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Analysis of *Zfhx1a* mutant mice reveals palatal shelf contact independent medial edge epithelial differentiation during palate fusion

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Abstract

Cleft palate is a common birth defect that involves disruptions in multiple developmental steps such as growth, differentiation, elevation and fusion. Medial edge epithelial (MEE) differentiation is essential for palate fusion. An important question is that the MEE differentiation during fusion is induced by palate shelf contact or is programmed intrinsically by the palate shelf itself. Here, we report that the loss of *Zfhx1a* function in mice leads to a cleft palate phenotype that is mainly due to a delay in palate elevation. *Zfhx1a* encodes a transcription regulatory protein that modulates several signaling pathways including those activated by members of the TGF- β superfamily. Loss of *Zfhx1a* function in mice leads to a complete cleft palate with 100% penetrance. *Zfhx1a* mutant palatal shelves display normal cell differentiation and proliferation and are able to fuse in an in vitro culture system. The only defect detected was a 24–48 hour delay in palatal shelf elevation. Using the *Zfhx1a* mutant as a model, we studied the relationship between medial edge epithelial differentiation and palate contact/adhesion. We found that down regulation of *Jag2* expression in the medial edge epithelial cells, a key differentiation event establishing palate fusion competence is independent of palate contact/adhesion. Moreover, the expression of several key factors essential for fusion, such as TGF- β 3 and MMP13, are also down regulated at stage E16.5 in a contact independent manner, suggesting that differentiation of the medial edge epithelium is largely programmed through an intrinsic mechanism within the palate shelf.

Keywords

mouse secondary palate; *Zfhx1a* mutant mice; palate elevation; MEE differentiation; cleft palate

Introduction

The formation of the continuous secondary palate involves multiple developmental steps including palatal growth, elevation and fusion (Ferguson, 1988; Hilliard et al., 2005; Gritli-Linde, 2007). In mouse embryonic development, a group of cells within the bilateral maxillary

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processes are specified to be palatal mesenchymal cells and form two palatal outgrowths on embryonic day 11.5 (E11.5) (Murray and Schutte, 2004). From E12.5 to E13.5, the two developing palate shelves grow vertically along the lateral aspects of the tongue (Ferguson, 1988; Murray and Schutte, 2004). Each palate shelf is composed of a block of neural crest derived mesenchymal cell mass surrounded by epithelial cells that are cranial ectoderm in origin (Ferguson, 1988; Chai et al., 2000; Chai and Maxson, 2006).

On E14.5, however, the vertically growing palate shelves undergo elevation and position themselves above the dorsal level of the tongue (Ferguson, 1988; Hilliard et al., 2005). The two elevated palate shelves grow horizontally until they make contact along the facial midline followed by medial edge epithelial (MEE) cell degeneration and mesenchymal confluence leading to the formation of a single continuous palate, a process referred to as palate fusion (Ferguson, 1988; Murray and Schutte, 2004; Hilliard et al., 2005).

Recent studies with mouse embryos have uncovered a number of genes and signaling pathways that are important for palate growth and fusion (Hilliard et al., 2005). Inactivation of *Msx1* function in mice leads to a complete cleft palate due to impaired cell proliferation that can be rescued by trans-expression of the *BMP4* gene, indicating a pathway mediated by BMP and *Msx1* plays a critical role in palate growth (Satokata and Maas, 1994; Zhang et al., 2002; Hilliard et al., 2005). Consistently, loss of BMP receptor 1A (Alk3) in the mouse facial region leads to maxillary growth defects and cleft palate (Liu et al., 2005). TGF- β 3 plays an essential role in palate fusion (Kaartinen et al., 1995; Proetzel et al., 1995). On E14.5, TGF- β 3 is specifically expressed in the MEE cells of the developing palate shelves (Fitzpatrick et al., 1990; Pelton et al., 1990) and inactivation of TGF- β 3 function in mice results in cleft palate due to the defects in palate fusion (Kaartinen et al., 1995; Proetzel et al., 1995; Taya et al., 1999; Cui et al., 2005). Further study indicated that metalloproteinase 13 (MMP13) is involved in TGF- β 3 mediated palate fusion (Blavier et al., 2001). In contrast, *Fgf-10* and *Jagged2* (*Jag2*) mutant embryos display aberrant and premature fusion of the palatal shelves with the tongue or mandible, which physically prevents elevation of the palate shelves. (Jiang et al., 1998; Rice et al., 2004; Alappat et al., 2005; Casey et al., 2006).

