unpublished observations). In *Osr2-Cre;pMes-Nog* mice, we observed a greatly reduced expression of *Hand2* not only in the MEE but also in the mesenchyme of the anterior palate (Fig. 8E, 8F). Associated with this change in *Hand2* expression is a down-regulation of *Shh* in the MEE (Fig. 8I, 8J). However, a down-regulation of *Fgf10* expression in the *Osr2-Cre;pMes-Nog* palate may also contribute, at least partially, to this down-regulation of *Shh* in the MEE (data not shown). In addition, we also observed a repression of *Hand2* expression in the epithelial cells of the lateral junction of the posterior palatal shelf and mandible (Fig. 8G, 8H). Consistent with this repressed *Hand2* expression is an aberrant fusion of posterior palate and mandible, identical to that observed in *Hand2*$^{LoxP/-}$ embryo (Xiong, W. and Chen, Y.P., unpublished results). BMP activities appear necessary for *Hand2* expression in the developing palate.

Discussion

In this study we investigated the cellular and molecular etiology of cleft palate in *Hand2* hypomorphic mice. Our results reveal an indispensable role for *Hand2* during palate development. Down-regulation of this transcription factor results in a number of cellular and molecular defects, including reduced cell proliferation and an aberrant fusion of the palatal shelf with mandible, which contribute to the formation of a cleft palate.

Epithelial expressed *Hand2* is essential component regulating palatogenesis

During embryonic development, *Hand2* is expressed in the developing pharyngeal arch and other organs (Srivastava et al., 1995; 1997; Charite et al., 2000; Clouthier, 2000; Fernandez-

Teran et al., 2000; Yelon et al., 2000; McFadden et al., 2002; Miller et al., 2003; Barbosa et al., 2007). While *Hand2* has been shown to play a critical role primarily in the mesenchymal component of a number of organs during the development, our work now demonstrates a role for *Hand2* in the epithelium component during organogenesis. A role for *Hand2* in palatogenesis was suggested from the observations that *Hand2* is strongly expressed in the mandibular mesenchyme and a specific deletion of the *Hand2* branchial arch-specific enhancer leads to mandibular hypoplasia and a cleft palate defect (Yanagisawa et al., 2003). However, the cleft palate phenotype in the mutant mice was considered as a secondary defect to the malformed mandible. In addition, the relatively weak, restricted and dynamic expression of *Hand2* in the developing palate may have been overlooked due to its strong expression in the tongue and mandible. In this report, we show unambiguous *Hand2* expression in both epithelial and mesenchymal components of the developing palate. In addition, our real-time PCR results further confirm the expression of *Hand2* the developing palatal shelves, and a reduction in *Hand2* expression in the palatal shelves of *Hand2* hipomorphic mice. By using a conditional inactivation approach, it is evident that mesenchymally expressed *Hand2* is dispensable during palate development. One possibility is that *Hand1*, another bHLH family member related to *Hand2*, is also expressed in the neural crest-derived mesenchyme and may functions redundantly with *Hand2* to regulate palate development. In contrast, specific deletion of *Hand2* in the palatal epithelium phenocopies the cleft palate defect seen in *Hand2* hypomorphic mice. Although we cannot exclude the possibility that mandibular defects may contribute to a cleft palate formation in certain circumstance, the fact that a cleft palate defect is not associated with mandibular hypoplasia in *Wnt1-Cre;Hand2*$^{\mathrm{LoxP/LoxP}}$ mice further support a direct role for *Hand2* in palate development.

Hand2 acts downstream of BMP signaling to regulate *Shh* expression in the MEE

In developing limbs, *Hand2* has been shown to regulate *Shh* expression in the ZPA (Charite et al., 2000; Fernandez-Teran et al., 2000). Although the underlying regulatory mechanisms remain to be determined, our results demonstrate the existence of similar genetic regulatory hierarchy in the developing palate. In *Hand2* hypomorphic mice, the level of *Shh* expression in the MEE is significantly reduced but not completely abolished, as are the *Shh* downstream targets *Ptc* and *Bmp2*. The unaltered expression of *Bmp4* and *Fgf10* in *Hand2*$^{\mathrm{LoxP/-}}$ palate further supports a role for *Hand2* in the regulation of *Shh* expression. It is possible that *Hand2* does not regulate *Shh* expression directly in the MEE. The excess periderm cell death in the MEE of *Hand2*$^{\mathrm{LoxP/-}}$ palate may impair the MEE leading to a down-regulation of *Shh* in the MEE. Arguing against this, expression of *Jagged2* in the *Hand2*$^{\mathrm{LoxP/-}}$ palatal epithelium including the MEE was not affected, suggesting a normal epithelial structure. Although Shh has been shown to stimulate proliferation in many vertebrate organs, its mitotic effect is mediated by Bmp2 in the developing palate (Zhang et al., 2002). Therefore, the down-regulation of *Bmp2* expression accounts for the reduced cell proliferation rate in the palatal mesenchyme of *Hand2* hypomorphic mice. This reduced cell proliferation rate causes retarded growth of the palate, and eventually contributes to, at least partially, the formation of cleft palate in *Hand2* hypomorphic mice.

Shh expression in the MEE is controlled by multiple signaling pathways, including BMP and FGF signaling (Zhang et al., 2002; Rice et al., 2004; reviewed in Grtili-Linde, 2007). As a transcriptional factor, Hand2 acts upstream of *Shh*, possibly directly regulating its expression. As demonstrated in our transgenic model in which Noggin, an antagonist of BMPs, is ectopically expressed in the palatal mesenchyme, *Hand2* expression is inhibited, as is *Shh*, in the anterior palatal shelf. Interestingly, *Hand2* expression is repressed in both the palatal epithelium and mesenhcyme, suggesting a similar regulatory mechanism in both tissue components. In addition, overexpression of *Noggin* also inhibits *Hand2* expression in the epithelial junction of the posterior palate and mandible, leading to an abnormal fusion of palate

and mandible identical to that seen in *Hand2* hypomorphic embryos (unpublished data). The down-regulation of *Fgf10* in the *Osr2-Cre;pMes-Nog* palate also suggests that *Fgf10* may act as an intermediate between BMP action and *Hand2* expression. Nevertheless, our results demonstrate a requirement of BMP activities for *Hand2* expression in the developing palate. This regulatory pathway appears to be common during organogenesis, operating in several other developing systems, such as the heart and sympathetic neurons (Howard et al., 2000; Schlange et al., 2000; Liu et al., 2004).