The vertebrate *Zfhx1a* and *Zfhx1b* genes encode two transcription regulators that are closely related to *Drosophila* Zfh-1 (Fortini et al., 1991; Lai et al., 1991; Funahashi et al., 1993; Cabanillas and Darling, 1996; Sekido et al., 1996; Postigo and Dean, 1997). The vertebrate *Zfhx1a* gene is variously known as *δ EF1* (Funahashi et al., 1993), *Zfh* (Darling et al., 1998; Yen et al., 2001; Darling et al., 2003) and *ZEB-1* (Genetta et al., 1994; Postigo and Dean, 1997; Postigo, 2003; Postigo et al., 2003). The *Zfhx1b* is also known as *SIP1* or *ZEB-2* (Postigo and Dean, 1997; Verschueren et al., 1999; Postigo, 2003; Postigo et al., 2003). We refer to these two genes herein as *Zfhx1a* and *Zfhx1b* according to the nomenclature used by the Mouse Genome Database (MGD) and NCBI Entrez Gene, two commonly used databases. Both Zfhx1a and Zfhx1b proteins contain multiple DNA and protein interacting motifs including seven zinc fingers organized into two regions in the N- and C- terminus, a homeodomain, a Smad-binding domain, and three CtBP binding motifs (Furusawa et al., 1999; Postigo and Dean, 1999; Postigo et al., 2003), suggesting that they may function as modulators of other pathways such as the TGF- β signaling pathway. Indeed, in vitro cell line-based studies have shown that Zfhx1a and Zfhx1b modulate TGF- β and BMP activities (Postigo, 2003). In vivo analysis demonstrated that Zfhx1a is involved in TGF- β signaling in vascular smooth muscle cell differentiation (Nishimura et al., 2006). Through genetic studies, *Zfhx1a* is found to be involved in Shh signaling during mouse limb development as the semidominant extra toes defect in *Gli3* mutants was rescued when crossed into a *Zfhx1a*^{-/-} background (Moribe et al., 2000). *Zfhx1a* is highly expressed in the neural tube and brain, mesoderm and neural crest-derived tissues including the limb buds, somites and branchial arches (Takagi et al., 1998). Zfhx1a protein is also strongly expressed in proliferating neuroblasts of the embryonic brain, but not

in post-mitotic cells, with the exception of discrete nuclei of the hindbrain (Darling et al., 2003). Loss of *Zfhx1a* function in mice leads to impaired T cell development and multiple skeletal defects in the limbs, vertebrae and ribs (Higashi et al., 1997; Takagi et al., 1998). In addition, the *Zfhx1a* null mutant displays cleft palate, thus suggesting a role for *Zfhx1a* in secondary palate development (Takagi et al., 1998).

In this study, we report that *Zfhx1a* plays a specific role in promoting palate shelf elevation and that loss of *Zfhx1a* function causes a delay of up to 48 hours in palate elevation, which subsequently leads to cleft palate. No defects in palate growth, differentiation or fusion competence were detected in *Zfhx1a* mutant mice. Importantly, the *Zfhx1a* mutant mice provide an excellent model to study the relationship between palatal MEE differentiation and palate contact, a critical question in MEE differentiation and palate fusion that is difficult to be addressed in wild type embryos in vivo. We demonstrated that MEE differentiation is largely programmed by the palatal shelf itself and not induced by palatal contact.

Material and Methods

Mice

The *Zfhx1a* mutant mouse line used in this study was reported previously by Takagi *et al.* (Takagi et al., 1998). In this line, a *LacZ-Neo* cassette was inserted into the first exon of the *Zfhx1a* gene, which leads to the disruption of *Zfhx1a* protein, except for the first 15 amino acids in the N-terminal region (Takagi et al., 1998). This line is maintained in a C57BL/6 background.

In vitro palate fusion assay

In vitro palate fusion was assayed using a palatal organ culture system described by Carette and Ferguson (Carette and Ferguson, 1992). Wild type and *Zfhx1a* mutant palatal shelves were dissected from E13.5 and E16.5 mouse embryos in cold α -MEM medium buffered with 25 mM Hepes (PH 7.4). The dissected palates were paired and transplanted onto MF-Millipore membranes (Fisher Cat # AABP 04700). The palatal shelf pairs, together with the membranes, were then placed on autoclaved grilles in organ culture dishes (Fisher Cat# 08-772-12) filled with BGJb medium (Invitrogen/Gibco Cat# 12591038) containing 0.1 μ g/ml ascorbic acid. The explants were cultured at 37°C with 5% CO₂ for 72 hours with medium changes every 24 hours.

Preparation of E13.5 palatal sagittal sections

To obtain proper sagittal sections of vertically oriented palate shelves on E13.5, palatal shelves were dissected from E13.5 embryos and placed on MF-Millipore membrane paper without pairing. The palate shelves were then incubated for 1 hour to allow the tissue to adhere to the membrane followed by fixation. The fixed samples were processed for paraffin embedding and histology sectioning together with the membrane filter papers.

Cell proliferation assay

A bromodeoxyuridine (BrdU) incorporation assay was used to determine cell proliferation rates. Pregnant mice were administrated BrdU (Sigma) by intraperitoneal injection at 100mg/kg and euthanized 1 hour later (for Fig. 4D–I) or at 200mg/kg and euthanized 2 hours later (for Fig. 4 B and C). Embryonic tissues were collected and subjected to routine paraffin embedding and sectioning. Incorporated BrdU was detected by immunocytochemistry using an anti-BrdU antibody from BD Bioscience (Cat # 347580). The strong signal in the brain was used as an internal positive control. To determine the density of BrdU positive cells, a series of images

were taken and imported into Adobe Photoshop, the numbers of BrdU positive cells in palate shelves were counted and normalized by the size of the palate.

In situ hybridization

Whole mount and cryo-section based section situ hybridizations using DIG labeled antisense RNA probes were performed according to the protocol described by Shen (Shen, 2001).