Hand2 regulates epithelial adhesion/fusion by controlling cell apoptosis

It is noteworthy that ectopic cell death is always associated with *Hand2* deficiency (Srivastava et al., 1997; Thomas et al., 1998; Yamagishi et al., 2001). This is also true in the developing palate when *Hand2* levels fall below a threshold. We observed aberrant cell apoptosis in the MEE of the anterior palate and the epithelia of the lateral junction of the palatal shelf and mandible of *Hand2* hypomorphic mice. Most interestingly, we observed two distinct steps of cell death in the epithelial junction of the palatal shelf and mandible: apoptosis of periderm cells before epithelial contact, and death of basal layer epithelial cells after epithelial contact/adhesion to create mesenchyme confluence, leading to fusion of the palatal shelf with the mandible. During normal palate fusion process, the periderm cell are shed before contact of the palatal shelves (Fitchett and Hay, 1989). This removal of periderm cells is thought to be essential for normal palate fusion, as artificial removal of periderm cells leads to degradation of underlying basal layer cells in MEE, a key step of palate fusion, and inhibition of cell death results in persistence of the medial edge seam (Cuervo and Covarrubias, 2004). It appears that similar to palate fusion, removal of periderm cells from surface of the palatal epithelium and oral epithelium allows abnormal adherence of the basal layer epithelial cells, and subsequently fusion of the palatal shelf and mandible.

Among various causes of cleft palate is pathological palate-mandible or palate-tongue fusion (Gritli-Linde, 2007). Such aberrant fusions have been reported in mice carrying mutation in either *Jagged2*, *Fgf10*, or *Irf6*, causing cleft palate defect (Jiang et al., 1998; Rice et al., 2004; Alappat et al., 2005; Ingraham et al., 2006; Casey et al., 2006; Richardson et al., 2006). The delayed palate elevation in the anterior palate and failed elevation in the posterior palate of *Hand2* hypomorphic mice further supports the notion that the abnormal palate-mandible fusion represents a major cause of cleft palate formation. It was previously shown that *Jagged2* expression is required in the epithelium of oral cavity to prevent abnormal epithelial adhesion, while *Tgfβ3* is needed for cell apoptosis in the MEE for palate fusion (Taya et al., 1999; Casey et al., 2006). In *Fgf10* mutant palate, altered expression of *Jagged2* and *Tgfβ3* is associated with the abnormal palate-mandible fusion (Alappat et al., 2005). However, we did not detect a change in the expression of either gene in the adhesion/fusion region of the *Hand2* hypomorphic palate, suggesting that *Hand2* could either regulate a distinct pathway or act downstream of these two genes in the regulation of epithelial adhesion/fusion during palate development.

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References

Alappat SR, Zhang Z, Suzuki K, Zhang X, Liu H, Jiang R, Yamada G, Chen YP. The cellular and molecular etiology of the cleft secondary palate in *Fgf10* mutant mice. Dev Biol 2005;277:102–113. [PubMed: 15572143]

Barbosa AC, Funato N, Chapman S, McKee MD, Richardson JA, Olson EN, Yanagisawa H. Hand transcription factors cooperatively regulate development of the distal midline mesenchyme. Dev Biol 2007;310:154–168. [PubMed: 17764670]

Casey LM, Yu L, Eui-Sic C, Maltby KM, Thomas G, Jiang R. Jag2-Notch1 signaling regulates oral epithelial differentiation and palate development. Dev Dyn 2006;235:1830–1844. [PubMed: 16607638]

Chai Y, Jiang X, Ito Y, Bringas P, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 2000;127:1671–1679. [PubMed: 10725243]

Charite J, McFadden DG, Olson EN. The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. Development 2000;127:2461–2470. [PubMed: 10804186]

Clouthier DE. Signaling pathways crucial for craniofacial development revealed by endothelin-A receptor-deficient mice. Dev Biol 2000;217:10–24. [PubMed: 10625532]

Clouthier DE, Williams SC, Yanagisawa H, Wieduwilt M, Richardson JA, Yanagisawa M. Signaling Pathways Crucial for Craniofacial Development Revealed by Endothelin-A Receptor-Deficient Mice. Dev Biol 2000;217:10–24. [PubMed: 10625532]

Cuervo R, Covarrubias L. Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis. Development 2004;131:15–24. [PubMed: 14645125]

D'Autreaux F, Morikawa Y, Cserjesi P, Gershon MD. Hand2 is necessary for terminal differentiation of enteric neurons from crest-derived precursors but not for their migration into the gut or for formation of glia. Development 2007;134:2237–2249. [PubMed: 17507395]

Danielian PS, Puccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol 1998;8:1323–1326. [PubMed: 9843687]

Ferguson MWJ. Palate development. Development 1988;103:41–60. [PubMed: 3074914]

Fernandez-Teran M, Piedra ME, Kathiriya IS, Srivastava D, Rodriguez-Rey JC, Ros MA. Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the Sonic hedgehog pathway. Development 2000;127:2133–2142. [PubMed: 10769237]

Fitchett JE, Hay ED. Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. Dev Biol 1989;131:455–474. [PubMed: 2463946]

Gritli-Linde A. Molecular control of secondary palate development. Dev Biol 2007;301:309–326. [PubMed: 16942766]

Gu S, Wei N, Yu XY, Jiang Y, Fei J, Chen YP. Mice with an anterior cleft of the palate survive neonatal lethality. Dev Dyn 2008;237:1509–1516. [PubMed: 18393307]

Halford MM, Armes J, Buchert M, Meskenaite V, Gail D, Hibbs ML, Wilks AF, Farlie PG, Newgreen DF, Hovens CM, Stacker SA. Ryk-deficient mice exhibit craniofacial defects associated with perturbed Eph receptor crosstalk. Nat Genet 2000;25:414–418. [PubMed: 10932185]

Hogan, B.; Beddington, R.; Costantini, F.; Lacy, E. Manipulating the mouse embryo: A laboratory manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1994.