Results

Zfhx1a mutant mice display delayed palate

Previous studies have reported that the *Zfhx1a* protein is strongly and specifically expressed in the mesenchymal cells of mouse secondary palate shelves on E11.5 with decreasing expression through E14.5 (Darling et al., 2003). Consistently, using whole mount in situ hybridization followed by sectioning, we detected *Zfhx1a* mRNA in palatal mesenchymal cells on E12.5 and the expression is decreased on E13.5 (see supplemental figure). By E14.5, the expression of *Zfhx1a* mRNA is mostly absent in the palate (data not shown). Takagi et al. previously reported a *Zfhx1a* mutant mouse line that displays cleft palate at stage E18.5, indicating a functional requirement for *Zfhx1a* in palate development (Takagi et al., 1998). To determine the cause of the cleft palate in *Zfhx1a* mutant mice and the role of the *Zfhx1a* gene in palate development, we carried out a detailed analysis of *Zfhx1a* null embryos with respect to palate development. We found that *Zfhx1a* null embryos on a C57BL/6 background display a complete cleft palate on E16.5 and E17.5 with 100% penetrance (n=18) (Fig. 1b). Histological examination of palates from *Zfhx1a* mutant mice from E13.5 to E16.5 revealed that the mutant palate shelves were significantly delayed in elevation. As shown in Figure 2, the mutant palate shelves were morphologically normal at stage E13.5 compared with their wild type littermates (Fig. 2a, e). From E14.5, however, all of the wild type palate shelves elevated above the dorsal level of the tongue (Fig. 2b), whereas all the mutant palate shelves remained vertical (n=12) (Fig. 2f). On E15.5, wild type embryos formed a continuous palate shelf (Fig. 2c). In contrast, 77% of the mutant palate shelves (10/13) remained vertical (Fig. 2g). The remaining 23% mutant palate shelves (3/13) elevated at stage E15.5, but the two shelves did not contact as determined by either paraffin sections or cryo-sections (see the in situ data in Fig. 6f, g, j). On E16.5, all of the mutant palate shelves elevated above the tongue, but remained widely separated (Fig. 2h). The shape of the palate shelf in mutants at stage E16.5 was normal (Fig. 2h). Therefore, the loss of *Zfhx1a* function leads to a 48 hour delay in palate elevation in 77% of mutant embryos and a 24 hour delay in the remaining 23%. No aberrant adhesion was observed between the mutant palate shelves and the tongue, as was seen in *Jag2* and *Fgf-10* mutants (Jiang et al., 1998; Alappat et al., 2005). In addition, the *Zfhx1a* mutant palate shelves were separated from the tongue and well above the dorsal level of the tongue on E14.5 and on E15.5 as visualized by both paraffin sections (Fig. 2f, g) and cryo-sections, which rule out the possibility of tissue shrinkage (data not shown). Therefore, the delay of palate elevation is unlikely caused by defects, if any, in tongue development such as delay in flattening of the tongue as previously suggested (Seegmiller and Fraser, 1977; Barrow and Capecchi, 1999). The palate defects in *Zfhx1a* mutant mice closely resemble the defects observed in *Osr2* mutants, except that the shape of E16.5 palate shelf in *Osr2* mutant embryo is abnormal, which is most likely due to the reduced cell proliferation in the medial edge mesenchyme (Lan et al., 2004). To determine whether there was any alteration in *Osr2* expression in the *Zfhx1a* mutants, we examined the expression of *Osr2* in *Zfhx1a* mutant palates and found no significant difference of *Osr2* expression between wild type and *Zfhx1a* mutant palates (data not shown).

As mentioned above, over 75% of *Zfhx1a* mutant shelves were not elevated at E15.5. Detailed analysis of these mutants revealed that the anterior and middle regions were vertical (Fig. 3a, b), whereas the extreme posterior region was horizontal (Fig. 3c), indicating that the elevation

or re-orientation of posterior palate is not necessarily dependent on the elevation of anterior palate.