Howard MJ, Stanke M, Schneider C, Wu X, Rohrer H. The transcription factor dHAND is a downstream effector of BMPs in sympathetic neuron specification. Development 2000;127:4073–4081. [PubMed: 10952904]

Ingraham CR, Kinoshita A, Kondo S, Yang B, Sajan S, Trout KJ, Malik MI, Dunnwald M, Goudy SL, Lovett M, et al. Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). Nat Genet 2006;38:1335–1340. [PubMed: 17041601]

Ito Y, Yeo JY, Chytil A, Han J, Bringas P Jr, Nakajima A, Shuler CF, Moses HL, Chai Y. Conditional inactivation of Tgfr2 in cranial neurla crest causes cleft palate and calvaria defects. Development 2003;130:5269–5280. [PubMed: 12975342]

Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze DV, Weinmaster G, Gridley T. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. Genes Dev 1998;12:1046–1057. [PubMed: 9531541]

Kaartinen V, Volcken JK, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J. Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial-mesenchymal interactions. Nat Genet 1995;11:415–421. [PubMed: 7493022]

Lan Y, Wang Q, Ovitt CE, Jiang R. A unique mouse strain expressing Cre recombinase for tissue-specific analysis of gene function in palate and kidney development. Genesis 2007;45:618–624. [PubMed: 17941042]

Liu W, Selever J, Wang D, Lu MF, Moses KA, Schwartz RJ, Martin JF. Bmp4 signaling is required for outflow-tract septation and branchial-arch artery remodeling. Proc Natl Acad Sci USA 2004;101:4489–4494. [PubMed: 15070745]

Massari ME, Murre C. Helix-Loop-Helix Proteins: Regulators of Transcription in Eucaryotic Organisms. Mol Cell Biol 2000;20:429–440. [PubMed: 10611221]

McFadden DG, McAnally J, Richardson JA, Charite J, Olson EN. Misexpression of dHAND induces ectopic digits in the developing limb bud in the absence of direct DNA binding. Development 2002;129:3077–3088. [PubMed: 12070084]

Miller CT, Yelon D, Stainier DYR, Kimmel CB. Two endothelin 1 effectors, hand2 and bapx1, pattern ventral pharyngeal cartilage and the jaw joint. Development 2003;130:1353–1365. [PubMed: 12588851]

Morikawa Y, Dai YS, Hao J, Bonin C, Hwang S, Cserjesi P. The basic helix-loop-helix factor Hand2 regulates autonomic nervous system development. Dev Dyn 2005;234:613–621. [PubMed: 16145670]

Morikawa Y, D'Autreaux F, Gershon MD, Cserjesi P. Hand2 determines the noradrenergic phenotype in the mouse sympathetic nervous system. Dev Biol 2007;307:114–126. [PubMed: 17531968]

Naiche LA, Papaioannou VE. Cre activity causes widespread apoptosis and lethal anemia during embryonic development. Genesis 2007;45:768–775. [PubMed: 18064676]

Peters H, Neubuser A, Kratochwil K, Balling R. *Pax9*-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev 1998;12:2735–2747. [PubMed: 9732271]

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MWJ, Doetschman T. Transforming growth factor-β3 is required for secondary palate fusion. Nat Genet 1995;11:409–414. [PubMed: 7493021]

Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DPC. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions cause cleft palate. J Clin Inves 2004;113:1692–1770.

Richardson RJ, Dixon J, Malhotra S, Hardman MJ, Knowles L, Boot-Handford RP, Shore P, Whitmarsh A, Dixon MJ. Irf6 is a key determinant of the keratinocyte proliferation-differentiation switch. Nat Genet 2006;38:1329–1334. [PubMed: 17041603]

Ruest LB, Dager M, Yanagisawa H, Charite J, Hammer RE, Olson EN, Yanagisawa M, Clouthier DE. Dhand-cre transgenic mice reveal specific potential functions of dHAND during craniofacial development. Dev Biol 2003;257:263–277. [PubMed: 12729557]

Schlange T, Andree B, Arnold HH, Brand T. BMP2 is required for early heart development during a distinct time period. Mech Dev 2000;91:259–270. [PubMed: 10704850]

Shiratori H, Sakuma R, Watanabe M, Hashiguchi H, Mochida K, Sakai Y, Nishno J, Saijoh Y, Whitman M, Hamada H. Two-step regulation of left-right asymmetric expression of Pitx2: Initiation by Nodal signaling and maintenance by Nkx2. Mol Cell 2001;7:137–149. [PubMed: 11172719]

Soriano P. Generalized LacZ expression with the ROSA26 Cre reporter strain. Nat Genet 1999;21:70–71. [PubMed: 9916792]

Srivastava D, Cserjesi P, Olson EN. A subclass of bHLH proteins required for cardiac morphogenesis. Science 1995;270:1995–1999. [PubMed: 8533092]

Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet 1997;16:154–160. [PubMed: 9171826]

St Amand TR, Zhang Y, Semina EV, Zhang X, Hu Y, Nguyen L, Murray JC, Chen YP. Antagonistic signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. Dev Biol 2000;217:323–332. [PubMed: 10625557]