***Zfhx1a* mutant embryos display normal growth, differentiation and fusion competence during palate development**

To determine if any growth and/or differentiation defects in addition to the elevation defect observed in *Zfhx1a* mutant mice, we carried out shelf size comparison, cell proliferation assays, mesenchymal/epithelial marker analyses and in vitro fusion assays. No obvious difference in size was observed in E13.5 palate shelves in vivo (Fig. 4a) or in dissected palate shelves (data not shown) between the null mutant and heterozygote/wild type littermates (number of mutant shelves > 10, number of heterozygous/wild type shelves > 16). Bromodeoxyuridine (BrdU) incorporation assays on sagittal sections showed that *Zfhx1a* mutant palate tissues are as proliferative as the wild type controls on E13.5 from the anterior to posterior (Fig. 4b, c). BrdU assays on frontal sections showed no significant difference in medial edge cell proliferation between wild type and mutant palate shelves from E13.5 to E15.5 (Fig. 4d–i). Thus, no measurable growth defects in the development of *Zfhx1a* mutant palate were detected. We then examined cell differentiation in *Zfhx1a* mutant palates. *Msx1* and *Shox2*, two anterior markers (Zhang et al., 2002; Yu et al., 2005), were expressed only in the anterior region of the *Zfhx1a* mutant palate at stage E13.5 (Fig. 5d, e), but not in the posterior region (data not shown). *Meox-2*, a marker for posterior palate mesenchyme in C57BL/6 embryos (Jin and Ding, 2006), was expressed only in the posterior mesenchymal cells of both mutant and wild type palates at stage E13.5 (Fig. 5c, f). No *Meox2* expression was observed in the anterior region of the mutant palate (data not shown). In addition, both wild type and *Zfhx1a* mutant palates expressed *BMP4* in mesenchymal cells at stage E13.5 (data not shown). As to the differentiation of epithelial cells, we found that the E13.5 mutant palates express epithelial markers *IRF-6* (Xu et al., 2006) and *Pitx2* (Dong et al., 2006) in a normal pattern (Fig. 5g, h, j, k). Moreover, the mutant palates at stage E13.5 displayed normal expression of *TGF-β3*, a key medial edge epithelium marker essential for palate fusion (Fig. 6i, l). In addition, both wild type and mutant palate shelves displayed the same pattern of *Jag2* expression from E13.5 to E15.5 (see Fig. 6d–f). To determine whether the palate epithelium in *Zfhx1a* mutant embryos differentiates normally and enables fusion, we performed an in vitro palate fusion assay to examine whether the mutant palate shelves can undergo fusion in culture. As shown in figure 5, both wild type and *Zfhx1a* mutant palate shelves dissected from E13.5 embryos underwent complete fusion with no residual seam remaining after 3 days in culture (16 mutant pairs and 7 wild type pairs) (Fig. 5m, n). In addition, mutant palate shelves dissected from E16.5 embryos retained the ability to fuse after 3 days of culture (Fig. 5o). Both marker analyses and in vitro fusion assays showed that epithelial differentiation in *Zfhx1a* mutant palate shelves was largely normal. Therefore, the cleft palate in *Zfhx1a* mutant mice is predominately due to the delay of palate elevation that leads to shelves failing to contact because of the rapid growth of the skull around E14.5. In that regard, *Zfhx1a* mutant mice represent a unique case for cleft palate, in which the timing of shelf elevation is the major cause of the cleft.

Relationship between medial edge epithelial differentiation and palate contact/adhesion

The differentiation of the medial edge epithelium is critical for palate fusion. The timing of medial edge epithelial differentiation must be regulated precisely in order to prevent premature and/or aberrant fusion. In this regard, the expression of *Jag2* in palate epithelial cells is of particular importance since the loss of *Jag2* causes aberrant palate fusion with the tongue around E13.5–E14.5 (Jiang et al., 1998). We therefore examined the expression of *Jag2* in palate development from E13.5 to E15.5 and found that the *Jag2* expression domain covered the entire palate epithelium up to early E14.5 (Fig. 6a, b). At stage E15.5, however, the expression was decreased specifically in the medial edge area (Fig. 6a–c). Other investigators have also reported that the expression of *Jag2* is down-regulated in the medial edge epithelium

when the two palate shelves are tightly adhered (Casey et al., 2006). Presumably, the expression of *Jag2* before E14.5 is to prevent premature fusion (Jiang et al., 1998), since the loss of *Jag2* is sufficient to cause premature fusion as early as E13.5–E14.5 (Jiang et al., 1998). This also indicates that down regulation of *Jag2* in the MEE cells at stages E14.5–E15.5 is sufficient to establish the fusion competence. A critical question is if the down regulation of *Jag2* expression in the MEE occurs autonomously or is induced by palate contact. This question is difficult to address in wild type embryos, since the two processes occur simultaneously. We addressed this question using *Zfhx1a* mutant embryos, and found that down-regulation of *Jag2* expression in the MEEs around E15.0 was completely independent of palate contact. As shown in figure 6, the expression of *Jag2* is in the entire epithelium in the mutant palate from E13.5 to E14.5, similar to wild type palate (Fig. 6d, e). The expression is specifically abolished in the MEE region of *Zfhx1a* mutant palate at stage E15.5 even without palate contact, whereas the expression was maintained in both the nasal and oral epithelium at the same stage (Fig. 6f). Therefore, the down regulation of *Jag2* expression in the MEEs is programmed intrinsically by the palate shelf rather than induced by contact/adhesion. The timing of this intrinsic program for MEE differentiation matches that for contact/adhesion/fusion precisely.

Previous studies (Lavrin et al., 2001) have shown that the MEE retain in vitro fusion competence until E16.5. From E17.5, however, the fusion efficiency drops dramatically (Lavrin et al., 2001). We suspect that the loss of fusion ability after E16.5 is because of the down regulation of the key components of the fusion machinery. The *Zfhx1a* mutant mouse line provides a good model to test this hypothesis, as the mutant palates retain MEE cells at stage E16.5, whereas the MEE cells in wild type embryos are removed before E16.5. We therefore examined the expression of several key genes that have been previously demonstrated to be important for palate fusion such as *TGF-β3* (Karttinen et al., 1995; Proetzel et al., 1995; Taya et al., 1999), *MMP13* (Blavier et al., 2001) and *Jag2* (Jiang et al., 1998) in the *Zfhx1a* null embryos from E15.5 to E17.5. *TGF-β3* and *MMP13* were highly expressed in the MEE at E15.5 (Fig. 6g, j). The expression continues into stage E16.5 albeit with less intensity (Fig. 6h, k). At stage E17.5, the expression of *TGF-β3* and *MMP13* were almost absent in palate epithelium (Fig. 6i, l). The expression of *Jag2* was absent in the MEE on E15.5 (Fig. 6c, f), and abolished in the entire palate epithelium by E16.5 in *Zfhx1a* mutants (data not shown). Thus, it appears that there is an intrinsic differentiation program that is limited to a specific time window from E13.5 to E16.5 as demonstrated by the expression of the genes critical for fusion. Interestingly, a recent study from Bloch-Zupan's group showed that down-regulation of *Snail* expression in palate mesenchyme is also independent of palate fusion in vitro, suggesting a fusion-independent gene expression program may also operate in palate mesenchyme as well (Pungchanchaikul et al., 2005).

Discussion

Zfhx1a null mice display a cleft palate with 100% penetrance. The palate shelves of *Zfhx1a* mutant mice have a normal cell proliferation rate, undergo normal mesenchymal and epithelial differentiation and are competent to fuse. The only defect detected was a delay of 24–48 hours in palate elevation with no abnormal fusion of the palate shelves with the tongue or mandible. It has been proposed that mandibular growth defects can cause a delay in flattening of the tongue, which may subsequently interfere with palate elevation (Seegmiller and Fraser, 1977; Barrow and Capecchi, 1999). However, the *Zfhx1a* mutant palate shelves were separated from the tongue and well above the dorsal level of the tongue on E14.5 and on E15.5 (Fig. 2f, g). Therefore, it is unlikely that the delay in palate elevation is due to physical obstruction by the tongue, but rather due to a defect within the palate shelf itself. As to how a delay in palate elevation causes cleft palate, it is most likely that the continuing growth of the skull on E14.5 and E15.5 increase the distance between the two palate shelves, which prevents the two shelves from contacting after elevation on E15.5–E16.5. Therefore, the *Zfhx1a* null mutant represents

a unique model for cleft palate, in which the timing of a developmental event is a major, if not the only, cause of cleft palate