Taya Y, O'Kane S, Ferguson MWJ. Pathogenesis of cleft palate in TGF-β3 knockout mice. Development 1999;126:3869–3870. [PubMed: 10433915]

Thomas T, Kurihara H, Yamagishi H, Kurihara Y, Yazaki Y, Olson EN, Srivastava D. A signaling cascade involving endothelin-1, dHAND and msx1 regulates development of neural-crest-derived branchial arch mesenchyme. Development 1998;125:3005–3014. [PubMed: 9671575]

Yamagishi H, Yamagishi C, Nakagawa O, Harvey RP, Olson EN, Srivastava D. The Combinatorial Activities of Nkx2.5 and dHAND Are Essential for Cardiac Ventricle Formation. Dev Biol 2001;239:190–203. [PubMed: 11784028]

Yanagisawa H, Clouthier DE, Richardson JA, Charite J, Olson EN. Targeted deletion of a branchial arch-specific enhancer reveals a role of dHAND in craniofacial development. Development 2003;130:1069–1078. [PubMed: 12571099]

Yelon D, Ticho B, Halpern ME, Ruvinsky I, Ho RK, Silver LM, Stainier DY. The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development 2000;127:2573–2582. [PubMed: 10821756]

Yu L, Gu S, Alappat S, Song Y, Yan M, Zhang X, Zhang G, Jiang Y, Zhang Z, Zhang Y, Chen YP. *Shox2*-deficient mice exhibit a rare type of incomplete clefting of the secondary palate. Development 2005;132:4397–4406. [PubMed: 16141225]

Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen YP. Rescue of cleft palate in *Msx1*-deficient mice by transgenic *Bmp4* reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 2002;129:4135–4146. [PubMed: 12163415]

Zhang Z, Yu X, Zhang Y, Geronimo B, Lovlie A, Fromm SH, Chen YP. Targeted misexpression of constitutively active BMP receptor-IB causes bifurcation, duplication, and posterior transformation of digit in mouse limb. Dev Biol 2000;220:154–167. [PubMed: 10753507]

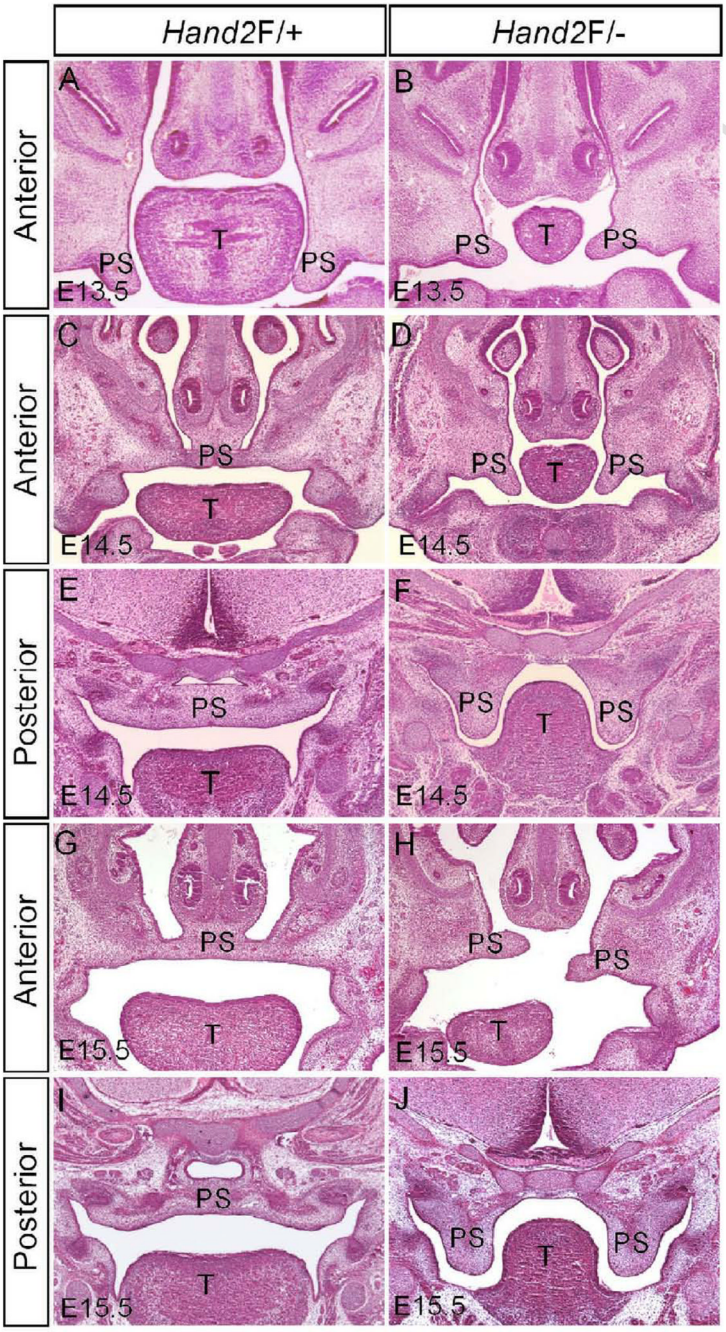

Figure 1.

Hand2$^{LoxP/-}$ mice exhibit hindrance of palate shelf elevation. (A) A section of an E13.5 wild type embryo shows normal developing palatal shelves. (B) The palatal shelves of an E13.5 *Hand2*$^{LoxP/-}$ embryo appear relatively small. (C–F) At E14.5, palatal shelves in the wild type control have already elevated to the position above the tongue and fused at the midline (C, E). However, in *Hand2*$^{LoxP/-}$ mice, the palatal shelves remain in a vertical position (D, F). (G–J) Wild type controls at E15.5 are shown in (G) and (I). In the mutant, the anterior portion of the palatal shelves elevates to the horizontal position above the tongue, but exhibits a misalignment (H), while the posterior portion remains in the vertical position (J). T, tongue; PS, palatal shelf.

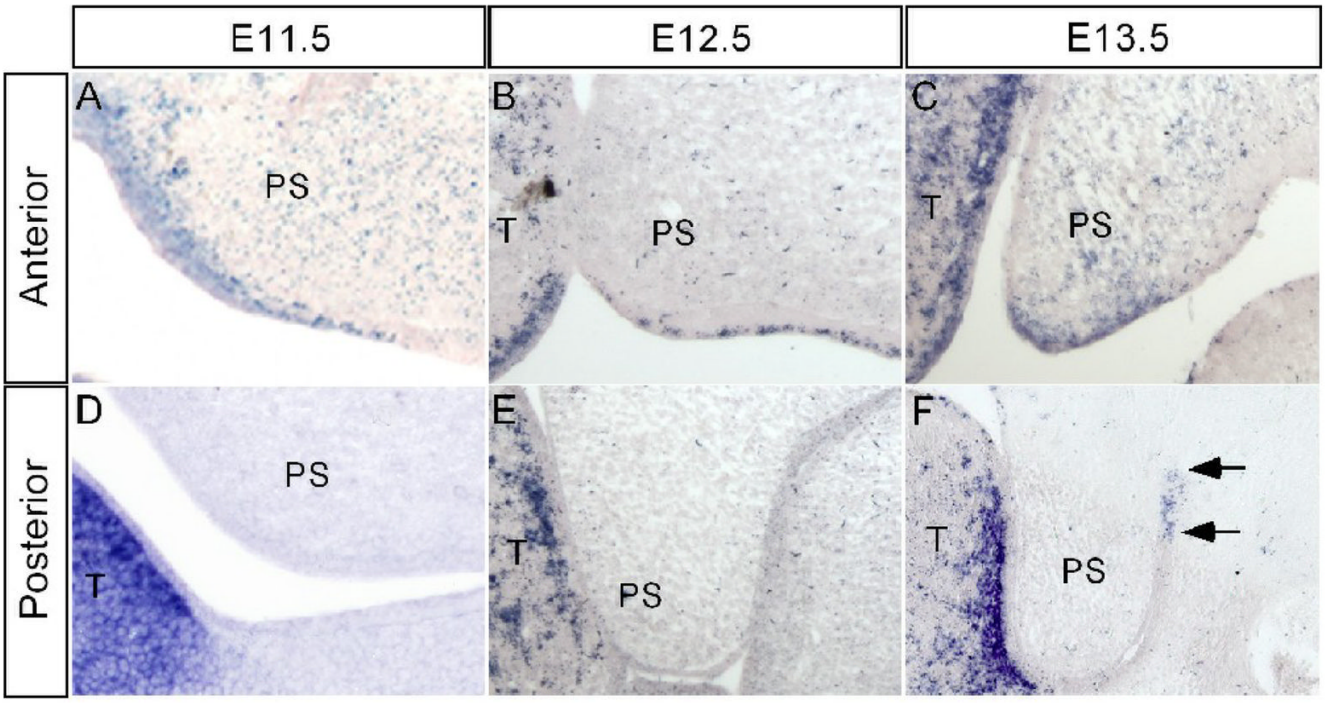

Figure 2.

Expression of *Hand2* in the early developing palatal shelf. (A–C) In the anterior palatal shelf, *Hand2* is expressed in the epithelium including MEE and mesenchyme at E11.5 (A), E12.5 (B) and E13.5 (C). (D–F) In the posterior palatal shelf, *Hand2* expression is not detectable at E11.5 (D) and E12.5 (E). However, at E13.5, *Hand2* transcripts (arrows) are specifically detected in the epithelium of the lateral junction of the palatal shelf and mandible (F). T, tongue; PS, palatal shelf.

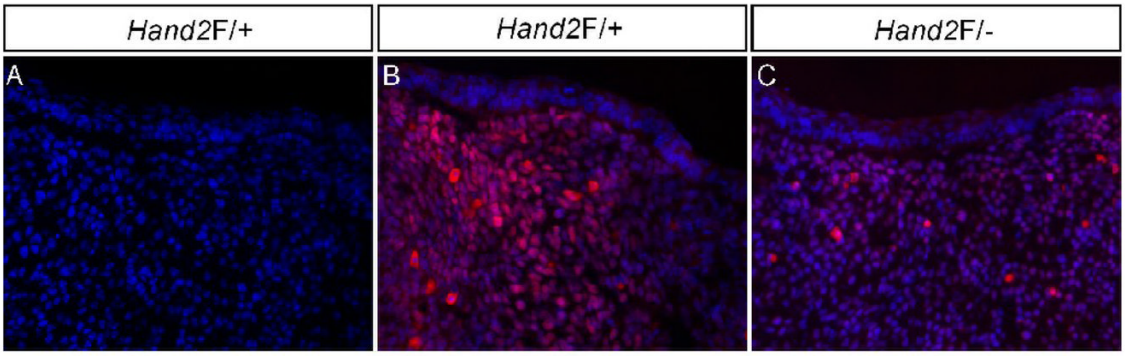

Figure 3.

Reduction of Hand2 protein in *Hand2*$^{LoxP/-}$ mandible. (A) A negative control for immunohistochemical staining. (B) Immunohistochemical staining shows abundant Hand2-positve cells in an E13.5 *Hand2*$^{F/+}$ mandible. (C) An E13.5 *Hand2*$^{F/-}$ mandible exhibits significantly reduced immunostaining signals for Hand2 protein in the mesenchymal cells.