The *Zfhx1a* mutant line used in this study has a *LacZ-Neo* insertion in the first exon that disrupts the entire protein product except for the first 15 amino acids at the N-terminus (Takagi et al., 1998). Higashi and colleagues generated another *Zfhx1a* mutant line by targeted insertion (Higashi et al., 1997) that produces a truncated protein containing several zinc finger domains and the Smad binding domain in the N-terminal region (Higashi et al., 1997; Takagi et al., 1998). Unlike the null allele, the truncated allele caused defects in T-Cell development without cleft palate (Higashi et al., 1997). Takagi and colleagues proposed a domain-specific regulatory model for *Zfhx1a* function, in which the N-terminal region is responsible for skeletal development and C-terminal region is responsible for thymus development (Takagi et al., 1998). As an extension of this model, the N-terminal zinc fingers and Smad binding domains may be important for *Zfhx1a* function in palate elevation. Interestingly, TGF- β 2 mutant mice display cleft palate caused by a failure of palate elevation without aberrant fusion (Sanford et al., 1997), and *Zfhx1a* has been reported to modulate TGF- β signaling in vitro and in vivo (Postigo, 2003; Postigo et al., 2003; Nishimura et al., 2006), it is possible that *Zfhx1a* affects palate elevation by modulating the TGF- β signaling pathway. The down stream targets for *Zfhx1a* protein are not yet clear. Interestingly, the *Osr2* mutant mice closely resemble the *Zfhx1a* mutant mice in palate defects (Lan et al., 2004). However, the expression of *Osr2* was not altered significantly in *Zfhx1a* mutant mice. Since the cellular mechanism of palate elevation is poorly understood at present time, it is difficult to address how *Zfhx1a* function affects palate elevation now.

The medial edge epithelial differentiation is a continuous process starting as early as E13.5 with the regional expression of *TGF- β 3* in palate epithelium (Fitzpatrick et al., 1990; Pelton et al., 1990). Further differentiation on E14.5 is marked by the expression of *MMP13* specifically in the medial edge epithelium (Blavier et al., 2001). Around late E14.5 (Casey et al., 2006) and early E15.5 (Fig. 6C), epithelial expression of *Jag2* is down-regulated specifically in the medial edge area. Since loss of *Jag2* function leads to premature fusion around E13.5–E14.5 (Jiang et al., 1998), the expression of *Jag2* in the palatal epithelium before palate contact may be to prevent premature or aberrant fusion, and down regulation of *Jag2* expression in the medial edge epithelium at E14.5–E15.5 should be sufficient to establish a “ready-to-go” status for palate fusion. In that regard, down regulation of *Jag2* marks the completion of medial epithelial differentiation program with respect to fusion. Considering that down regulation of *Jag2* expression precisely matched the palate contact both temporally and spatially, one would assume that the decrease in *Jag2* expression and the fusion competence are induced by palate contact. However, our study revealed that down regulation of *Jag2* in medial edge epithelium occurs normally in the separated *Zfhx1a* mutant palate shelves, demonstrating that the medial edge epithelial differentiation is programmed intrinsically within the palate shelf, and is coordinated with palate contact and adhesion. This is not to suggest that palate contact does not induce any changes in medial edge epithelium. There must be numerous changes in MEE cells induced by palate contact in order to remove the MEE seam, for example. Moreover, the expression of key genes required for fusion are down-regulated autonomously in the MEE cells by E16.5 also in a contact independent manner, suggesting a limited time window for fusion that is programmed intrinsically by the palate shelves. Consistently, a recent in vitro explant-based study showed that the down regulation of *Snail* expression in palate mesenchyme is also independent of palate fusion (Pungchanchaikul et al., 2005).

Supplemental Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. Whole mount view showing a complete cleft palate in *Zfhx1a* mutant embryos
(a) A wild type mouse head at stage E16.5 with fused palate along the midline (arrowhead and the insert). (b) A *Zfhx1a* homozygous mutant littermate of (a) bearing open secondary palate (arrowhead and the insert) from anterior to posterior.