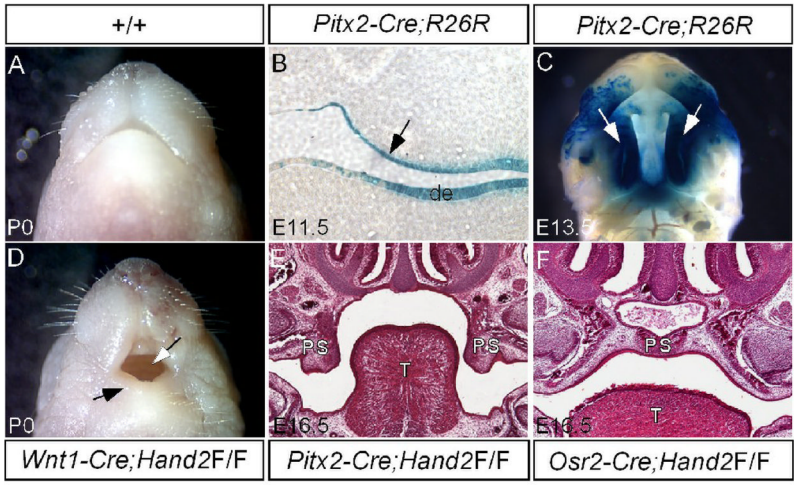

Figure 4.

Epithelial specific inactivation of *Hand2* leads to cleft palate formation. (A) A newborn wild type mouse shows a normal mandible. (B) A section through oral cavity of an E11.5 *Pitx2-Cre;R26R* embryo shows specific LacZ staining in the palatal epithelium (arrow) and dental epithelium (de). (C) Whole mount LacZ staining of an E13.5 *Pitx2-Cre;R26R* embryo demonstrates *Cre* activity in the entire palatal shelf (arrows). (D) A newborn *Wnt1-Cre;Hand2*$^{LoxP/LoxP}$ mouse (labeled as *Wnt1-Cre;Hand2*F/F) exhibits a hypoplastic mandible (black arrow) and an intact palate (white arrow). (E) A section through oral cavity of an E16.5 *Pitx2-Cre;Hand2*$^{LoxP/LoxP}$ embryo (labeled as *Pitx2-Cre;Hand2*F/F) shows cleft palate defect. Note the palatal shelves remain in a vertical position. (F) A section through oral cavity of an E16.5 *Osr2-Cre;Hand2*$^{LoxP/LoxP}$ embryo (labeled as *Osr2-Cre;Hand2*F/F) shows a normal formed palate. T, tongue; de, dental epithelium; PS, palatal shelf.

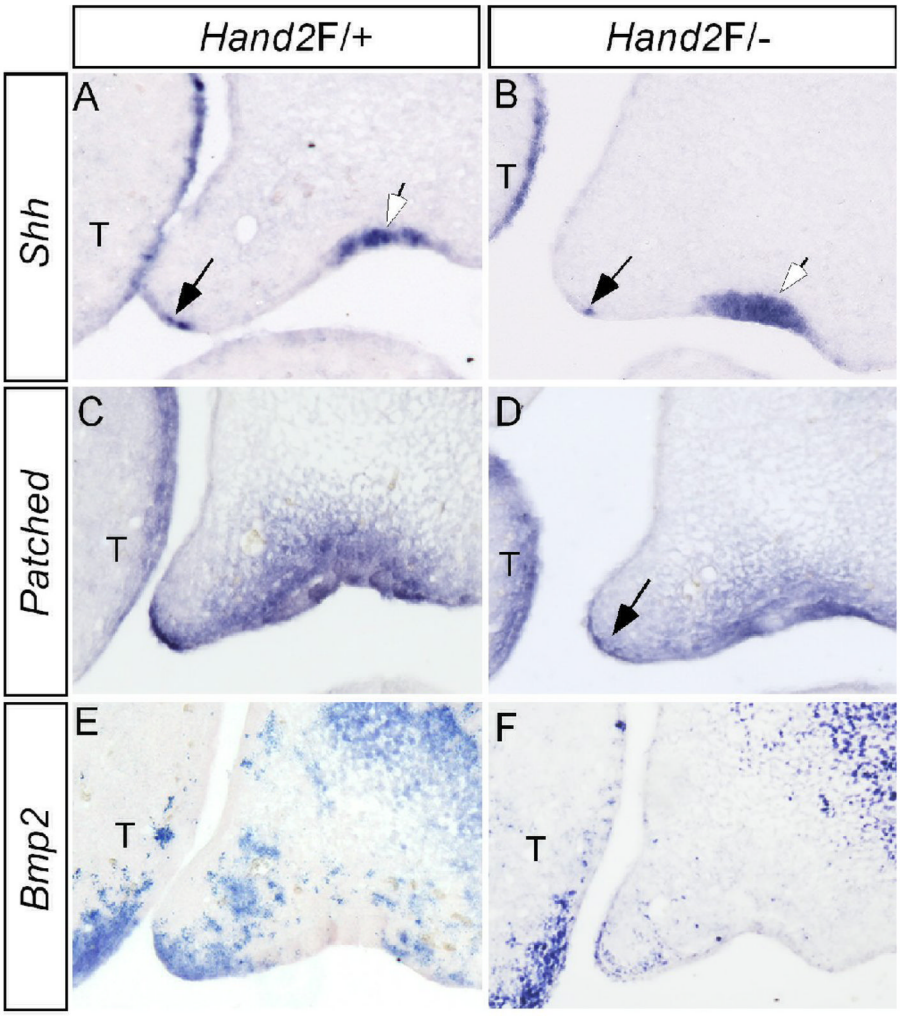

Figure 5.

Down-regulation of *Shh* and its downstream genes in the palatal shelf of *Hand2* hypomorphic mice. (A) Expression of *Shh* is detected in the MEE (black arrow) and the rugal epithelium (white arrow) of an E13.5 control palate. (B) *Shh* expression is significantly down-regulated in the MEE (black arrow), but remains unaffected in the rugal epithelium (white arrow) of an E13.5 *Hand2*$^{\mathrm{LoxP/-}}$ embryo. (C, E) Expression of *Ptc* (C) and *Bmp2* (E) in E13.5 control palatal shelves. (D) *Ptc* expression is down-regulated in the palatal shelf of an E13.5 *Hand2*$^{\mathrm{LoxP/-}}$ embryo. Note specific gene down-regulation in the palatal mesenchyme adjacent to the MEE (arrow). (F) *Bmp2* expression is reduced in the palatal shelf of an E13.5 *Hand2*$^{\mathrm{LoxP/-}}$ embryo. Note the expression in the maxillary mesenchyme and the tongue is not affected. T, tongue

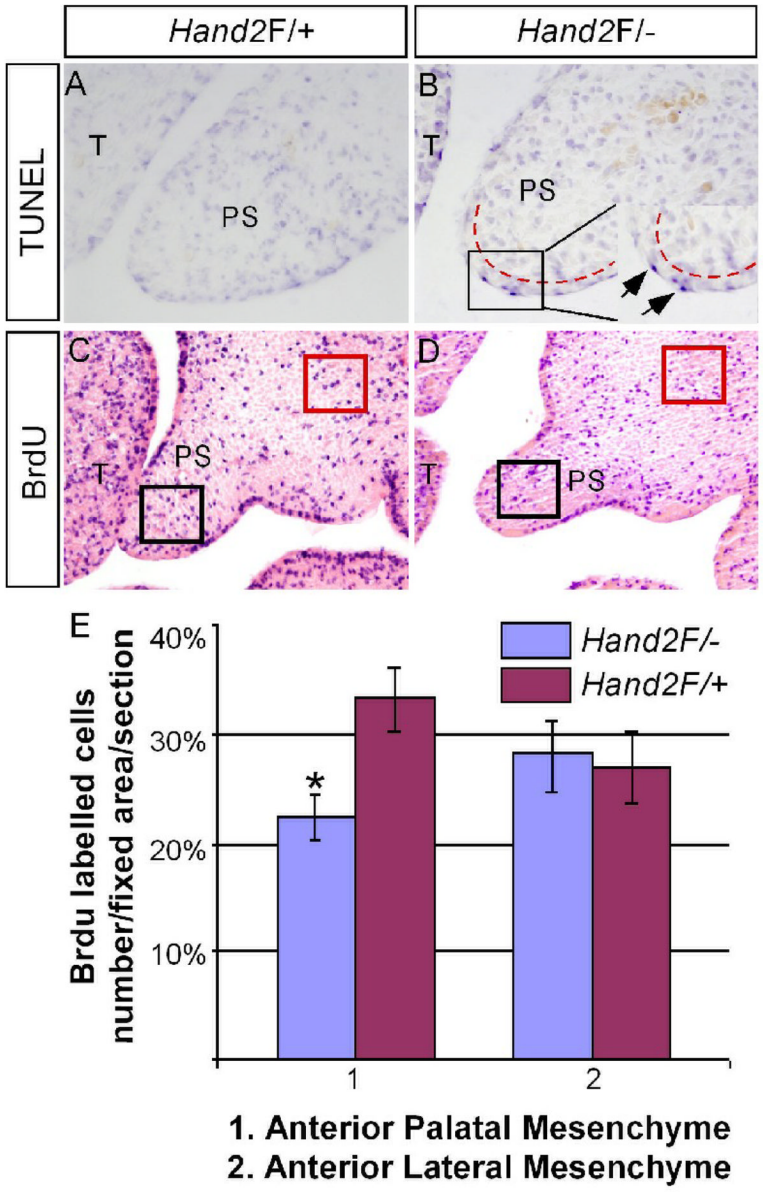

Figure 6.

Aberrant cell proliferation and apoptosis in the palatal shelf of *Hand2* hypomorphic mice. (A, B) TUNEL assay on the anterior portion of palate from E13.5 control (A) and *Hand2*$^{LoxP/-}$ (B) embryos shows abnormal apoptotic cells (arrows in the insert of panel B) in the MEE of the mutant. Note the apoptotic cells are periderm cells. The red dash lines demarcate the boundary of epithelium and mesenchyme. (C, D) BrdU labeling assays show a reduced level of cell proliferation in the anterior palate of E13.5 *Hand2*$^{LoxP/-}$ embryo (D), as compared to the control (C). (E) Comparison of BrdU-labeled cells in fixed areas of palate and lateral maxillary mesenchyme in controls and *Hand2*$^{LoxP/-}$ embryo. Standard deviation values were indicated as the error bars. *: $P < 0.01$. T, tongue; PS, palatal shelf.

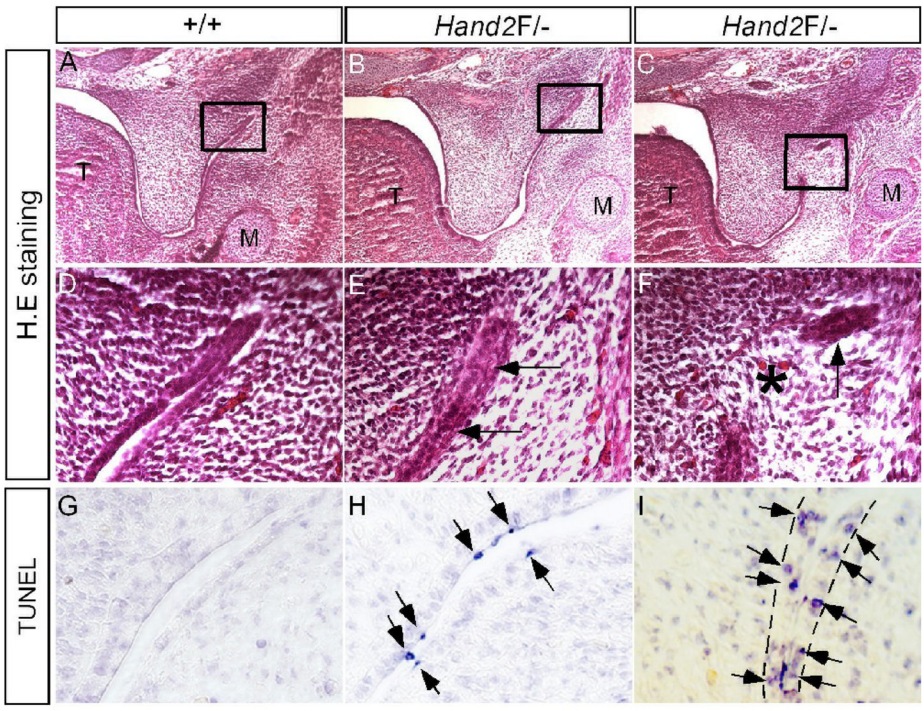

Figure 7.