Figure 2. Histological analyses showing delayed palate elevation in *Zfhx1a* mutant embryos (a, e) On E13.5, wild type palate (arrows in a) and *Zfhx1a* mutant palate (arrows in e) were morphologically indistinguishable. (b, f) On E14.5, wild type palate shelves (arrows in b) elevate above the level of the tongue, whereas the *Zfhx1a* mutant palate shelves (arrows in f) remained vertical. (c and g) On E15.5, wild type embryos have formed a continuous secondary palate above the tongue (arrow in c), and over 75% of *Zfhx1a* mutant palates (arrows in g), by contrast, were vertical. (d, h) At stage E16.5, the fused palate was further developed (arrow in d), and all the *Zfhx1a* mutant palate shelves have elevated above the tongue, but the two shelves remained separate (arrows in h). t: tongue. Scale bars represent 100μm.

Figure 3. Histology sections of E15.5 *Zfhx1a* mutant embryonic palate across anterior (a), middle (b) and posterior (c) showing the posterior palate shelves were horizontally orientated while the anterior and middle palate shelves were still in the vertical orientation. t: tongue. Scale bars represent 100µm.

Figure 4. Size comparison and BrdU incorporation assay showing no growth defect in *Zfhx1a* mutant palate

(a) Wild type and mutant palate shelves at stage E13.5 were similar in size. The white dotted lines indicate the edge of the palate shelf. (b, c) BrdU incorporation assay on sagittal sections of wild type palate (b) and *Zfhx1a* mutant palate (c) showing similar cell proliferation intensities between wild type and mutant palate shelves from anterior to posterior. (d–i) BrdU incorporation assay on frontal sections of palates on E13.5 (d, g), E14.5 (e, f) and E15.5 (f, i) showing no obvious difference in cell proliferation between the palate shelves (arrows) of wild type (d, e, f) and *Zfhx1a* mutant (g, h, i). The white dotted line indicates the position of the midline. *Wt*: wild type; *-/-*: *Zfhx1a*^{-/-}.

Figure 5. Marker analyses and in vitro fusion assay showing normal differentiation and in vitro fusion in *Zfhx1a* mutant palate shelves

(a–f) Palate shelves (arrows) of both wild type embryos (a–c) and *Zfhx1a* mutant embryos (d–f) on E13.5 express *Msx1* (a, d) and *Shox2* (b, e) in the anterior region, and *Meox2* (c, f) in the posterior palate. (g–l) Both wild type (g, h, i) and *Zfhx1a* mutant (j, k, l) palate shelves on E13.5 (arrows) expressed pan epithelial markers, *IRF6* (g, j) and *Pitx2* (h, k) as well as the medial edge epithelial marker, *TGF-β3*. (m–o) Palate shelves from wild type embryos (m) and *Zfhx1a* mutant embryos (n, o) at E13.5 (m, n) and E16.5 (o) underwent in vitro fusion in culture with concomitant degeneration of the seam (arrowheads). **Wt**: wild type; **-/-**: *Zfhx1a*^{-/-}.

Figure 6.

(a–f) The expression of *Jag2* covered the entire epithelium of both wild type palates (arrows in a, b) and *Zfhx1a* mutant palates (arrows in d, e) on E13.5 (a, d) and E14.5 (b, e), whereas the expression on E15.5 was specifically down-regulated in the medial edge epithelium (arrowheads) of both wild type (c) and *Zfhx1a* mutant (f). (g–h) *TGF-β3* expression in *Zfhx1a* mutant palate was strong on E15.5 (arrowhead in g), medial on E16.5 (h) and almost absent on E17.5 (i). (j–l) *MMP13* was highly expressed in E15.5 *Zfhx1a* mutant palate (arrowhead in j), and the expression was decreased at stage E16.5 (arrowhead in k) and eventually vanished by stage E17.5 (arrowhead in l). *Wt*: wild type; *-/-*: *Zfhx1a*^{-/-}.