Pathological adhesion/fusion of the palatal shelf and mandible in *Hand2* hypomorphic mice. (A, D, G) E13.5 wild type controls show normal histological structure (A, D) and absence of apoptotic cells in the lateral junction of the posterior palate and mandible. (B, E) An E13.5 *Hand2*$^{LoxP/-}$ embryo shows adherence of epithelia (arrows in E) of the lateral junction of the posterior palate and mandible. (C, F) An E13.5 *Hand2*$^{LoxP/-}$ embryo shows fusion of the palatal shelf and mandible at the lateral junction. Star denotes a confluence of mesenchyme, and arrow points to a remanent epithelium. (H) An E13.5 *Hand2*$^{LoxP/-}$ embryo shows apoptotic periderm cells (arrows) in the lateral junction right before epithelial contact/adherence. (I) An E13.5 *Hand2*$^{LoxP/-}$ embryo shows apoptotic basal layer epithelial cells (arrows) in the lateral junction fusion of the palatal shelf and mandible. The dash lines demarcate the epithelial boundary.

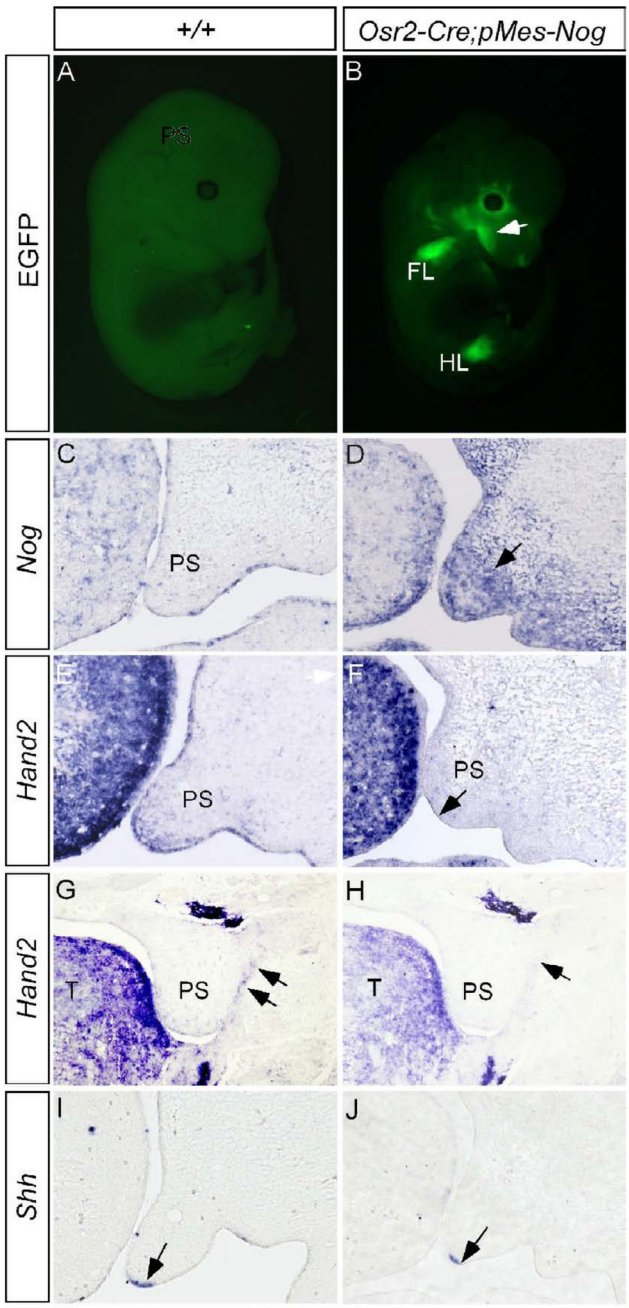

Figure 8.

Overexpression of *Noggin* to the palatal mesenchyme down-regulates the expression of *Hand2* and *Shh*. (A) An E13.5 wild type control embryo shows undetectable fluorescent signals. (B) An E13.5 *Osr2-Cre;pMes-Nog* embryo shows EGFP signal in the craniofacial region (arrow) and forelimb FL) and hindlimb (HL). (C) An E13.5 wild type control shows barely detectable *Noggin* transcripts in the developing palatal shelf. Note *Noggin* expression in the tongue. (D) Strong *Noggin* expression (arrow) is detected in the palatal mesenchyme of an E13.5 *Osr2-Cre;pMes-Nog* embryo. (E, G, I) The expression of *Hand2* in the anterior palate (E) and the epithelial junction of posterior palate (G) and *Shh* expression (I) in the MEE are detected in E13.5 controlled palatal shelves. (F, H, J) The expression of *Hand2* is down-

regulated in the anterior palate (F) and the epithelial junction of posterior palate (H) of E13.5 *Osr2-Cre;pMes-Nog* embryo. *Shh* expression is also down-regulated in the MEE of E13.5 *Osr2-Cre;pMes-Nog* palate (J). Note unaltered strong *Hand2* expression in the tongue in (F). Arrows in (F, G, H) point to the MEE, and in (G, H) point to the epithelial junction of posterior palate and mandible.